CUCURBITACEAE 2014

PROCEEDINGS

October 12-16, 2014

Bay Harbor, Michigan

MICHIGAN STATE UNIVERSITY

WISCONSIN
UNIVERSITY OF WISCONSIN–MADISON

Cucurbitaceae 2014 Proceedings

October 12–16, 2014

Bay Harbor, Michigan, USA

Organizing Committee

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ISBN 978-0-9830932-2-0

Cucurbitaceae 2014 Proceedings

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Preface

On behalf of the organizing committee, we welcome you to Cucurbitaceae 2014. This conference is the 14th biennial meeting of the international cucurbit research community. The first meeting was held in Avignon France in 1988, with subsequent meetings in the United States (1990, 1994, 1998, 2002, 2006, and 2010), Poland (1992), Spain (1996), Israel (2000), the Czech Republic (2004), France (2008), and Turkey (2012). We look forward to continuing this tradition of open communication and collaboration among researchers in the cucurbit community at this meeting and the next meeting planned for Poland in 2016.

Many people contributed to the planning for and organization of Cucurbitaceae 2014. We thank the staff of ANR Event Services at Michigan State University whose help was instrumental in the success of this meeting, especially the experience and organizational skills of Betsy Braid and Mary Slevin. We also thank Sylvia DeMar at the American Society of Horticultural Science for her efforts organizing and producing the Proceedings of our meeting.

Cucurbitaceae 2014 would not have been possible without the financial support of Michigan State University and our corporate sponsors Monsanto, Syngenta, Bejo, Hazera, HM-Clause, Rijk Zwaan, Sakata, East-West Seed, Bayer, and Magnum Seeds. Please take the opportunity to thank representatives of these companies for their generous support of our conference.

Rebecca Grumet

Brad Day

Michael Havey

Yiqun Weng

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A Transcriptomic Approach to Analyzing Genetic Pathways Involved in *Cucumis sativus* Resistance to *Pseudoperonospora cubensis*

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Additional index words. downy mildew, resistance, transcriptome, RNA-Seq

Abstract. Downy mildew is an economically relevant pathogen of cucumbers and other cucurbits caused by the obligate oomycete, *Pseudoperonospora cubensis*. In recent years, host resistance has been overcome by this pathogen leaving many crops, namely cucumber, vulnerable to this foliar disease. In order to better understand the cucumber-downy mildew interaction at a molecular level, recent efforts have been made to sequence the genome of *Ps. cubensis* as well as to sequence the transcriptome of both *Ps. cubensis* and the susceptible cucumber cultivar "Vlaspik" over the time course of infection from 0-8 days post inoculation. Current work is focused on uncovering the transcription-related regulation of resistance by comparing the transcriptome of a susceptible host (Vlaspik) to a resistance cucumber host (PI 197088) over the time course of *Ps. cubensis* infection from 0–6 days post inoculation. Previous research has shown that PI 197088 shows resistance to downy mildew in field-based studies, and work in this study confirms this resistance through the use of controlled time course studies, which include leaf phenotype, microscopy, and real-time PCR data to show the level of resistance. Through this study, significantly differentially regulated genes in the resistant line were identified, and future work will examine the relationship between the susceptible and resistant cucumber transcriptomes and the roles that SNPs and small RNAs play in mediating resistance.

Pseudoperonospora cubensis, the causal agent of cucurbit downy mildew, is an obligate pathogen of *Cucumis sativus* (cucumber) and many other hosts in the Cucurbitaceae. Similar to many other plant pathogenic oomycetes, *Ps. cubensis* has a broad host range (>20 Genera), a wide geographical distribution, exhibits increasing tolerance to chemical management, and is highly adapted to variations in environment (Savory et al., 2010). For decades, cucurbit-producing regions in Europe and Asia have faced production pressures from *Ps. cubensis*. Historically, in the U.S. however, *Ps. cubensis* was successfully managed through plant resistance, conferred by the recessive locus, *dm-1*. In recent years, increasing virulence and environmental tolerance of *Ps. cubensis* has proven to be a primary factor limiting U.S. cucurbit production; indeed, in 2004, resistance in cucumber to *Ps. cubensis* was overcome (Savory et al., 2010).

Downy mildew symptoms on cucumber include angular chlorotic lesions on the upper leaf surface that expand and coalesce, with sporulation occurring on the lower leaf surface. Wind-dispersed sporangia germinate in the residual water on leaf surfaces, and biflagellate zoospores swim to stomata. The zoospores encyst, produce appressoria, and form hyphae throughout host tissue. During infection, virulence factors are secreted through developing haustoria, which in turn function to manipulate the physiology and primary defense processes of their host. In total, this process functions to subvert immune signaling such that the pathogen can persist, develop, and acquire nutrients. In the later stages of the life cycle, the pathogen will sporulate and release asexually produced sporangia into the air to spread and infect new hosts (Burkhardt & Day, 2013).

Recently, a draft genome assembly of *Ps. cubensis* isolate MSU-1 inventoried the virulence capacity of *Ps. cubensis*, as well as to began functional characterization of host range and adaptation (Tian et al., 2011). From these initial studies, we have developed a foundation to support the further study of this important obligate oomycete-plant interaction. Protein coding genes in the assembly were annotated using a combination of *ab initio* gene predictions, including protein and transcript evidence from other sequenced oomycete genomes. Through this work, we approximated 23,500 gene loci and models. The size of the *Ps. cubensis* genome is similar to other oomycetes with more streamlined genomes; however, the number of genes exceeds predictions of other comparable oomycete genome annotations.

In two follow-up publications, RNA-Seq was used to profile genome-wide changes in gene expression during various stages in the development of *Ps. cubensis* on susceptible cucumber 'Vlaspik' (Adhikari et al., 2012; Savory et al., 2012). Using a gene correlation network analysis, more than 3,000 differentially expressed genes were grouped into six modules that correlate to important stages of infection. Most of the candidate RXLR-containing effectors were expressed between 2–4 dpi and the candidate host-targeting hydrolytic enzyme-encoding genes were expressed between 4–8 dpi. Additionally, we identified 440 putative transcription-factor encoding genes. Of these, ~250 were expressed throughout the course of infection, suggesting an extensive transcriptional module during host infection.

Current work is focused on expanding upon the bioinformatic resources previously established in the Day lab in order to address the challenge of uncovering the transcriptomic networks

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that regulate cucumber resistance to *Ps. cubensis*. Through a collaboration with Todd Wehner (North Carolina State University), we obtained seeds for cucumber cultigen PI 197088, which was found to be resistant to downy mildew (Call et al., 2012). Controlled inoculation time courses were performed for both PI 197088 (resistant) and 'Vlaspik' (susceptible) in order to directly compare which genes and gene networks were differentially expressed in a resistant vs. susceptible interaction. Through the utilization of next-generation sequencing and quickly advancing bioinformatics tools, the goal of this research is to decipher the genetic interactions that contribute to downy mildew resistance.

Materials and Methods

C. sativus growth and Ps. cubensis inoculation. Ps. cubensis isolate MSU-1 was propagated on *Cucumis sativus* 'Vlaspik' as previously described (Tian et al., 2011). Healthy cucumber plants were grown at 22 °C in 16h light/8h dark cycles. Infected plants were grown at 22 °C in 12h light/dark cycles and maintained at near 100 % humidity. Time course experiments were performed on the susceptible 'Vlaspik' and the resistant plant line PI 197088 by inoculating 10 μL of 1×10^5 sporangia/ml onto the underside of 4-week-old cucumber leaves, followed by sampling the inoculated areas at regular time points using a #3 cork borer.

Microscopy. Samples collected for each time point were cleared in ethanol and stained with trypan blue. Microscopy of destained tissue was performed using an Olympus IX71 inverted light microscope (Savory et al., 2012).

RNA and total RNA isolation. RNA was extracted from sporangia and from flash-frozen leaf tissue using the RNeasy Plant Mini Kit (Qiagen). Genomic DNA was removed from the sample with the on-column DNase treatment (Qiagen). Total RNA was extracted from sporangia and flash-frozen leaf tissue for mRNA and small RNA sequencing using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen). RNA quality was analyzed with the 2100 Bioanalyzer (Agilent) and the Agilent RNA 6000 Pico kit.

Quantitative real-time PCR. cDNA was prepared using the USB first-strand cDNA synthesis kit with random hexamer primers (USB Affymetrix), as described above, using 1 μg of total RNA extracted from sporangia and corresponding infected leaf tissue. Samples were prepared using the HotStart SYBR Green qPCR Master Mix (2x; USB). A Mastercycler ep Realplex real-time PCR (Eppendorf) was used to perform quantitative real-time PCR. To normalize for the level of *Ps. cubensis* in infected tissue samples, internal transcribed space (ITS) primers were used to detect the amount of pathogen (Tian et al., 2011) relative to the amount of plant tissue quantified with cucumber-specific actin primers. The following cycling parameters were used: 1 hold of 95 °C for 2 minutes, 40–50 cycles of 95 °C (15 s), 56 °C (15 s), and 72 °C (30 s). Relative expression for each splice variant was calculated where relative expression = $2^{(-\Delta Ct)}$ and where $\Delta Ct = Ct_{ITS} - Ct_{actin}$ (Porter et al., 2012). Error bars represent the standard error from four biological replicates. Data were analyzed and processed using Prism (GraphPad Software).

Next-generation sequencing. Libraries from two biological replicates each of Vlaspik and PI 197088 cucumber leaves that were mock-inoculated or collected 1,2,3,4, or 6 days post inoculation (dpi) with *Ps. cubensis* were prepared using the Illumina TruSeq Stranded mRNA Library Preparation Kit LT or the Illumina Small RNA Library Preparation Kit for mRNA and small RNA, respectively. Each mRNA sample was sequenced with at least 20 million 50 bp SE reads and each small RNA sample was sequenced with at least 10 million 50 bp SE reads on Illumina HiSeq at the Michigan State University Research Technology Support Facility.

Bioinformatic analysis of next-gen sequencing. mRNA-Seq reads were trimmed, quality-evaluated by FastQC, and aligned to the annotated *C. sativus* reference genome of Chinese long (ftp://www.icugi.org/pub/genome/cucumber/Chinese_long/v2/) using the short read aligner Bowtie v.1.0.0 and the splice-aware mapper TopHat v1.4. Accepted BAM hits from TopHat were converted to SAM files using SAMTools v0.1.18. The number of reads that align to each annotated gene of the cucumber genome were counted using HT-Seq count, which is part of the HT-Seq Python package; these counts were used as the data input to measure differential gene expression using DESeq (Anders et al., 2013; Seyednasrollah et al., 2013).

Results and Discussion

Time course phenotypes show resistance to Ps. cubensis in PI197088. Infection time courses of the cucumber varieties Vlaspik and PI 197088 confirm that Vlaspik is susceptible to *Ps. cubensis* while PI 197088 has a resistant phenotype in that it does not allow the pathogen to sporulate and thus reproduce (Fig 1A). Leaf phenotypes as shown in Fig 1A also indicate that the mechanism of resistance in PI 197088 is likely not a direct gene-for-gene interact in which R genes directly recognize a pathogen effector, as no hypersensitive response (HR) is observed. Microscopy data (Fig. 1B) confirms that, unlike on Vlaspik at 6 dpi and 8 dpi, *Ps. cubensis* is not able to produce sporangiophores or sporangia when grown on PI 197088, which further supports the conclusion that this cucumber line is resistant. Furthermore, microscopy data indicates that *Ps. cubensis* is able to enter PI 197088 and establish hyphae, which indicates that the mechanism to resistance is not a physical barrier. Finally, Fig. 1C indicates that *Ps. cubensis* is only able to briefly survive on PI 197088, as shown by a barely detectable pathogen level; in contrast *Ps. cubensis* rapidly increases in biomass on Vlaspik, as inferred from the level of detected pathogen ITS product normalized to the level of plant actin product. The real-time PCR data indicate that *Ps. cubensis* growth is somehow halted early during infection; however it is unclear from this assay at what point and how this is accomplished.

From the phenotypic data (Fig. 1) several hypotheses regarding the mechanism(s) of cucumber resistance to downy mildew were made and are addressed to some extent with the following data regarding transcriptomic changes during infection. First, given that no HR was observed, transcriptomic sequencing at multiple time points was completed because the resistant interaction is likely not a simple gene-for-gene situation. Second, given that the pathogen is able to enter PI 197088, briefly makes hyphae, but is not able to establish sporangiophores, it was hypothesized that multiple genes and or gene networks related to pathogen nutrition might be involved in establishing host resistance.

Differentially expressed genes during PI197088 infection with Ps. cubensis. Significantly differentially expressed genes were identified throughout the time course of infection of PI 197088 with MSU-1 compared to the mock-inoculated time point. At each time point, thousands of significantly differentially expressed genes were identified in PI 197088 at each time point post infection compared to the mock. The 30 most significantly differentially expressed genes were plotted in a heat map in Fig. 2 to see patterns that may exist in the grouping of some genes by

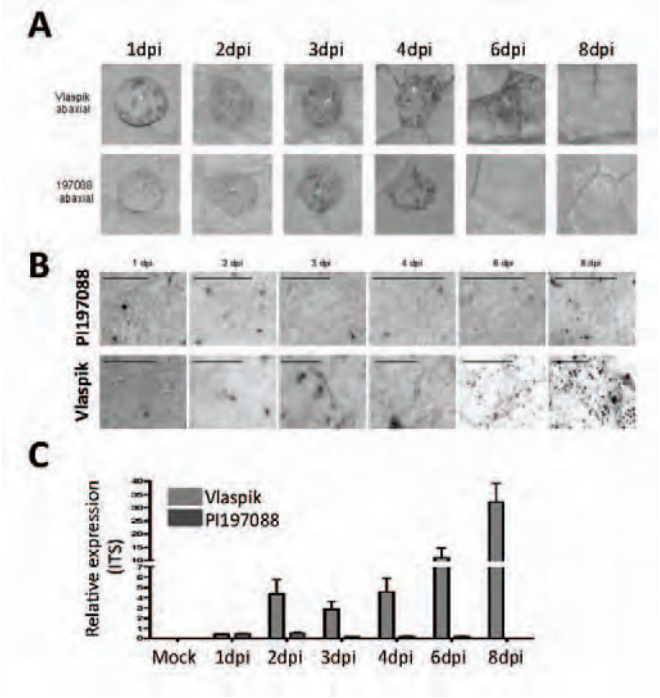

Fig. 1. Time course inoculation of cucumber cultivars Vlaspik (susceptible) and PI197088 (resistant). **A)** Abaxial-surface images of point of inoculation. **B)** Trypan blue staining of infected leaves showing pathogen infection structures. **C)** Real time PCR quantification of *Ps. cubensis* from infected leaf material using strain-specific ITS primers. Error bars show the standard error of the mean.

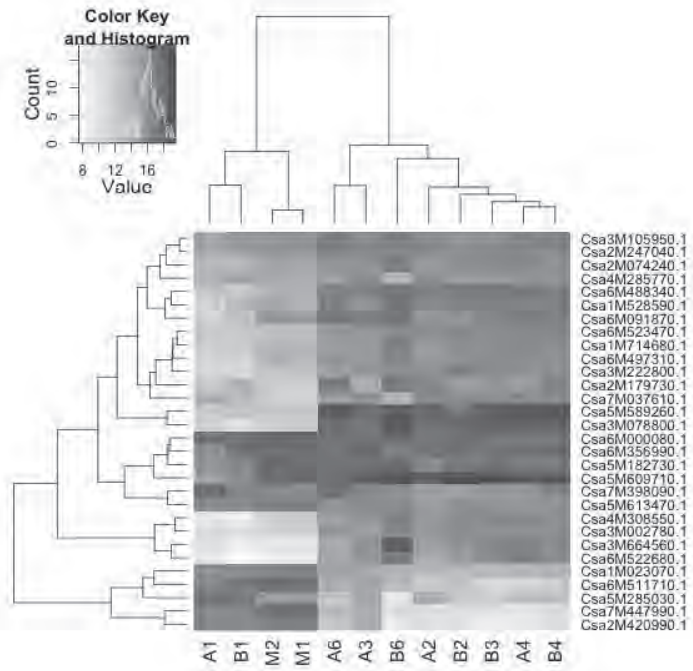

Fig. 2. Expression heatmap of the relationship between 30 cucumber genes over our time-course of inoculation with *Ps. cubensis* Biological replicates are indicated with A or B, numbers correspond to time points, M1 and M2 represent the mock replicates. Blue to green indicates high to low, respectively.

expression over time. Of interest is gene Csa3M078800, which encodes a thiamine thiazole synthase, responsible for thiamine biosynthesis, an essential vitamin which *Ps. cubensis* and other oomycetes obtain from their hosts (Kemen et al., 2011). Other commonly upregulated genes included photosynthesis-related genes, which might be coordinated with an increased energy demand as the host fights pathogen infection.

Future studies. Future studies are aimed at identifying differentially expressed genes within the Vlaspik time course and between the Vlaspik and PI 197088 plant lines before and during infection. Additional bioinformatic studies using this data will include the completion of weighted genome co-expression network analysis (WGCNA) (Childs et al., 2011), SNP analysis, and small RNA analysis. SNP analyses will be used to analyze the mRNA data from both Vlaspik and PI 197088 to determine if nucleotide changes result in protein changes that could have an effect on the function of the protein. Small RNAs will be examined in both *Ps. cubensis* and in infected plant tissue to determine if small RNAs from the pathogen have a role in modulating host defense in this specific host-pathogen interaction. Ultimately, the goal of this research is to shed light on the mechanism of cucumber resistance to downy mildew with the long-term vision of improving crops.

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Differential Analysis of Gene Expression Profiling in Different Cucumber Cultivars Inoculated With *Pseudoperonospora cubensis*

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Additional index words. *Cucumis sativus L., Pseudoperonospora cubensis,* downy mildew, RNA-Seq, R gene

Abstract. Cucumber downy mildew is a destructive leaf disease caused by *Pseudoperonospora cubensis*. Exploiting the resistant genes and developing resistant varieties has been the most effective way to control diseases in cucumber. Therefore, further analysis of the defense mechanism and identification of the key genes resistance to cucumber downy mildew are particularly important. In this study, cucumber cultivars "M801-3-1" and "M302-3" infected by downy mildew were used for transcriptome sequencing. The sequencing results showed obvious differences of gene expression profiling between resistant and susceptible varieties after downy mildew infection. There were 3599 genes highly expressed in the resistant variety "M801-3-1", including auxin related proteins, calcium binding protein, heat shock protein, glutathione peroxidase, glutathione reductase, glutathione S-transferase, chloroplast binding protein, ubiquitin, and proteasome protein. We found 47 genes only expressed in M801-3-1, including DNA damage induced protein and ascorbic acid peroxidase, in which a CC-NBS-LRR like gene in resistant varieties may be the resistance R gene in resistance variety. In the susceptible cultivar "M302-3", 1727 genes showed high expression after downy mildew infection, such as ethylene oxidase gene, ethylene related transcription factor, and WRKY transcription factor. There were 168 genes expressed only in the susceptible cultivar M302-3, including the genes of TIR-NBS-LRR disease resistance protein, ribosomal protein, ethylene forming enzyme, ubiquitin protein. The results suggested that two differential expression TIR-NBS-LRR-like genes and a TIR-NBS-LRR-like gene which highly expressed in the susceptible cultivars may be the susceptible R gene.

Cucumber downy mildew is a destructive leaf disease caused by *Pseudoperonospora cubensis*. Downy mildew was observed on cucumbers and melons as early as the 19th century, but it has not received sufficient attention until the mid-1980s when it caused large-scale economic losses. In 1984, a severe outbreak of downy mildew occurred on melons in France. In 1985, the disease reached epidemic levels in cucumbers production in central-eastern Europe. It has been a serious problem in the United States since 2004. Yearly downy mildew occurrences now threaten cucumber production in more than 80 countries and muskmelon production in over 50 countries, causing significant economic loss (Helenao et al., 2011). In recent years, this disease is accelerated with the variation of pathogen. Alternative splicing of a multi-drug transporter from *Pseudoperonospora cubensis* generates an RXLR effector protein that elicits a rapid cell death (Savory, et al., 2012). *Pseudoperonospora cubensis* has 271 RXLR and RXLR-like effector proteins, which likely function was as virulence or avirulence determinants during the course of host infection. Cohen and Eyal (1987) demonstrated that the wild melon PI 124111F (PI) was highly resistant to all pathotypes of *Pseudoperonospora cubensis*. Taler (2004) found a 45-kD protein (P45) that was not present in the susceptible varieties and sequencing results showed that it was a peroxisomal aminotransferase enzyme (At1 and At2). The sequencing project of 'Chinese long' cucumber inbred line 9930 has been completed in 2009 (Huang S.W. et al., 2009). This sequencing work provides an effective platform for researching important genes resistant to cucumber downy mildew. We analyzed the expression patterns of NBS-LRR and related genes in inbred line 9930. Some candidate resistance genes have been identified in the cucumber genome.

Materials and Methods

Two cucumber inbred lines "M801-1-3" (resistant to downy mildew) and "M302-3" (susceptible to downy mildew) were used in this study. Field experiments were conducted in Harbin during the summer of 2012.

Pathogen was collected from cucumber leaves when downy mildew occurrence in the field and purified on the susceptible inbred line "M302-3". Healthy "M801-1-3" and "M302-3" cucumber seedlings were treated with 1×10^5 sporangium/mL pathogen suspension on the back of true leaves at the two-leaf and one-bud stage. Water was as the control instead of pathogen. Seedlings were covered by plastic bags under the condition of humidity 100%, temperature 22 °C, in light culture chamber. After dark treatment for 24 h, the condition changed to 12h light/12h dark respectively. The materials were collected after 72h inoculation and preserved in liquid nitrogen.

Total RNA was extracted with RNAiso Reagent kit (Takara). Total RNA was digested with 10U Dnase at 37 °C for 1h; then

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purified with Micropoly (A) Purist™ mRNA purification kit (Ambion, USA); RNA was diluted up to 250 μl,according to reagent kit operation steps (Cat. No: 1919). Finally, mRNA was eluted with 100 μl pre-heated buffer, then quantified by NanoDrop.

cDNA synthesis is according to the published method (Ng, 2005). The first strand synthesis of cDNA utilizated GsuI-oligodT as reverse transcription primer, 10 μg mRNA as template, 1000U Superscript II reverse transcriptase (Invitrogen, USA), 42 °C incubation for 1h; subsequently utilize NaIO4 (Sigma, USA) to oxidate mRNA 5-end cap structure, then linked with biotin; mRNA/cDNA. linked to Biotin was selected by Dynal M280 magnetic beads (Invitrogen, USA), alkaline lysis to release the first strand of cDNA; 5-end of the the first strand cDNA was ligated with an adaptor by DNA ligase (TaKaRa, Japan), the second strand of cDNA was synthesized by Ex-Taq polymerase (TaKaRa, Japan). Finally, using *Gsu* I digested to remove the adaptors and polyA from cDNA.

cDNAs were broke into 300–500bp range fregments by ultrasound machine (Fisher), purified by Ampure beads (Agencourt, USA). The cDNAs library were constructed by TruSeq™ DNA sample Prep Kit (illumina, USA), then amplified by Tru-Seq PE Cluster Kit (illumina, USA). Sequencing reaction was performed in illumina machine.

Assembled contigs from sequencing data were performed to gene prediction using 'GetORF' in EMBOSS and obtained the encoding protein sequence. The best matched proteins,compared by blastp in NR, GO, KEGG, KOG databases, were selected as the annotation information. GoPipe (Chen et al., 2010) was performed to GO-analysis. TrEMBL database were performed to sequence assignment. Condition is blastp, E value < 1e-5, obtained the GO information of predicted protein. KEGG database (Kanehisa et al., 2010) were used to sequences assignment, Condition is double-directional blast, E value < 1e-3; obtained the KO number of predicted protein, according to the KO number, obtained the information of predicted protein metabolic pathways. Expression abundance analysis: First obtaining clean reads, then mapping to splicing contig, the number of reads from each contig was counted, then transformed into RPKM (Mortazavi et al, 2008), by utilizing the MAPS (MA-plot-based method with random sampling model) model in DEGseq program package (Wang et al., 2010), the difference of gene expression abundance that each contig represent from two samples were calculated. Significant differences were considered when FDR value < 0.01.

Results and Discussion

Solexa sequencing results showed that the reads numbers (pair) from M302-2 was 11268160. A total of 11101492 reads were valid and accounted for 98.5% of read numbers. The average length of reads was 100 bp. The read numbers (pair) from M801-3-1 was 11218220 in which valid reads were 11078365 and accounted for 98.75% of read numbers. The average length of reads was 100 bp.

Assembly was performed by using Trinity and 41553 EST clusters (contigs) were obtained. The contigs length ranged from 201 to 18678.

Predicted protein-encoding sequences according to the NR, GO, KEGG, and KOG databases using blastp, E value < 1e-5.The best matched protein was selected as the annotation information. Detailed results were showed in the annotation.xls file including contig name, length of contig, gene function annotation and KOG classification.

GoPipe was used to GO-analysis. We obtained 7982 predicted proteins in cucumber which matched with 42980 GO terms. (Figs. 1, 2, and 3)

By using KEGG database, 6256 proteins in cucumber were found to have KO numbers. These proteins participate in multiple metabolic pathways, which including biosynthesis of the secondary metabolites, amino acids metabolism, carbohydrate metabolism, energy metabolism, xenobiotics biodegradation and metabolism, lipid metabolism, glycan biosynthesis and metabolism, metabolism of terpenoids and polyketides, nucleotide metabolism, metabolism of cofactors and vitamins, membrane transport, signaling molecules and interaction, signal transduction,

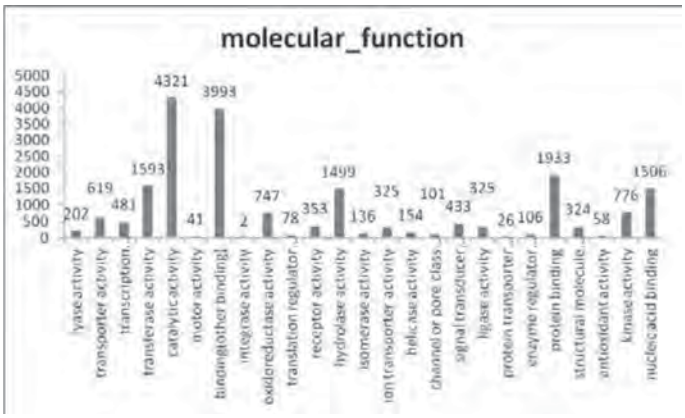

Fig. 1. Cucumber expressed genes molecular function.

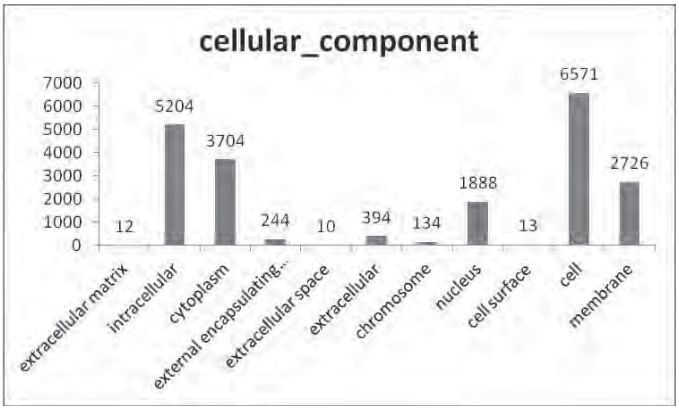

Fig. 2. Cucumber expressed genes in cellular component.

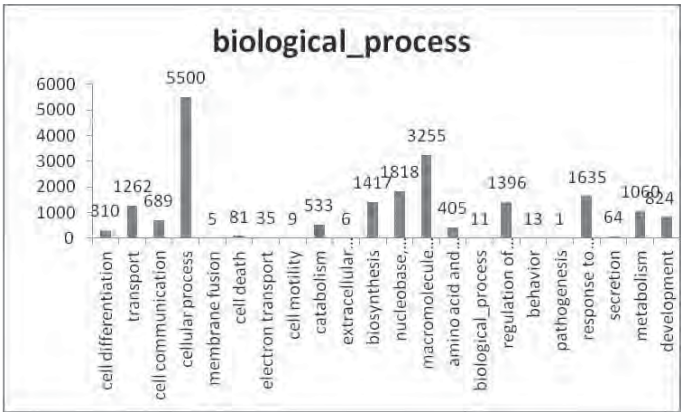

Fig. 3. Cucumber expressed genes involved in biological process.

cell communication, transport and catabolism, cell growth and death, transcription, translation, genetic material replication and repair. The metabolic pathways shown in Figs. 4 and5.

The results showed that 1727 genes highly expressed in susceptible variety M302-3, which encoded ethylene oxidase, ethylene-responsive transcription factor, salicylic acid carboxyl methyltransferases, and WRKY transcription factor. There were 168 proteins founded only in "M302-3", including TIR-NBS-LRR resistance protein, ribosomal composition protein, ethylene synthetase, ubiquitin proteins and effector protein. It was confirmed that three TIR-NBS-LRR class R genes specifically expressed in susceptible varieties, which called susceptible R gene (Timothy, 2010). The discovery of these genes may play an important role in improving the mechanism of cucumber resistance to downy mildew (Jones and Dangl, 2006). The proteins that involved in plant-pathogen interaction metabolic pathways and higher expression in susceptible varieties included: calcium-dependent protein kinase (CDPK), calmodulin (CaM), WRKY, PR1, PBS1, RIN4, MYC2. The high expression genes in susceptible varieties are homologous with those in resistant varieties, but not the same genes. Genes mentioned above may involved in the susceptible performance of susceptible varieties. The expression of ethylene oxidase, ethylene synthetase, ethylene receptor transcription factor, salicylic acid carboxyl methyltransferases were much more higer in susceptible varieties. The expression of ethylene and alicylic acid related genes may be related to the formation of susceptible phenotype. The formation of yellow spots caused by downy mildew in susceptible varieties may be related to the regulation of ethylene and salicylic acid. Antagonism existed between ethylene and salicylic acid. The high expression of auxin related proteins in resistant varieties may reduce the occurrence of disease spots.

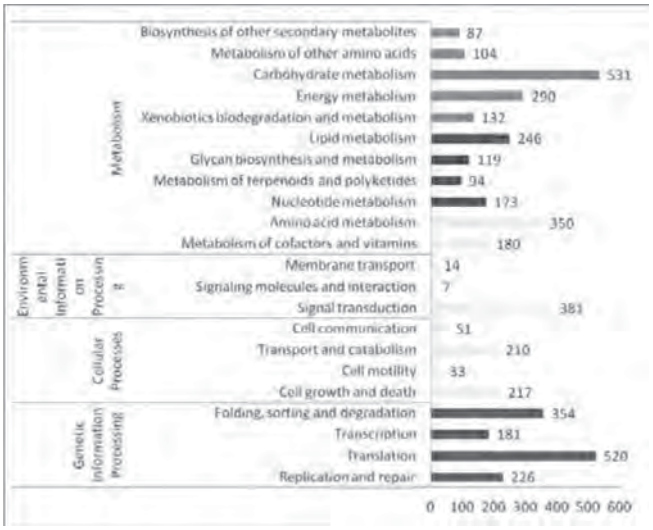

Fig. 4. The metabolic pathways category of which cucumber expression gene encoding proteins involved in after infected with downy mildew.

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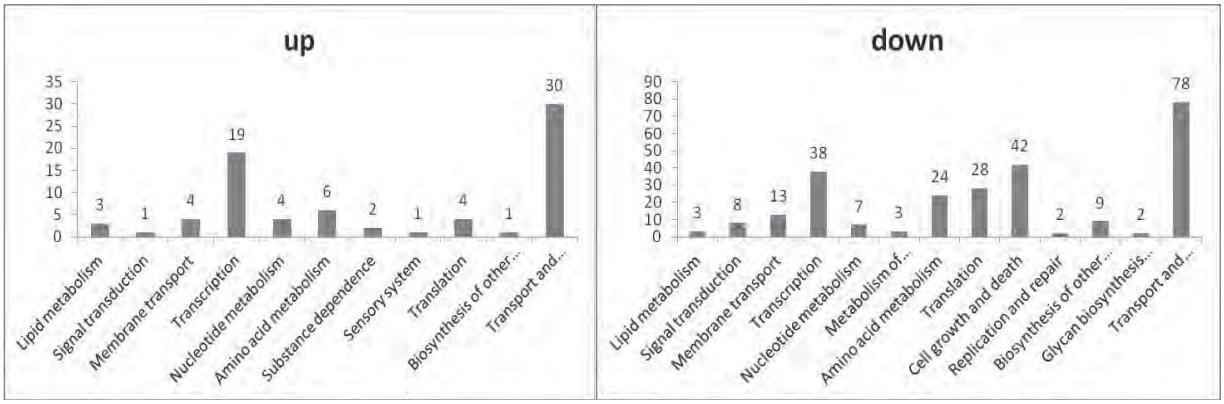

Fig. 5. The changes of Cucumber M302 / M801 up-regulated and down-regulated gene (abscissa for gene, ordinate for statistics)/

Effects of Bacterial Fruit Blotch Pathogen on Treated Melon Seed

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Additional index words. *Acidovorax avenae* subsp. *Citrulli*, melon seed, heat sterilization, coating agent

Abstract. Melon seeds contaminated with the pathogen (*Acidovorax avenae* subsp. *citrulli*) of bacterial fruit blotch (BFB) were treated with 70 °C, 75 °C and 80 °C dry heat, a coating agent (Vitavax) or 70 °C dry heat plus a coating agent. The presence of the pathogen was evaluated using PCR with specific primers (BX-L1/BX-S-R2) against the *A. avenae* subsp. *citrulli* genome. These results showed that the target fragment (279 bp) could be amplified in groups of seeds treated with only dry heat sterilization or the coating agent. However, no positive amplicon was detected in seeds treated with 70°C dry heat plus a coating agent. However, real-time quantitative PCR results indicated that the extent of contamination of BFB pathogens in seeds processed with different treatments decreased in varied degrees. The BFB pathogens could not be detected in seeds treated with 70 °C dry heat sterilization plus coating agent. Furthermore, BFB pathogens could only be isolated from the seeds treated with 70 °C dry heat sterilization. Finally, the seed germination rate and germination potential were slightly decreased after treatment with 75 °C or 80 °C dry heat in the seed germination test but were not affected by the other treatments.

The phytopathogenic bacterium *Acidovorax avenae* subsp. *Citrulli* (*Ac*) (Schaad et al., 1978; Willems et al., 1992) causes bacterial fruit blotch (BFB), a devastating disease of cucurbits (Cheng et al., 2000; Isakeit et al., 1998; Langston et al., 1999). It is well known that *A. avenae* subsp. *Citrulli* is a serious seed-borne pathogen of watermelon (*Citrullus lanatus* (Thunb.) Matsumara and Nakai) and melon (*Cucumis melo* L.) worldwide (Hopkins, 1989; Isakeit et al., 1997; Latin and Hopkins, 1995; Schaad et al., 2003). Seed-borne pathogens represent a serious concern to both melon growers and seed producers, and seed producers are usually under threat of litigation from farmers accusing them of selling pathogen-contaminated seeds. Thus, rapid and accurate methods of detecting the pathogen prior to seed sale and distribution are urgently needed. At present, PCR technology has been commonly used to detect *Ac* (Walcott et al., 2003; Bahar et al., 2008; Tian et al., 2010; Hui et al., 2007). In this study, melon seeds spontaneously infected by the pathogen (*Ac*) were sterilized with 70 °C, 75 °C, or 80 °C dry heat, a coating agent (Vitavax), or 70 °C dry heat plus a coating agent. PCR and real-time quantitative PCR were used to detect and evaluate the extent of contamination of *Ac*, and the presence of *Ac* was further detected by isolation from each seed extract. The effects of each treatment on seed germination and root elongation were finally analyzed. As a result, the best seed treatment protocol was proposed.

Materials and Methods

Melon (*Cucumis melo* L.) seeds of Dongfangmi 1 infected by the pathogen (*Acidovorax avenae* subsp. *citrulli*) of bacterial fruit blotch (BFB) were naturally harvested at Xinjiang province, China, in 2012. The standard strain (*Acidovorax avenae* subsp. *citrulli*) of bacterial fruit blotch pathogen was provided by the Zhengzhou Fruit Research Institute, Chinese Academy of Agriculture Sciences.

Different treatment of naturally infested melon seeds. Naturally infested melon seeds were processed using five different treatments (Table 1).

PCR detection. Three hundred seeds from five different treatments, or a check (CK) control, were placed in sterilized flasks, and 30 mL of sterile deionized water was added to each seed sample. The samples were then shaken at 220 rpm for 3 h at 28 °C. Next, 1 µL of extract from each sample and the specific primers (BX – L1 / BX – S – R2) (O. Bahar et al., 2008) were used in the PCR reaction as templates and primers, respectively. The analysis of each sample was repeated at least three times. The PCR reaction was performed in a total volume of 25 µL, which contained 1U of Taq DNA polymerase, 2.5 µL of 10× Taq DNA polymerase reaction buffer, 2.0 µL of $MgCl_2$ (25 mmol/l), 0.5 µL of dNTPs (10 mmol/l each), 1 µL of each primer (10 µmol/l) and 1 µL of template. Amplifications were performed using a BIO-Rad T100™ Thermal Cycler. The PCR products were subjected to gel electrophoresis at 100 V for 40 min in a 1.2 % agarose gel. The gels were stained with Goldview dye (5 µL Goldview was diluted to 100 ml with 1 × TBE) for 10 min and imaged under UV light.

Real-time PCR detection. Real-time PCR was performed using the Bio-Rad CFX96™ Real-Time PCR System with SYBR-green dye (Takara). The PCR reaction was run in 25-µL reaction volumes according to the instructions provided by Takara SYBR® P*remix Ex Taq*™. The reaction cycles included denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 30 s. Three replicates were performed with each sample. The relative content of *Ac* was calculated using the formula $2^{-\Delta Ct}$ [in which ΔCT = (Ct value of each treatment) – (Ct value of CK)].

Isolation of BFB pathogens. The seed extract of each treatment was incubated on KB agar medium and semi-selective KB agar

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Table 1. Different treatments of the naturally infested melon seeds

Number	Treatment
CK	Naturally infested melon seeds
NO.1	40 °C for 2 d, 50 °C for 1 d, 70 °C for 3 d, 50 °C for 1 d, 40 °C for 1 d
NO.2	40 °C for 2 d, 50 °C for 1 d, 75 °C for 3 d, 50 °C for 1d, 40 °C for 1 d
NO.3	40 °C for 2 d, 50 °C for 1 d, 80 °C for 3 d, 50 °C for 1d, 40 °C for 1 d
NO.4	Coating treatment (coating agent: Vitavax; active ingredient: carboxin 200 g/L, thiram 200 g/L)
NO.5	Treatment of NO.1 + treatment of NO.4

medium (containing ampicillin 20 mg/L, novobiocin 5 mg/L, actidione 25 mg/L) at 28 °C for 48 h. At least three plates of each treatment were incubated.

Acidovorax avenae subsp. *citrulli* was grown in KB liquid medium on a shaker for 48 h at 28 °C. Cells were centrifuged and washed using sterile distilled water, and the suspension was adjusted to three 10-fold dilutions ($2 \times 10^4 - 2 \times 10^2$ CFU/mL) according to the OD_{600} value. Next, 100 µL of each dilution was plated onto three replicates of KB agar medium (as a control medium) and semi-selective KB agar medium to observe the effects of semi-selective KB agar medium in response to *Ac*.

Total bacterial colony DNA extraction and PCR detection. Total bacterial colonies grew on semi-selective KB medium and were washed with sterile water. Next, the suspension was centrifuged, and the bacteria were collected for genomic DNA extraction. The DNA was used for PCR amplification.

Seed germination test. One hundred melon seeds from each treatment were incubated at 28 °C for 24 h. The germination rates and root length were applied to the statistical analysis. Three replicates of each analysis were performed.

Results and Discussion

PCR and real-time PCR detection. The results of PCR detection (Fig. 1) showed that the target band (279 bp) could be detected in the group of infected seeds treated with all four of the treatments (NO.1, NO.2, NO.3, and NO.4, according to Table 1) and the untreated check (CK). In contrast, no positive band was detected in the seeds treated with treatment NO.5.

In addition, the relative extent of *Ac* contamination in the melon seeds was also evaluated using real-time quantitative PCR. In contrast to untreated CK seeds, the relative content of *Ac* in seeds treated with treatments NO.1, NO.2, NO.3, and NO.4 decreased to various extents, while the presence of *Ac* in seeds treated with treatment NO.5 could hardly be detected (Fig. 2). This result is consistent with the results displayed in Fig. 1.

Isolation of the BFB pathogens. After 48 h of incubation on KB agar medium at 28 °C, bacterial colonies containing a large population of non-target bacteria from the seed extracts of CK and each treatment appeared. CK had the highest number of bacterial colonies, and this number decreased in varied degrees in the five treatment conditions (Fig. 3A). However, due to the existence of a large amount of other non-target bacteria, which strongly affected the DNA extraction and PCR detection of *Ac*, semi-selective KB agar medium was employed. The results (Fig. 3B) showed that after incubation on semi-selective KB agar medium for 48 h, no colony appeared except for the CK and treatment NO.1 samples. Next, the DNA of the total colonies of CK and treatment NO.1 was extracted, and the target band (279 bp) was detected by PCR amplification using the BX - L1 / BX - S - R2 primers (Fig. 4), although the presence of other species of bacteria spontaneously grown on the semi-selective KB could not be thoroughly inhibited.

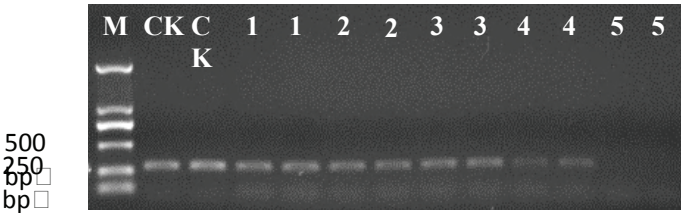

Fig. 1 PCR detection of infested melon seeds treated with five different treatments. Note: M, D2000 ladder; 1–5 represents treatments NO.1–NO.5, respectively.

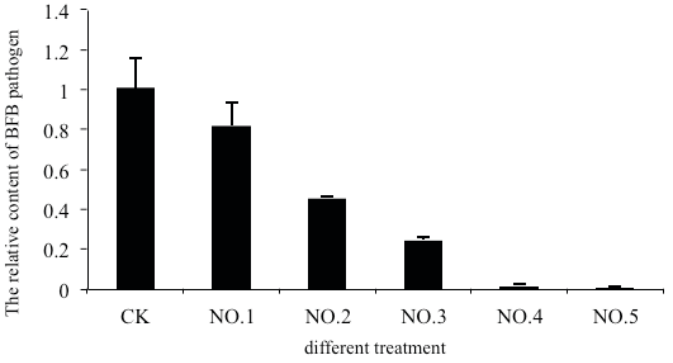

Fig. 2 Real-time quantitative PCR results of infested melon seeds treated using five different treatments

Next, 100 µL of three 10-fold dilutions were plated onto KB agar medium (as a control medium) and semi-selective KB agar medium. As shown in Fig. 5, the *Ac* colonies grown on KB agar medium and semi-selective KB agar medium demonstrated the same growth rate, colony size, and numbers. These results suggested that semi-selective KB agar medium had no effect on the growth of *Ac*.

Seed germination test. The seed germination test (Table 2) indicated that the seeds treated with treatment NO.3 showed the lowest germination rate (87%); however, the seeds from the other treatments and CK all showed germination rates higher than 90%. In addition, treatments NO.2 and NO.3 both significantly attenuated root elongation during seed germination, while treatment NO.5 had little effect on root elongation, and treatments NO.1 and NO.4 resulted in an even longer root length compared to CK.

Conclusion

Taken together, our results demonstrate that treatment NO.5 (70 °C dry heat plus coating agent) most effectively reduced the contamination rate of *Acidovorax avenae* subsp. *citrulli*, and the treated seeds maintained standard seed germination and root

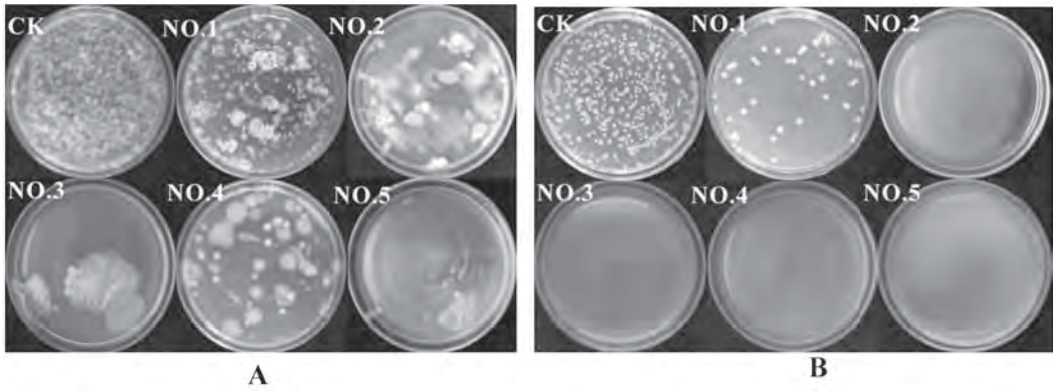

Fig. 3 Seed extracts from the different treatment groups incubated on KB agar medium (**A**) and semi-selective KB agar medium (**B**) for 48 h.

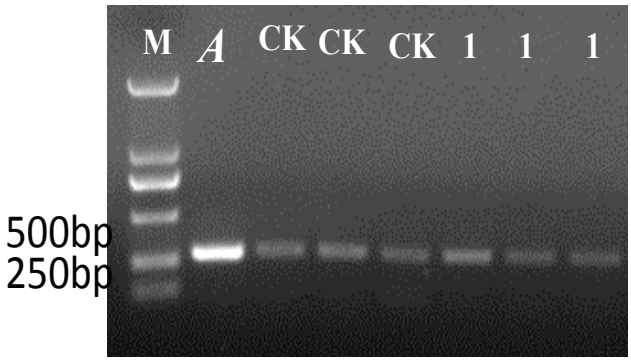

Fig. 4 PCR detection of *Acidovorax avenae* subsp *citrulli* isolated from the melon seeds of CK and treatment NO.1. M, D2000 ladder; *Ac*, *Acidovorax avenae* subsp. *citrulli* strain; 1, NO.1 treatment.

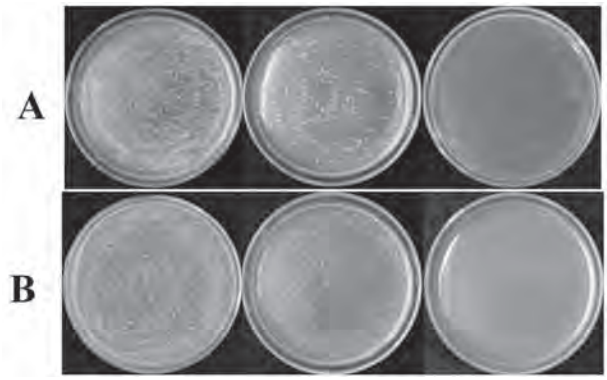

Fig. 5 Three 10-fold dilutions (2×10^4 – 2×10^2 CFU/mL) of *Acidovorxs avenae* subsp. *citrulli* incubated on semi-selective KB agar medium (**A**) and KB agar medium (**B**) for 48 h.

Table 2. Effect of different treatments on seed germination

Treatment	Total No. (grain)	Germination No. (grain)	Germination rate (%)	Average root length (mm)
CK	100	97	97	7.69 ± 2.27^{c}
NO. 1	100	96	97	8.76 ± 2.24^{d}
NO. 2	100	91	91	3.86 ± 1.16^{b}
NO. 3	100	87	87	2.12 ± 0.60^{a}
NO. 4	100	95	95	9.79 ± 2.55^{e}
NO. 5	100	94	94	7.18 ± 2.15^{c}

Values within a column followed by a different letter are significantly different ($P < 0.05$) based on Duncan's multiple range test.

length, suggesting that this method is most preferable for melon seed treatment against bacterial fruit blotch contamination prior to marketing and planting.

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Cucumber Mosaic Virus Resistance in Melon: Learning Lessons from the Virus

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Additional index words. Recessive resistance, *cmv1*, virus movement, movement protein, phloem transport

Abstract. *Cucumber Mosaic Virus* (CMV) infections can cause complete harvest loss in more than 1000 species. Resistance to CMV in melon is scarce. One of the two resistant genotypes reported is the exotic accession Sonwang Charmi, PI161375 (SC) that shows a complex mixture of qualitative and quantitative resistance, depending on the virus strain. Based on phylogenetic studies, CMV strains have been classified in two major subgroups (I and II) sharing about 75% nucleotide identity. *Cmv1* is a recessive gene present in SC that confers total resistance against some strains, but not to others, mainly of subgroup I. The identity of *cmv1* is still unknown, but we can infer its function by studying the behavior of the virus in the resistant plant. Both sets of strains, responsive and non-responsive to *cmv1*, must be different in their virulence determinant. Studies on RNA re-assortment and chimeric constructs between CMV-FNY (subgroup I) and CMV-LS (subgroup II) have showed that the virulence determinant that governs the response to *cmv1* is the Movement Protein (MP). Further studies have revealed that the strain LS is able to replicate and move cell-to-cell in the *cmv1* resistant line, but is not able to enter the phloem. Therefore, the fate of the virus in the resistant line indicates that *cmv1* is a host factor involved in the transport of the virus into the phloem.

Cucumber mosaic virus is able to infect more than 1200 species worldwide from over 100 families (Edwardson and Christie, 1991) and has evolved to develop a vast genetic diversity (Palukaitis and Garcia-Arenal, 2003). This diversity can be grouped in two subgroups to which all CMV isolates belong, subgroup I and II, defined by their serological and biological properties. Genetically, CMV has three genomic and two sub genomic RNAs. The RNA1 encodes for the 1a, a protein with two domains, one methyltransferase and one helicase. RNA 2 encodes for the 2a, an RNA-dependent RNA polymerase, that together with the 1a are involved in the replication of the viral RNA. RNA2 also encodes the 2b, a suppressor of RNA silencing that is also involved in long distance movement. RNA 3 is bi-cistronic and encodes for the 3a, the Movement Protein (MP), and the 3b, the Coat Protein (CP) (Palukaitis and Garcia-Arenal, 2003). All CMV proteins have been found as determinants of virulence in different systems (Mochizuki and Ohki, 2011).

There are few sources of resistance in melon reported until now. One of these sources is the Korean accession 'Sonwang Charmi' PI161375 (SC), which is one of the parental lines of our populations. It shows a resistance recessive, oligogenic (Karchi et al., 1975) and quantitative (Dogimont et al., 2000). We addressed the study of this resistance and have defined one gene, *cmv1*, able to produce total resistance against some CMV strains (Essafi et al., 2009) and at least three QTLs, *cmqw3.1*, *cmqw10.1* and *cmqw12.1,* that need to act together to produce total resistance against a second set of CMV strains. Furthermore, *cmqw12.1*co-locates with *cmv1*, indicating that for this second set of strains, *cmv1* is necessary but not sufficient (Guiu-Aragonés et al., 2014). We have addressed the map-based cloning of *cmv1* from a F_2 population between SC and 'Piel de Sapo' (PS) and, after screening more than 3000 individuals, we have reached an interval of 135Kb were, according to the melon genome, reside three genes (Giner et al, unpublished results). We are currently working on the identification of *cmv1* by genetic and molecular methods. However, we have been analyzing the virus itself and the differences between both sets of strains, the ones that overcome the resistance provided by *cmv1* and the ones that are unable to do it. We have defined the Movement Protein (MP) of the virus as the determinant of virulence that allows some strains to infect systemically the *cmv1*-encoding plant. Additionally, we have found that the resistance mediated by *cmv1* is able to block the viral life cycle at the level of phloem entry. Therefore, the virus has revealed that the mechanism underlying *cmv1*-mediated resistance must be related to impairing the transport of the virus into the phloem to produce a systemic infection.

Materials and Methods

Plant and virus materials. Genotypes of *Cucumis melo* used for the study of the resistance to CMV were: the Korean accession PI161375 cultivar 'Sonwang Charmi' (SC) and the Spanish cultivar PS (Piel de Sapo) as resistant and susceptible controls, respectively. The near isogenic line (NIL) SC12-1-99 was derived

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from the NIL SC12-1 (Essafi et al., 2009), carrying a shorter introgression of SC on the linkage group XII that contains *cmv1* gene. For the inoculation experiments, seeds were pre-germinated and the plants were grown as previously indicated (Guiu-Aragonés et al., 2014). Viruses used in this study were the infectious clones of CMV-LS, belonging to subgroup II, and CMV-FNY, belonging to subgroup I (Rizzo and Palukaitis, 1990; Zhang et al., 1994).

Inoculations. Viral inocula were freshly prepared from infected zucchini squash and inoculated as previously reported (Essafi et al., 2009).

Infectious RNAs of the pseudo-recombinants and chimeric viruses were inoculated to *Nicotiana benthamiana*. RNAs were generated from 1µg of the linearized infectious cDNA clones by in vitro transcription using T7 RNA polymerase (Roche Diagnostics, Germany) and Cap Analog (Roche Diagnostics, Germany) according to manufacturer's protocols. The three transcribed RNAs, without further purification, were rub-inoculated together onto *Nicotiana benthamiana* producing a systemic infection. Sap produced from infected leaves was used to inoculate melon cotyledons or first leaf. Symptoms in melon plants were scored visually 20 days after inoculation.

Viral detection. Viral detection was performed by Reverse Transcriptase (RT)- Polymerase Chain Reaction (PCR) or by Northern blot hybridization. RT-PCR was performed from young newly developed leaves as described (Essafi et al., 2009). Combinations of primers from both strains, CMV-LS and CMV-FNY, were used to confirm the infection when necessary. Northern blot hybridization was performed for tissue printings and detection of virus in the phloem. For tissue printings, freshly cut foliar discs were cut and printed onto positively charged nylon membranes (Roche Diagnostics, Barcelona, Spain) according to (Mas and Pallás, 1995). For phloem detection, petiole and stems were freshly cut with a razor blade and, immediately after, the cross-sections were blotted onto positively charged nylon membrane. Hybridization was done using an "in vitro" transcribed digoxygenin-11-UTP- labeled RNA probe synthesized from a construct containing partial sequences of CMV-LS and CMV-FNY coat protein genes. In vitro transcription of the probe was performed using SP6 RNA polymerase (Amersham Pharmacia Biotech, little Chalfont, U.K).

Results

The MP is the determinant of virulence against cmv1. CMV has the ability of combine or recombine in nature RNAs of different strains and be viable (Palukaitis and Garcia-Arenal, 2003). We have used this property to combine the molecular clones of the strains LS and FNY. In vitro transcribed independent RNAs of each strain were combined to produce all possible pseudo-recombinants for ulterior inoculation into melon plants. The only combination that resulted virulent in the plant SC12-1-99, resistant to CMV-LS, carried RNA3 from FNY and RNA1 and 2 from LS. The other combinations were infectious in PS and none was in SC. Therefore, the determinant that confers virulence to CMV- LS in the presence of the gene *cmv1* was encoded by RNA3. Then, chimaeras exchanging either the three untranslated regions (UTRs) independently or the two open reading frames, MP or CP genes, were generated and inoculated into SC12-1-99 plants. As shown in Fig. 1, the only chimaera producing a virulent phenotype carried the MP of FNY into the background of CMV-LS (clone L3(MPFNY)). The infection was confirmed by RT-PCR using specific primers to detect the recombinant chimaera.

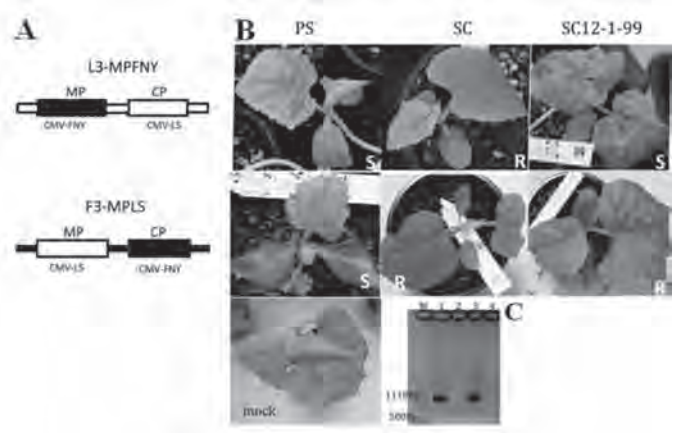

Fig. 1. The MP determines the virulence in the *cmv1* plant. **A)** Schematic representation of the clones where the MP has been exchanged between CMV-LS and CMV-FNY. **B**) Pictures of virus symptoms of F3-MPLS and L3-MPFNY at 16dpi in *C.melo* genotypes: Piel de Sapo (PS), Sonwang Charmi (SC) and SC12-1-99 (*cmv1*). Plants were systemically resistant (R) or susceptible (S). Mock: Mock-infected PS plant. **C)** Chimaeric viruses were detected by RT-PCR. Lanes 1,2,3: PS, SC and SC12-1-99 inoculated with L3-MPFNY; lane 4: mock-inoculated plant; M: pUC Mix Marker 8 (Fermentas).

Given that the viral element interacting directly or indirectly with *cmv1* is the MP, the protein involved in both cell-to-cell and long distance movement (Jacquemond, 2012), this result indicate that the gene *cmv1* is involved in controlling the movement of the virus. The MP of LS might not be able to interact with the product of the resistance gene *cmv1*, and only would be able to interact with the product of *CMV1*, the PS allele, to be transported. On the other hand, the MP of FNY would be able to interact with both of them, being able to produce a systemic infection in both, PS and the resistant line SC12-1-99.

The resistance mediated by cmv1 is manifested by impairment of phloem entry. To characterize the resistance provided by the gene *cmv1*, and given that the determinant of virulence was de MP, we have investigated if CMV-LS was able to move cell-to-cell. We inoculated the first melon leaf of susceptible and resistant plants and, at 3 dpi we inspected for local spread of the virus by tissue printing of leaf discs. As shown in Fig. 2A, CMV-LS can be detected both in the susceptible PS line and in the resistant NIL SC12-1-99. The virus was located mainly in the veins of both lines indicating that it is able to move cell-to-cell until the veins independently of the presence of the gene *cmv1* in the line SC12-1-99. Therefore, this result indicates that the resistance is not acting at the level of cell-to-cell movement and that *cmv1* is not involved in this process.

Despite moving and accumulating in the veins of the inoculated leaf, the virus did never produce a systemic infection in the resistant plant. To know if the virus was able to enter the phloem or remained in some cell type of the vein, the first leaf of PS and SC12-1-99 plants was inoculated with CMV-LS and after 12 dpi the presence of the virus in the phloem was inspected by blotting sections of the petiole of the inoculated leaf and stem in a membrane. Northern blot hybridization showed that whereas CMV-LS was detected in all sections of the PS plant, it was never detected in the resistant SC12-1-99 plant, even in the petiole of the inoculated leaf. Therefore, the virus was not invading the sieve elements of the phloem to be transported and establish a systemic infection. This result indicated that the PS allele *CMV1*

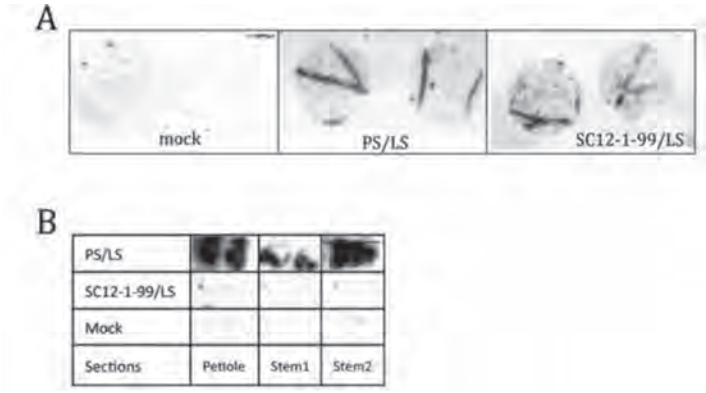

Fig. 2. Characterization of CMV-LS movement in Piel de Sapo (PS) and SC12-1-99 (*cmv1*). **A**) Tissue printing analysis of the localization of CMV-LS in discs of inoculated leaf. First true leaves were inoculated with CMV-LS of Piel de Sapo (PS) and SC12-1-99. Samples were taken at 3dpi. R: systemically resistant. S: susceptible. **B**) Virus detection of CMV-LS in sections of PS and SC12-1-99 in the petiole of the inoculated leaf and in two sections of the main stem. Mock: mock- inoculated plant.

is necessary in some cell type of the phloem tissue to transport the virus to the sieve elements and only when the resistant allele *cmv1* is present in homozygosity, the virus cannot enter the phloem and establish a systemic infection. It remains to be established the cell type of the phloem where CMV1 is necessary and what mechanism it is impairing, if the intracellular travel towards the plasmodesmata (PD), or it is needed in some particular PDs to open the size exclusion limit and allow the virus to enter the sieve elements.

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Methanolic Extracts of Cucumber Fruit Peel Inhibit Growth of *Phytophthora capsici*

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Abstract. *Phytophthora capsici* is a soil-borne, oomycete plant pathogen that infects many cucurbit crops. In cucumber (*Cucumis sativus*), an age-related resistance (ARR) to *P. capsici* was previously observed in select cultivars. Young fruits are highly susceptible to infection but become resistant as they reach full size at approximately 12-16 days post pollination (dpp). Our previous studies indicated that cucumber fruit surface was associated with resistance, suggesting possible physical or chemical components of resistance. A preliminary assay showed that methanolic peel extracts from 16 dpp fruits had an inhibiting effect on *P. capsici* growth in vitro. To further test the inhibitory effects of fruit peel extracts on pathogen growth, a high-throughput screening bioassay was developed. Cucumber peels were sequentially extracted in water and methanol, lyophilized, and re-dissolved in water or 10% methanol. Centrifuged V8 Media was loaded into a 96-well plate, inoculated with 1 x 10^5 *P. capsici* zoospores per milliliter, and treated with peel extracts at 1 µg/µl. Inhibition of mycelial growth was observed in wells treated with methanolic extracts, but not with aqueous extracts. Expression analyses of flavonoid synthesis-related genes showed specific up-regulation of the flavonol branch-point enzyme, flavonol synthase (FLS) in peels of 16 dpp fruit that express ARR to *P. capsici*.

The oomycete pathogen, *Phytophthora capsici,* infects a broad range of crop species including several members of the *Cucurbitaceae* family (Babadoost, 2004). Many cucumber growing areas are contaminated with *P. capsici* resulting in loss of productive land and rejection of loads of harvested cucumbers (Hausbeck and Lamour 2004). In cucumber*, P. capsici* primarily causes fruit rot. Studies of the cucumber - *P. capsici* pathosystem showed that cucumber fruit exhibit an age-related resistance (ARR) to *P. capsici* (Gevens et al., 2006; Ando et al., 2009). Fruits that completed the period of rapid fruit elongation [approximately 10-12 days post pollination (dpp)] became less susceptible to the pathogen. The underlying basis for ARR is not fully understood, and study of ARR in different pathosystems suggests possible involvement of a variety of mechanisms (Develey-Reviere and Galiana, 2007).

Examination of *P. capsici* zoospore germination on the surface of cucumber fruit harvested at 8 and 16 dpp showed short germ tubes and a higher number of appresoria on 8 dpp fruit and more frequent occurrence of long or aberrant germ tubes on 16 dpp fruit (Ando, 2009). Long germ tubes and aberrant appressoria-like structure have been associated with inability of the pathogen to infect the host plant (Grenville-Briggs, 2008). When peels of 15 dpp fruit were placed on top of 8 dpp intact fruit and inoculated with *P. capsici*, the 15 dpp fruit surface section showed resistance similar to intact 15 dpp fruit and protected the underlying 8 dpp fruit from infection (Ando, 2009). These results suggest that the fruit surface has properties that inhibit *P. capsici* growth.

The physiological basis of resistance of plants to various pathogens has been associated with both pre-formed and infection-induced antimicrobial compounds (Hammerschmidt, 1999; Mert-Türk, 2002). Developmental regulation of the biosynthesis of such compounds may play a role in ARR to *P. capsici* in cucumber. Several studies have demonstrated antimicrobial activity of biochemical compounds in cucumber, including methanol-soluble C-glycosyl flavonoid phytoalexins (McNally et al., 2003a,b; Fofana et al., 2005) and glycoside-linked phenolic compounds from cucumber leaves (Lin et al., 2009), sphingolipids isolated from crude methanol extract of cucumber stems (Tang et al., 2010), and antimicrobial volatiles from cucumber fruit (Sotiroudis et al., 2010).

Initial petri-dish bioassay of crude methanolic extracts from cucumber peels showed inhibition of growth of *P. capsici* (Grumet et al., 2014). Examination of cucumber fruit peel transcriptomes showed that putative homologs of key flavonoid biosynthesis genes, *phenylalanine amonia-lyase* (*PAL*), *flavonol synthase* (*FLS*), *flavanone-3-hydroxylase* (*F3H*) and *flavonoid 3'-monooxygenase (also flavanoid-3'-hyrdroxylase, F3'H)* were nearly exclusively expressed in fruit peel, and were more highly expressed in peels from 16 dpp than 8 dpp fruit (Grumet et al., 2014). In addition to the cucumber examples cited above, flavonoid production and increased flavonoid biosynthesis gene expression have been associated with resistance to pathogens in several plant species such as avocado (Ardi et al., 1998), chickpea (Cho et al., 2005), soybean (Cheng et al., 2009) apple (Slatnar et al., 2010) and grape (Ali et al., 2011).

The objectives of this study were to evaluate the effect of aqueous and methanolic extracts from 8 dpp and 16 dpp cucumber fruit peel on *P. capsici* growth, and to develop a microtiter plate bioassay method for more efficient and quantitative analysis. We also examined the expression of selected flavonoid biosynthesis-

Acknowledgments. This work was in part supported by the Agriculture Research Fund, Pickle Packers International and the USDA National Institute of Food and Agriculture Special Research Grant No. 2008-34572-19339 (*Phytophthora* Research, MI). We thank Dr. Mary Hausbeck for providing *P. capsici* isolate OP97 and Dr. Chris Smart (Cornell University) for fluorescent strains of isolate NY0664-1. We thank Dr. Muralee Nair and his laboratory for assistance with the extraction methods and the G. Sundin and S-Y. He labs for use of the spectrophometric plate readers.

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related genes in 8 dpp and 16 dpp cucumber fruit peel and in ARR-expressing and non-ARR expressing cucumber cultigens.

Materials and Methods

Preparation of peel extracts and pathogen. Pickling cucumber plants cv. 'Vlaspik' (ARR expressing; ARR+) and breeding line 'Gy14' (non-ARR expressing; ARR-) were grown in the greenhouse as described by Ando et al. (2010). Hand pollinations were performed sequentially to allow for simultaneous harvest of fruit at 8 and 16 dpp. To avoid the effects of interfruit competition, only one fruit per plant was allowed to develop. Fruit exocarp (1-2mm thick) was collected from the middle section of each fruit by razor blade. Frozen peel samples from fruits of the same developmental stage were pooled and used immediately for sequential extraction with water followed by methanol (Fig. 1) based on the procedure by Jayaprakasam et al. (2003). Each extract was concentrated by rotary evaporation (BUCHI Rotavapor, BUCHI, Corp., Newcastle, DE) and freeze-dried using Genesis Pilot Freeze Dryer (SP Scientific Industries, Stoneridge, NY). Zoospore suspensions were prepared from 7-day old cultures of *P. capsici* isolate OP97 (Gevens et al., 2006) or NY0664-1 expressing either GFP or RFP (Dunn et al., 2013) grown on diluted V8 media and flooded with 6–10 ml sterile distilled water to release zoospores as described by Gevens et al. (2006).

Bioassay of peel extracts. The aqueous and methanolic extracts were redissolved in water and 10% methanol, respectively, to a final concentration of 25 µg/ul. A 96-well clear (Thermo Fischer Scientific Inc., Waltham MA) or black microtiter plate (Griener Bio-One, Orlando, FL) was prepared with 200 µl clarified V8 media (centrifuged at 10,000 rpm for 10 min) per well. Samples were treated with 10 µl crude extract solution or solvent controls, and inoculated with 20 µl of 1 x 10^5 zoospores/ml suspension of either *P. capsici* isolate OP97, NY0664-1G (GFP) or NY0664-1R (RFP) and incubated at 25 °C with a 16h light/ 8h dark cycle for 72 hours. Visual ranking was performed on a 1-5 scale as illustrated in Fig. 2C. Fluorescence values were measured at 485nm (excitation) and 530nm (emission) for NY0664-1G (GFP) and at 530nm (excitation) and 590nm (emission) for NY0664-1R (RFP) using SpectraMax M2e (Molecular Devices, Sunnyville, CA) at 0, 24, 48 and 72 h post-inoculation. Mean fluorescence measurements from the control (media with aqueous/methanolic extracts) were subtracted from the mean fluorescence values for the corresponding treatments. Each experiment was repeated two or three times with five replicate samples per treatment.

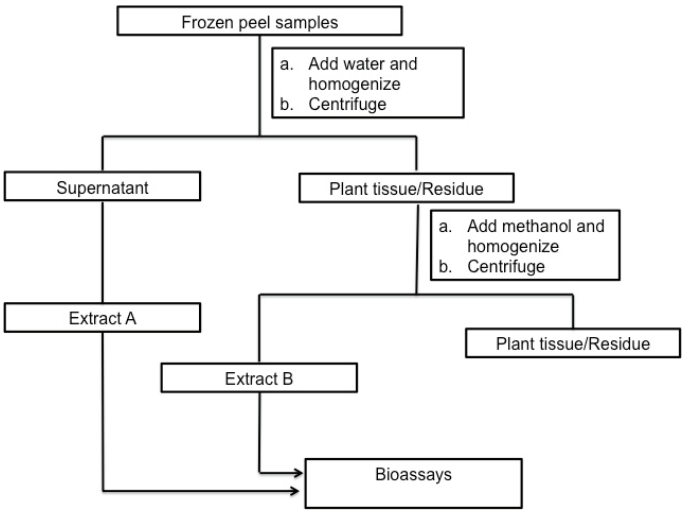

Fig. 1. Schematic of extraction protocol for cucumber peel samples.

Expression analysis of flavonoid synthesis pathway genes. Expression of genes in the flavonoid synthesis pathway was tested using RT-PCR analysis. 'Vlaspik' and 'Gy14' fruits were grown as described above. For each genotype and age, three biological replicates were prepared; each replicate included peel tissue pooled from three fruits. Fruits were harvested, immediately peeled, and pericarp and peel samples were frozen in liquid nitrogen and stored at –80 °C for total RNA extraction. RNA preparation was as per Ando et al. (2012) and samples were loaded in a 384-well plate for analysis with an ABI Prism 7900HT (Life Life Technologies, Inc., Gaithersburg, MD) using rEVAlution Master Mix (Syzygy Biotech, Grand Rapids, MI). Primers for flavonoid synthesis pathway gene were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Product specificity and reaction efficiencies were verified for each primer pair. A standard curve dilution series (20, 4, 0.8 and 0.16 ng/µl) was made from a pool of 2 µl of each of the cDNA samples. PCR conditions were as described in Ando et al. (2012). Threshold Cycle (Ct) levels were translated to cDNA concentration using the relevant standard curve for each gene and normalized to the expression level of *C. sativus Ubiquitin 3* (*CsUBQ3*). Statistical analyses were performed on log 2 transformed data.

Results and Discussion

A microtiter plate assay was developed to provide a replicable, quantitative method to test effects of cucumber fruit peel extracts on growth of *P. capsici* (Fig. 2A). Aqueous and methanolic extracts from fruit peels of susceptible (8dpp) and resistant (16dpp) age cucumber, cv. 'Vlaspik' (ARR+) and breeding line 'Gy14' (ARR-), were evaluated against three isolates of *P. capsici* (OP97, NY0664-1RFP, and NY0664-1GFP). For all isolates tested, growth of *P. capsici* was greatly inhibited in wells with methanolic extracts as evidenced visual pathogen growth or fluorescence assay at 48-72 hours post-inoculation (hpi) (Fig. 2). Wells treated with aqueous extracts were comparable to the controls and were characterized by cottony and powdery appearance indicating extensive mycelial growth (Fig. 2A–C). Though methanolic extracts from 8dpp and 16dpp peels were both inhibitory, 16dpp peel extracts showed greater inhibition, than 8dpp peel extracts (Fig. 2B). There was not an obvious difference in growth inhibition between extracts from the ARR+ and ARR- genotypes (Fig. 2B, D).

Antimicrobial effects of methanolic extracts from cucumber also have been observed from cucumber leaves and stems. Epidermal tissue of cucumber leaves showed induced resistance to powdery mildew due to production of methanol soluble C-glycosyl flavonoid phytoalexins (McNally et al. 2003a,b), and inhibitory glycoside-linked phenolic compounds were found to accumulate in cucumber leaf cells beneath penetrating appressoria of *Colletotrichum orbiculare* (Lin et al., 2009). Sphingolipids isolated from crude methanol extract of cucumber stems exhibited antifungal activity and antibacterial activity (Tang et al., 2010).

Previous work in our laboratory, comparing fruit peel and pericarp tissue in cv. 'Vlaspik', revealed that several genes in the flavonoid synthesis pathway were specifically up-regulated in peel tissue of 16 dpp fruit (Grumet et al., 2014). Consequently, we compared expression of several flavonoid biosynthetic genes in the peels of 'Vlaspik' and 'Gy14'. Expression of *F3H* and *F3'H* was similar (less than two-fold different) in all samples tested,

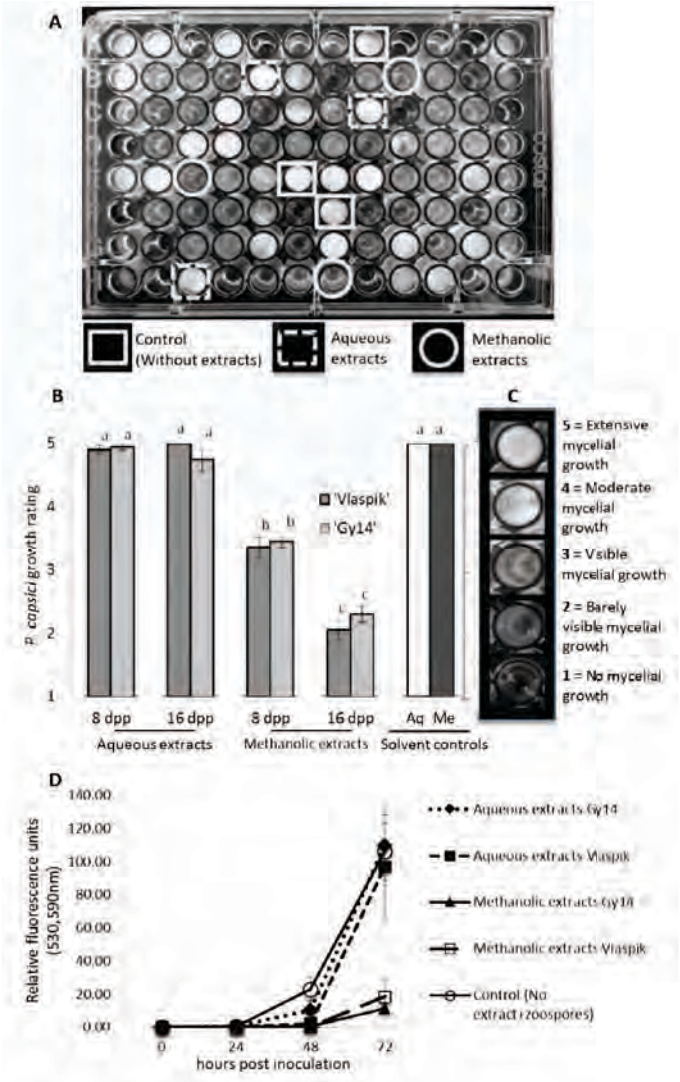

Fig. 2. Effect of aqueous and methanolic extracts from cucumber fruit peel on growth of *P. capsici* in vitro. (**A**) Photograph illustrating microtiter plate assay of *P. capsici* growth response to fruit peel extracts. (**B**) Effect of 8 dpp and 16 dpp fruit peel extracts of 'Vlaspik' and 'Gy14' on growth of isolate OP97. (**C**) Visual rating scale for *P. capsici* growth. (**D**) Effect of 8 dpp and 16 dpp fruit peel extracts of 'Vlaspik' and 'Gy14' on growth of isolate NY0664-1RFP. Each value is the mean of 4-5 replicate samples ± S.E. Bars marked with different letters are significantly different (LSD, $P < 0.05$). Each experiment was performed twice with equivalent results.

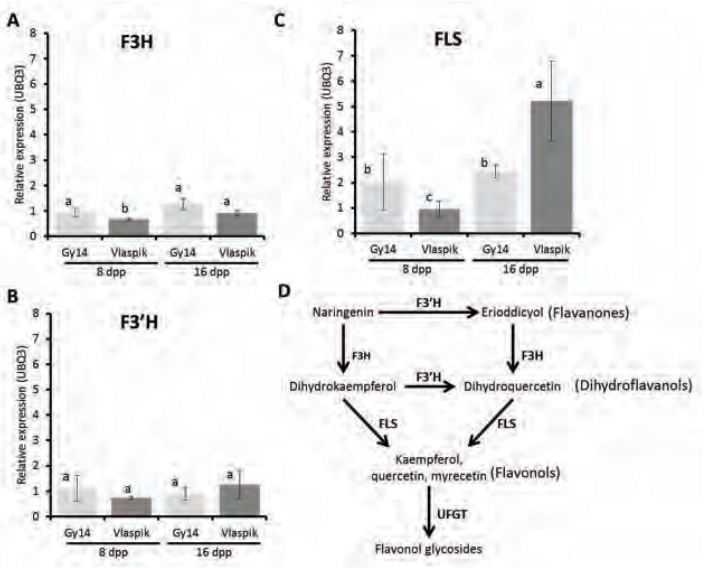

Fig. 3. Expression of flavonoid synthesis genes *F3H* (*flavanone-3-hydroxylase*) (**A**), F3'H (*flavonoid-3'-hyrdroxylase* also *flavonoid 3'-monooxygenase*) (**B**), and *FLS flavonol synthase* (**C**) in peels from 'Gy14' and 'Vlaspik' fruit at 8 and 16 days post pollination (dpp). Each value is the mean ± S.E. of three biological replicates with three technical replicates/biological replicate. Values marked by different letters are significantly different, LSD, $P < 0.05$. (**D**) Flavonoid biosynthesis pathway adapted from Winkel-Shirley (2001).

regardless of fruit genotype and age (Fig. 3A, B). However, the synthetic branch-point enzyme for flavonol glycosides, *FLS,* displayed five-fold greater expression in the peels of 16-day old 'Vlaspik' fruit than 8 dpp fruit (Fig. 3C, D). There were not significant differences in expression levels between peels of 'Gy14' 8 and 16 dpp fruit; levels in 'Gy14' were 2.5-fold lower than those of 'Vlaspik' 16 dpp peels.

Whether increased expression of *FLS* in 16 dpp peel of Vlaspik is a contributing factor to ARR remains to be determined. Flavonoid biosynthetic genes have been associated with resistance in other pathosystems. Expression analysis of flavonoid biosynthesis enzyme-encoding genes *Phenylalanine ammonia lyase* (*PAL)* and *F3H* in resistant avocado lines showed that high gene expression resulted in accumulation of epicatechin preventing *Colletotrichum gloeosporioides* to cause fruit decay (Ardi et al., 1998). High gene expression of *F3H* homolog in chickpea recombinant inbred lines was associated with resistance against *Ascochyta rabiei* (Cho et al., 2005) and linkage mapping in soybean supported the role of *F3H* in resistance to soybean mosaic virus (Cheng et al., 2009). Induction of *FLS* expression was observed in apple infected with *Erwinia amylovora* (Venisse et al., 2002) and *Venturia inaequalis* (Slatnar et al., 2010) and higher expression was observed in a variety of grape resistant to multiple pathogens (Ali et al., 2011)

In conclusion these results provide evidence that cucumber fruit peels are capable of producing methanol-soluble substances that can inhibit growth of *P. capsici* in vitro. While precedence from other cucumber tissues indicates that cucumber plants are capable of producing methanol-soluble antimicrobial flavonoid, phenolic and sphingolipid compounds, the chemical identity of the *P. capsici* inhibitory compound(s), remains to be determined.

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QTLs for Downy Mildew Resistance and Their Association with LRR-RLK Resistance Gene Analogs in Cucumber

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Abstract. Downy mildew (DM) caused by the obligate oomycete *Pseudoperonospora cubensis* is an important disease of cucumber and other cucurbit crops. In the United States, for more than 50 years, DM was effectively controlled in cucumber through host resistance conferred mainly by the *dm-1* derived from PI 197087, which was overcome in 2004 due to the appearance of a new pathotype. Resistance sources (for example, K8, PI 197088 and PI 330628) against the new DM strain have been identified and QTL mapping studies have revealed the recessive and quantitative nature of DM resistance in these materials. QTLs for DM resistance in K8, PI 197088 and PI 330628 were detected in five (1, 2, 4, 5, and 6) of the seven cucumber chromosomes. Previous studies in other plant species suggested that the leucine-rich repeat (LRR)-receptor-like kinases (RLKs) or proteins (RLPs) may be responsible for recessively inherited host resistance against oomycete pathogens. To better understand the molecular basis of DM resistance in cucumber, we performed genome-wide identification of LRR-RLK and LRR-RLP type resistant gene homologs (RGHs) in the cucumber genome, aiming for possible association of these RGHs with DM resistance QTLs. From the Gy14 and 9930 draft genome assemblies, we identified 192 and 178 LRR-RLK, and 42 and 56 LRR-RLP RGHs respectively. Several LRR-RLKs in chromosomes 4 and 5 were co-localized or near major-effect DM resistance QTLs, but further evidence is needed to establish true association of these RGHs with the QTLs for DM resistance.

Cucumber (*Cucumis sativus* L.) is an important vegetable crop and is widely grown in the world with total harvest of more than two million hectares in 2012 after tomato, onion, and cabbage (FAOSTAT, 2014). Downy mildew (DM hereinafter), caused by the obligate oomycete *Pseudoperonospora cubensis* [(Berkeley & M. A. Curtis) Rostoyzev] has a host range of more than 60 species in the Cucurbitaceae family including several economically important cucurbit crops such as cucumber, melon (*C. melo* L.), watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai], and squash/pumpkin (*Cucurbita* spp.) (Whitaker and Davis, 1962; Palti and Cohen, 1980). In the U.S., in the past, cucumber DM was well managed with deployment of resistant cultivars such as 'Polaris', 'Poinsett', and 'Chipper'. Most of these varieties carried the DM resistance gene *dm-1* from PI 197087 which, however, became less effective in 2004 due to the appearance of a new pathotype. Fortunately, large scale screening tests have identified resistant lines to this new DM strain (Criswell, 2008; Klosinska *et al*., 2010; Call *et al*., 2012a, b; Holdsworth *et al*., 2014).

The inheritance of DM resistance in cucumber has been investigated in a number of studies with varying results. Classical genetic analysis suggested that the resistance in cucumber line Gy14 was controlled by polygenes (McFerson, 1978), while three genes, *dm1*, *dm2*, and *dm3* derived from PI 197087 were reported to present in resistant cultivars 'Aojihai' and 'Poinsett' (Van Vliet and Meysing, 1974; Doruchowski and Lakowska-Ryk, 1992). One or two incompletely dominant genes were reported in the cucumber variety J-13, a derivative of Wisc 2843 (Petrov *et al*., 2000).

Among more than 1,300 Plant Introduction (PI) lines, Call *et al*. (2012b) identified Ames 2353, PI 197088, PI 330628, and PI 197085 as the four most resistant cultigens to DM. Genetic variance study for Ames 353 revealed a small number of genes controlling DM resistance, and one of them seems to be allelic to *dm-1*(Kozik *et al*., 2013). In a QTL mapping study for DM resistance in the cucumber line CS-PMR1 derived from PI 197088, Yoshioka *et al*. (2014) identified nine DM resistance QTLs, six of which were from PI 197088. On the other hand, Caldwell *et al*. (2011) reported three DM resistance QTLs contributed by PI 197088, which were located in cucumber chromosomes (Chr) 2, 4, and 5, respectively, and there was epistasis effect between the two QTLs in Chr4 and Chr5. In a north Chinese type cucumber (Chinese Long) inbred line K8, Zhang *et al*. (2013) mapped three DM resistance QTLs in Chr5 (*dm5.1*, *dm5.2* and *dm5.3*).

While a number of DM resistance QTLs have been identified, the molecular basis of DM resistance in cucumber is not known. So far most simply inherited dominant disease resistant (R) genes cloned in crop plants belong to the NB-LRR (nucleotide binding - leucine rich repeats) type R genes. NB-LRR type resistant gene homologs (RGHs) have been well characterized in the cucumber

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genome (Yang *et al*., 2013; Lin *et al*., 2013; Wan *et al*., 2013), which were significantly lower in number than most other plant species with draft genomes. Other important classes of R genes in plants include LRR receptor-like kinases (RLKs) and proteins (RLPs), which are key regulators of plant development and defense (Becraft, 2002; Diévart and Clark, 2004). LRR-RLKs/RLPs can be triggered by microbe-associated molecular patterns (MAMPs) during the interactions with fungal and bacterial pathogens (Shiu and Bleecker, 2001; Morris and Walker, 2003). Studies in different plant species indicated that LRR-RLKs/RLPs might be responsible for recessively inherited host resistance (Kessler *et al*., 2010; Srour *et al*., 2012). In *Arabidopsis*, *IOS1* (*IMPAIRED OOMYCETE SUSCEPTIBILITY* 1) an LRR-RLK type gene was found to contribute to downy mildew disease susceptibility (Hok *et al*., 2011); *CLV3* (LRR-RLKs) and *CLV1* (RLPs) were reported to interact with *RPP27* and contribute to Arabidopsis downy mildew resistance (Tör *et al*., 2004).

To better understand the molecular mechanisms of DM resistance in cucumber, we performed genome-wide identification of LRR-RLK/RLP type RGHs in the cucumber genome. We reviewed DM resistance QTLs identified in cucumber. The physical locations of the flanking markers of these QTLs were compared with predicted RGHs aiming for possible association between the two.

Materials and Methods

RGH prediction and classification. The Gy14 (Version 1.0) and 9930 (Version 2.0) cucumber draft genome assemblies and annotations were used for RGH prediction. There are three domains involved in LRR-RLK/RLP prediction: LRR, transmembrane (TM), and serine-threonine kinase (STK). Nine types of LRR domains documented in the LRR XML database (http://tollml.lrz.de:8081/exist/rest/lrrml/index.xq) were used for developing HMM matrices with HMMER (version 2.3.2) (Eddy, 1998) with "-f" option for maximum search sensitivities. Pkinase (Pfam: PF00069) were used to search for STK domains by HMMER. The threshold was $1e^{-5}$ for STK and $1e^{-1}$ for LRR. The TMHMM (Krogh *et al*., 2001) program was used to identify the TM domain with default options. Putative RGHs were grouped into either RLK or RLP type based on the presence or absence of the STK domain (Fig. 1).

Physical mapping of RGHs and DM QTLs in cucumber genome. The Gy14 and 9930 draft genome assemblies were used to determine the physical locations of predicted LRR-RLKs/RLPs RGAs and DM QTLs. Thirty-five molecularly tagged DM QTLs were selected through literature review and our QTL mapping results. For comparison purpose, the NB-LRR type RGHs identified by Yang *et al*. (2013) were also included on the resulting physical map. A custom PERL script was used to draw the map for each chromosome.

Results and Discussion

Identification of LRR-RLK and LRR-RLP sequences in the cucumber genome. Plant RLKs contain common features: a N-terminal extracellular signal domain, a single TM domain, and a cytoplasmic STK domain, while RLPs do not have the STK domain. The extracellular domains vary in structure and functions, while LRR comprises the largest class in plants RLKs/RLPs which have been shown to play critical roles in plant-pathogen interactions. Results of bioinformatic analysis of these two classes of RGHs in the Gy14 and 9930 draft genomes are shown in Fig. 1 and Table 1. A total of 192 and 178 LRR-RLK, and 42 and 56 LRR-RLP RGH were predicted from the Gy14 and 9930 draft genome assemblies, respectively. Distribution of the predicted LRR-RLKs/RLPs in seven chromosomes is presented in Table 1. The minor differences in predicted RGH numbers between the Gy14 and 9930 draft genomes may be due to incomplete genome coverage of the draft genome assemblies, incorrect annotation or copy number variations.

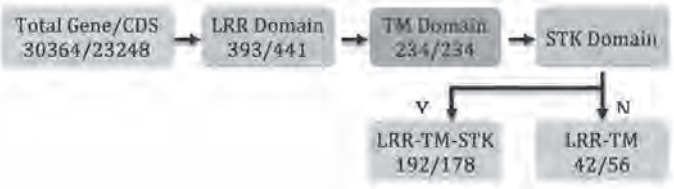

Fig. 1. Prediction and classification of LRR-RLK/RLP type resistance gene homologs in cucumber genome. RGHs were classified on the basis of presence or absence of the main functional domains. STK and LRR domains were predicted via *hmmpfam*; the TM domain was predicted with TMHMM. The numbers of RGHs before and after the slash were from the Gy14 and 9930 draft genome assemblies, respectively.

Table 1. Predicted LRR-RLK/RLP RGHs in the Gy14 and 9930 draft genomes.

		Chr1	Chr2	Chr3	Chr4	Chr5	Chr6	Chr7
LRR-RLKs	Gy14	30	15	48	23	25	24	20
	9930	26	18	40	23	24	21	26
LRR-RLPs	Gy14	11	3	5	3	8	11	1
	9930	15	1	7	6	8	13	6

Physical locations and LRR-RLK/RLP RGHs and DM resistance QTLs. Bioinformatic analysis identified 192 LRR-RLK and 42 LRR-RLP homologs in the Gy14 draft genome. Their physical locations among the seven cucumber chromosomes are plotted in Fig. 2. While both types of RGHs were distributed in all seven chromosomes, this distribution along the chromosome was not uniform, and clustering of these sequences was obvious. The three largest RLK clusters were located in Chr1, Chr3, and Chr7, respectively.

From several QTL mapping studies, 21 downy mildew resistance QTLs have been identified which were distributed in all seven chromosomes. Detailed information of these QTLs is presented in Table 2. In Table 2, flanking markers for these QTLs are also listed, and their physical locations in the chromosomes are shown in Fig. 2. It is obvious that a number of QTLs detected in different studies were location in the same region suggesting these QTLs are the same locus. Comparison of the chromosome locations of these RGHs and QTL flanking markers revealed co-localization of RGH members and DM resistance QTLs (Fig. 2). For example, in Chr4 and Chr5, several RGHs were within the regions where major DM resistance QTLs were detected in multiple studies.

The analysis in the present study on the distribution of RGHs in cucumber chromosomes provided a physical link between LRR-RPL/LRR-RLK RGHs and DM QTLs, which may be useful to identify casual genes responsible for DM or other pathogen resistances through the candidate gene approach. However, further analysis on the phylogeny, structure, and expression of these LRR-RLK/RLP RGHs is needed to explore their specific functions and establish true association with the QTLs for DM resistance.

Table 2. Molecularly tagged DM resistance QTLs in cucumber.

Chr	QTL	Resistant Source	Flanking markers	Reference
1	*dm1.1*	Santou	CU421-CSWTA05	Yoshioka et al. (2014)
	dm1.2	CS-PMR1	SSR19190-CU742	Yoshioka et al. (2014)
	dm1.3	CS-PMR1	SSR00231-SSR21747	Yoshioka et al. (2014)
	dm1.1	K8	SSR20705-SSR31116	Zhang et al. (2013)
2	*dm2.1*	PI 197088	SSR00289-SSR21276	Caldwell et al. (2011)
	dm2.1	PI 330628	11C03-72E09	Unpublished data
3	*dm3.1*	CS-PMR1	SSR03049-CSN251	Yoshioka et al. (2014)
4	*dm4.1*	PI 197088	SSR00012-SSR13159	Caldwell et al. (2011)
	dm4.1	PI 330628	14D06-1F03	Unpublished data
5	*dm5.1*	CS-PMR1	CSWTA04-CSWCT32	Yoshioka et al. (2014)
	dm5.2	CS-PMR1	SSR20165-SSR2693	Yoshioka et al. (2014)
	dm5.3	CS-PMR1	SSR00772-SSR02459	Yoshioka et al. (2014)
	dm5.1 /5.2/5.3	K8	SSR16110-SSR00772	Zhang et al. (2013)
	dm5	PI 197088	SSR02895-SSR07559	Caldwell et al. (2011)
	dm5	PI 330628	8E02-57C02	Unpublished data
6	*dm6.1*	Santou	SSR03940-SSR00259	Yoshioka et al. (2014)
	dm6.1	K8	SSR16882-SSR00723	Zhang et al. (2013)
	dm6	PI 330628	9D10-10E09	Unpublished data
7	*dm7.2*	Santou	SSR33278-SSR00477	Yoshioka et al. (2014)

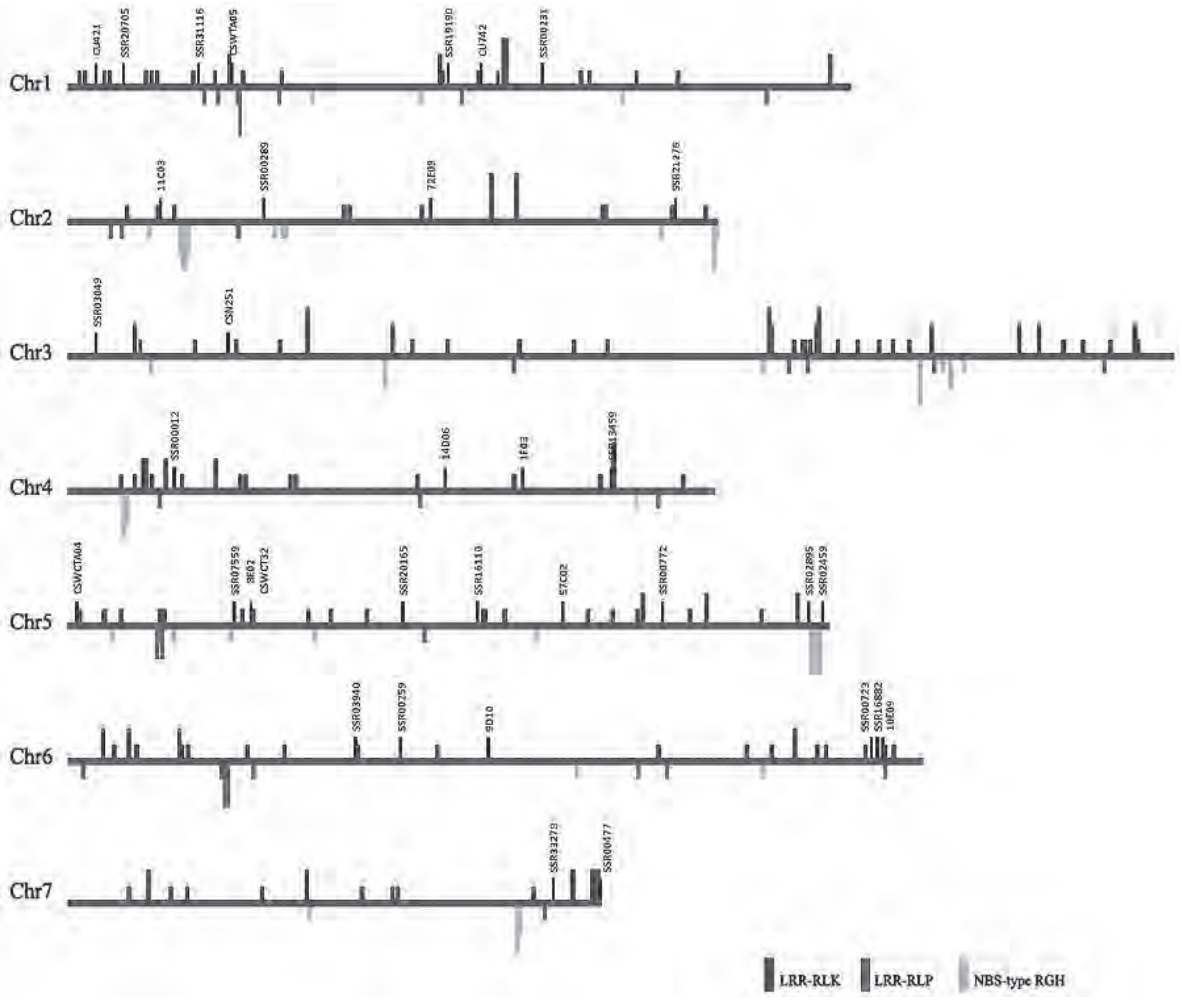

Fig. 2. Physical locations of RGHs and markers flanking DM QTLs in seven cucumber chromosomes. LRR-RLK = leucine-rich repeat receptor-like kinase (blue), LRR-RLP = leucine-rich receptor-like proteins (green), NB-LRR = nuclear binding – leucine rich repeats (yellow). Bar length represents # of copies of RGHs detected in 100 kb windows (short = 1 copy, medium = 2 copies, long = 3 or more copies).

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Occurrence of Bacterial Spot (*Xanthomonas cucurbitae*) in Cucurbit Fields in the Midwest of the United States

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Additional index words. bacterial spot, Cucurbita, cucurbits, pumpkin, squash, *Xanthomonas cucurbitae*

Abstract. Bacterial spot, caused by *Xanthomonas cucurbitae*, is an emerging disease in pumpkin and winter squash in the Midwestern states of the United States. During 2011 and 2012, field surveys were conducted in Illinois, Indiana, Iowa, Kansas, Michigan, Missouri, Nebraska, Ohio, and Wisconsin to assess the incidence and severity of bacterial spot in cucurbit fields. The disease was only observed in pumpkin and winter squash fields. During four weeks of the harvest, 111 and 133 fields in 2011 and 2012, respectively, were surveyed. Symptomatic fruit were observed in 95 (86%) and 118 (88%) of the fields surveyed, with overall 27 and 22% fruit infected, in 2011 and 2012, respectively. Severity of fruit infection (percent surface area of the fruit affected) ranged from 1 % to 20 %. In a field trial, effectiveness of 13 selected chemicals was evaluated for control of bacterial spot in pumpkin 'Howden'. To increase disease pressure, plants were inoculated twice with *X. cucurbitae* at the beginning of flowering and fruit setting stages. The first application of the chemicals was either 4 days prior to the first inoculation (pre-inoculation spray) or 3 days after the first inoculation (post-inoculation spray). Then, the chemicals were applied at 7-day intervals until two weeks to harvest. Incidence of the disease in fruit in pre-inoculated treated plots ranged from 11.1 % to 69.4%% compared to 88.9 % of control plots. Severity of the disease on fruit in pre-inoculated treated plots ranged from 0.07 % to 3.97 %, whereas severity of the disease in control plots was 4.33 %. In the post-inoculated treatments, the incidence and severity of bacterial spot ranged from 42.4 % to 82.2 % and from 0.73 % to 3.73%, respectively. The lowest incidence of bacterial spot on fruit was in the plots that received pre-inoculation spray of oxytetracycline (Mycoshield 40 WSP).

Approximately 75,000 ha are planted to commercial cucurbit crops (cantaloupe, cucumber, honeydew, pumpkin, squash, watermelon) annually in the Midwestern states (Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin) of the United States (U.S.) (USDA-NASS, 2013). In 2012, the Midwest produced approximately 16,000 and 4,000 ha of pumpkin and squash, respectively. Approximately 10,000 ha of pumpkins [*Cucurbita pepo* L., *C. maxima* Duchesne, and *C. moschata* (Duchesne) Duchesne ex Poir] are grown annually in Illinois (Babadoost and Ravanlou, 2012). More than 90% of commercial processing pumpkins (*C. moschata*) produced in the U.S. are grown and processed in Illinois.

Bacterial spot (also known as bacterial leaf spot), caused by *Xanthomonas cucurbitae* (ex Bryan) Vauterin et al. [syn. *Xanthomonas campestris* pv. *cucurbitae* (Bryan) Dye] (Saddler and Bradbury, 2005), has become a serious threat to production of pumpkins and winter squash in the Midwest (Babadoost and Ravanlou, 2012). Bacterial spot was first described on Hubbard squash in New York in 1926 (Bryan, 1958). Subsequently, the disease was reported on cucumbers, gourds, pumpkins, and summer and winter squash in Asia, Australia, Europe, and North America (Babadoost and Zitter, 2009; Blancards et al., 1994; Dutta et al., 2013; Latin and Rane, 1999; Pruvost et al., 2008; Pruvost et al., 2009; William and Zitter, 1996).

X. cucurbitae infects leaves and fruit of cucurbits throughout the growing season (Babadoost and Ravanlou, 2012; Latin and Rane, 1999; William and Zitter, 1996). Lesions on leaves are small (2 to 4 mm), angular, yellow to beige spots. Leaf infection can easily be overlooked because of the small size of the spots (Babadoost and Zitter, 2009; William and Zitter, 1996). The appearance and size of fruit lesions can vary, depending on rind maturity and the presence of moisture. Initial lesions are small, slightly sunken, circular spots (1 to 3 mm in diameter). In immature fruit, the lesions begin as light green with darker center. In mature fruit, the lesions have a beige center and dark brown halo. Fruit infected by *X. cucurbitae* is usually colonized by other fungi and bacteria, which results in rapid collapse of the fruit (Babadoost and Zitter, 2009).

During 2009–2013, *X. cucurbitae* caused severe leaf and fruit infections in pumpkin and squash fields in Illinois and other Midwestern states. Yield losses up to 90% occurred in some pumpkin and squash production (Babadoost and Ravanlou, 2012). The objectives of this study was to assess the occurrence of bacterial spot in pumpkin and squash fields in the Midwest and assess efficacy of selected chemicals for control of bacterial spot in pumpkin.

Materials and Methods

Disease occurrence. In 2011 and 2012 surveys were conducted in 65 and 70 pumpkin and winter squash fields, respectively, in Illinois to determine occurrence of bacterial spot on fruit at harvest. Also, surveys were conducted in 46 and 63 pumpkin and winter squash fields in Iowa, Indiana, Kansas, Michigan Missouri, Ohio, and Wisconsin in 2011 and 2012, respectively to assess occurrence of bacterial spot in these states. In each field, 12 locations in an M-shape pattern were chosen, and the sever-

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ity of the disease (percentage of fruit surface with bacterial spot lesions) in five fruit at each location (a total of 60 fruit in each field) was assessed.

Chemical field trial. A field trial was conducted at the University of Illinois Vegetable and Research Farm in Champaign, IL in 2012 to evaluate efficacy of the selected chemicals (Table 2) for control of bacterial spot in pumpkin. Seeds of jack-o-lantern pumpkin 'Howden' were sown on 6 June. Seeds were sown 46 cm apart in single-row plots, 6 m long. The design was randomized complete block design with three replications, and the plots were spaced 10 m apart. Plants were inoculated twice with *X. cucurbitae* (5×10^7 CFU/ml) mixed with carborundum powder (0.5 g carborundum/L inoculum) on 23 July (beginning of flowering stage) and 13 August (fruit setting stage) using a backpack sprayer. The first inoculation was for establishing infection in leaves and the second inoculation mainly aimed for establishing fruit infection.

Chemicals were spray-applied with a backpack sprayer using 470 liters of water per hectare. The first application of the chemicals was either on 19 July [4 days prior to the first inoculation (pre-inoculation spray)] or on 26 July [3 day after inoculation (post-inoculation spray)]. Then, the chemicals were applied at 7-day intervals until 6 September. Bacterial spots were observed on leaves on 2 August (10 days after the first inoculation) and severity of infection increased as the season progressed. Bacterial spots were observed on fruit on 17 August (25 days after the first inoculation), when fruit were approximately 15 cm in diameter. Incidence (percent symptomatic fruit) and severity (percent symptomatic area of fruit) of bacterial spot were assessed on 23 September. All of the fruit in the plots were evaluated for the occurrence of bacterial spot.

Results

Disease occurrence. In 2011, 95 of 111 (86%) pumpkin fields surveyed had fruit infected with *X. cucurbitae*, with 3-98% symptomatic fruit in a field (average 27%) (Table 1). Severity of bacterial spot on fruit ranged from 1 to 20%. Incidence of the infected fruit was highest in Indiana and lowest in Iowa. Similarly, in 2012, 118 of 133 (88%) pumpkin fields surveyed had fruit infected with *X. cucurbitae*, with symptomatic fruit ranging from 3 to 98% in a field (average 22%). Severity of bacterial spot on fruit ranged from 1 to 18%. Incidence of the disease was highest in Indiana and lowest in Michigan (Table 1).

Chemical field trial. Incidence and severity of bacterial spot on fruit in treated plots were lower than those of control plots (Table 2). Incidence of the disease in fruit in pre-inoculated treated plots ranged from 11.1 to 69.4% compared to 88.9% of control plots. Severity of the disease on fruit in pre-inoculated treated plots ranged from 0.07 to 3.97%, whereas severity of the disease in control plots was 4.33%. In the post-inoculated treatments, the incidence and severity of bacterial spot ranged from 42.4 to 82.2% and from 0.73 to 3.73%, respectively. The lowest incidence of bacterial spot on fruit was in the plots that received pre-inoculation spray of oxytetracycline (Mycoshield 40 WSP). The lowest severity of bacterial spot on fruit was in the plots which received pre-inoculation spray of copper (Phyton-016B).

Discussion

Bacterial spot, caused by *X. cucurbitae*, was economically an insignificant disease in the Midwestern states prior to 2005. But, in the recent years, the disease occurred widely in pumpkin and winter squash fields in the region. Bacterial spot was observed in more than 85% of all fields surveyed, causing up to 90% yield losses (Babadoost and Ravanlou, 2012). Bacterial spot has been reported from other pumpkin and squash growing areas in the U.S. (Blanchard et al., 1994; Bryon, 1958; Dutta et al., 2013; Latin and Rane, 1999; Pruvost et al., 2008; Pruvost et al., 2009; Thapa and Babadoost, 2013) and in Austria (Babadoost, unpublished data). Thus, it appears that bacterial spot (*X. cucurbitae*) is an emerging disease in cucurbits growing areas in the world.

Table 1. Occurrence of bacterial spot (*Xanthomonas cucurbitae*) in pumpkin and winter squash fields in the Midwest in 2011 and 2012[z].

Year	State	No. fields surveyed	No. fields with infected fruit	Infected fruit in a field (%)	Average infected fruit in the state (%)
2011	Illinois	65	57	3–87	24
	Indiana	7	7	22–98	62
	Iowa	8	4	8–28	8
	Kansas	5	5	15–50	30
	Missouri	6	3	7–57	14
	Nebraska	5	5	7–93	54
	Ohio	9	9	7–75	32
	Wisconsin	6	5	3–40	18
Total		111	95	3–98	27
2012	Illinois	70	60	2–92	19
	Indiana	10	10	13–98	56
	Iowa	14	14	3–50	13
	Kansas	10	10	7–30	16
	Michigan	5	3	3–15	5
	Missouri	5	3	55–82	43
	Ohio	13	13	15–92	43
	Wisconsin	6	5	3–40	15
Total		133	118	3–98	22

[z] In each field, 60 fruit in 12 locations (five fruit at each location) in an M-shape pattern were assessed for incidence of bacterial spot. Field surveys were within four weeks of fruit harvest.

Table 2. Incidence and severity of bacterial spot (*Xanthomonas cucurbitae*) in pumpkin fruit following application of chemicals in the field in 2012.

	Bacterial spot on pumpkin fruit			
	Pre-inoculation spray[v]		Post-inoculation spray[w]	
Treatment (product rate/Ha)[u]	Incidence (%)[x]	Severity (%)[y]	Incidence (%)[x]	Severity (%)[y]
Control (no chemical)	88.9 a[z]	4.77 a	88.9 a	4.77 a
Acibenzolar-s-methyl (ActiGard 50 WG, 70 g) + Copper hydroxide (Kocide-3000 46.1 DF, 1.40 kg)	33.3 a–c	0.20 c	82.2 ab	0.73 e
Copper (Agion E, 28 L)	58.3 a–c	0.83 bc	72.2 ab	1.23 de
Copper (Badge X2 DF, 1.40 kg)	25.0 bc	0.93 bc	80.5 ab	3.60 ab
Copper (Nordox 75 WG, 1.40 kg)	55.6 a–c	0.93 bc	64.4 a–c	3.03 a–d
Copper (Phyton-016B, 1.82 L)	23.3 bc	0.07 c	62.2 a–c	1.97 b–e
Copper hydroxide (Kocide-3000 46.1 DF, 1.40 kg)	69.0 a–c	1.20 a–c	64.4 a–c	3.37 a–c
Copper sulfate (Cuprofix Ultra 40DF, 2.24 kg)	58.3 a–c	2.31 a–c	53.3 bc	1.80 b–e
Famoxadone + cymoxanil (Tanos 50 DWG, 0.70 kg) + Copper hydroxide (Kocide-3000 46.1 DF, 1.40 kg)	26.7 bc	2.27 a–c	42.2 c	2.63 a–e
Kasugamycin (Kasumin 2L, 2.33 L)	44.4 a–c	0.13 c	60.0 a–c	1.43 c–e
Mancozeb (Dithane 75 DF, 2.80 kg) + Copper hydroxide (Kocide-3000 46.1 DF, 1.40 kg)	72.2 ab	3.77 ab	75.5 ab	2.97 a–d
Oxytetracycline (Mycoshield 40 WSP, 1.12 kg)	11.1 c	0.20 c	61.1 a–c	3.53 a–c
Quinolin (Quintec 2.08 SC, 0.44 L) + Copper hydroxide (Kocide-3000 46.1 DF, 1.40 kg)	69.4 a–c	3.03 a–c	77.4 ab	3.73 ab
Streptomycin (AgriMycin 17 WP, 0.56 kg)	46.7 a–c	3.97 ab	60.0 a–c	1.13 de
LSD ($P = 0.05$)	53.80	3.17	29.2	2.13

[u] Product rate per hectare.
[v] Pre = plants were treated on 19 July, 26 July, 2 August, 9 August, 16 August, 23 August, 30 August, and 6 September.
[w] Post = plants were treated on 26 July, 2 August, 9 August, 16 August, 23 August, 30 August, and 6 September.
[x] Incidence = percent symptomatic fruit.
[y] Severity = percent affected area of fruit.
[z] In each column values with a letter in common are not significantly different from each other according to the Fisher's protected LSD ($P = 0.05$).

Although the incidence and severity of bacterial spot on fruit were lower in the plots treated with chemicals, none of the treatments was highly effective in controlling the disease in pumpkin. Similar results have been reported by Lange and Smart (2013), indicating that Cueva, Regalia, Kocide, and ActiGard reduced incidence of black rot (*X. campestris* pv. *campestris*) in cabbage but did not control it effectively. In our study, application of chemicals began at flowering stage of plant growth. By that time, infection of plants by natural inoculum of *X. cucurbitae* may have taken place, although no symptoms were visible yet. It is possible that these treatments could have worked as better control of the bacterial spot if application of chemicals had begun from earlier stage of plant growth. Further studies are needed to determine disease development of bacterial spot from natural inoculum in the fields.

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Biological and Molecular Evidences About Changes in the Host Range and Virulence of *Pseudoperonospora Cubensis* Populations in the Czech Republic

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Additional index words. Host-pathogen interaction *Cucumis sativus, C. melo, Cucurbita* spp., cucurbit downy mildew, viruelence variability, genetic shift

Abstract. During the 2009–2012 growing seasons, disease prevalence and severity and the host range of *P. cubensis* were evaluated at more than 80 locations in the Czech Republic (CR). Infection by *P. cubensis* was observed primarily on *Cucumis sativus,* medium to high disease severity prevailed. During the years 2010 - 2012, *P. cubensis* infection was also frequently recorded on other cucurbit plants: *Cucumis melo*, *Citrullus lanatus, Cucurbita* spp. (*C. moschata, C. pepo, C. maxima, C. ficifolia*) and *Lagenaria siceraria*. *P. cubensis* infection on *C. moschata* and some other *Cucurbita* spp. was observed for the first time in the CR. Virulence shift (2009–2012) was studied in populations of 95 *P. cubensis* isolates. We observed substantial changes in the pathogen population virulence structure – from 2009 onwards. AFLP procedure and multilocus sequence analysis (MLSA) of six mitochondrial and nuclear DNA regions were used to detect changes in the genetic structure of *P. cubensis* populations. The analysed sample set contains 67 *P. cubensis* isolates collected during 1995 to 2012 in the Czech Republic and some other European countries. Both AFLP and MLSA revealed differences and changes in the genetic background of *P. cubensis* isolates sampled *before* and *after* 2009.

Cucurbit downy mildew is the most important foliar disease of cucurbit crops worldwide. Recently, more than 60 species were reported to be affected by *P. cubensis* (Lebeda and Cohen, 2011; Lebeda et al., 2012). In the Czech Republic, disease prevalence, host range and disease severity were evaluated from 2001-2009. The geographical distribution of *P. cubensis* was assessed on ca 80-100 locations per year in two main regions of the Czech Republic (Lebeda et al., 2011). Infection by *P. cubensis* was observed primarily on cucumber (*C. sativus*) but only on the leaves. The majority of *C. sativus* crops were heavily infected at the end of growing season (second half of August). Generally, *P. cubensis* was present at high or very high disease severity across the whole area of the Czech Republic studied. The loss of foliage from such severe infections results in the reduction of the quality and quantity of marketable fruits. Very rarely, in past years, we had also recorded infections on muskmelon (*Cucumis melo*) and *C. moschata* (Lebeda et al., 2011).

However, beginning in 2009, the pathogen population changed dramatically, and new hosts were recognized by new pathotypes that were able to establish serious infection of *Cucurbita moschata* (Pavelková et al., 2011), *C. maxima, C. pepo* and watermelon (*Citrullus lanatus*), not observed in 2001 to 2008 in the Czech Republic (Lebeda et al., 2011, 2012). Starting with the observation of devastating epidemics on cucumber in the Czech Republic in the mid-1980s (Lebeda and Schwinn, 1994), also in the first decade of the 21st century the re-emergence of *P. cubensis* as a serious threat to cucurbits was reported. New aggressive pathotypes appeared in Israel (Cohen et al., 2003), Italy (Cappelli et al., 2003) and highly aggressive isolates ravaged cucumber-growing areas of North Carolina, Delaware, Maryland and Virginia in 2004 in the United States (Holmes and Thomas, 2006; Holmes et al., 2006). In 2009 the next dramatic change in the virulence of pathogen population was observed in the Czech Republic, with new pathotypes which were not observed previously, but occurred repeatedly from 2009 onwards (Pavelková et al., 2011; Lebeda et al., 2011, 2013a,b).

The objectives of this study were: 1) to show changes in virulence variation (at the level of a pathotype) of *P. cubensis* in the Czech Republic in the period 2009-2011; 2) to support these data by molecular analyses (AFLP and MLSA analysis of nuclear and mitochondrial segments).

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Materials and Methods

P. cubensis virulence screening. A total of 398 isolates of *P. cubensis* were recovered and screened for virulence variability (pathotypes) from 2001 to 2010 (for details see Lebeda et al., 2013). The virulence of 70 isolates (collected in 2010 /37/ and 2011 /33/) was screened on a differential set of 12 cucurbit taxa (Lebeda and Widrlechner, 2003; Lebeda et al., 2013a). A leaf-disc method was used (Lebeda and Urban, 2010), with a visual 0-4 scale (Lebeda, 1991) used to evaluate sporulation intensity over a two-day period from 6 to 14 days after inoculation. The sporulation intensity was expressed as the percentage of maximum sporulation intensity (Lebeda and Urban, 2010). The virulence level of isolates was determined on the basis of the number of virulence factors, i.e. number of compatible reactions within the differential set of cucurbitaceous taxa. Pathotypes were designated with tetrad numerical codes (Lebeda and Widrlechner, 2003).

Spore collection, DNA-extraction, molecular and data analyses. After 7–9 days of cultivation, the spores were collected by transferring of whole leave into 25 ml cultivation tube with 5 ml of sterile deionized water and vortexing (20–25 seconds, 800 rps, IKA MS2 shaker). Washed leave was removed and spore suspension was allowed to settle freely for 20 minutes in a fridge at 4 °C. Upper part of solution was removed and spore concentrate with volume of ca. 1 ml was stored in 1.5 Eppendorf tube at –80 °C until DNA extraction. DNA was extracted using InnuPREP Plant DNA Kit (Analytik Jena AG, Germany) following the manufacture´s protocol. For AFLP analysis a set of 30 *P. cubensis* isolates collected throughout years 1995–2009 (Lebeda and Gadasová, 2002; Lebeda et al., 2013a). Enlarged set of 64 *P. cubensis* isolates have been used for multi-locus sequencing analyses. These isolates were collected during period 1995 to 2012 on the territory of the Czech Republic (Lebeda and Gadasová, 2002; Lebeda et al., 2013a), supplemented by a subset of isolates used in *P. cubensis* cryptic species concept by Runge et al. (2011) to pinpoint the classification and nature of Czech isolates.

The original AFLP procedure of Vos et al. (1995) has been used, following slightly modified protocol described in Kitner et al. (2008). Detailed info on core sequences of AFLP primers is described in Kitner et al. (2012) and the list of used primer combinations are available upon request. The products of 13 selective primer combinations were separated on a 6%, 0.4-mm-thick denaturing polyacrylamide gel (0.5x TBE buffer) using the T-REX (Thermo Scientific Owl Separation Systems, Rochester, NY, USA) sequencing gel electrophoresis apparatus. Subsequent silver staining was used for the visualization of AFLP patterns. Primer sequences used in selective amplification steps are available upon request. The visualized gels were scored for the presence (1) or absence (0) of bands. Because the main aim of our study was to reveal possible pattern of genetic variation related to the characteristics of isolates (pathotype code, geographical origin, sampling period) principal coordinate analysis (PCoA) to describe and visualize possible genetic structure of investigated sample-set. The binary matrix was constructed from primary data and subjected to FAMD ver. 1.23 beta (Schlüter and Harris, 2006) to visualize possible relationships among analyzed individuals by in PCoA plot.

For MLSA analysis six nuclear and mitochondrial segments have been tested in this study (cox1, cox2, cox spacer, *Nad1*, nrITS, ypt). Primers used for amplification and description of PCR conditions are described elsewhere (Choi et al. 2007; Runge et al. 2011; Kitner et al. submitted). All sequencing reactions were performed at Sequencing laboratory of the Biodiversity and Climate Research Centre (BiK-F), Frankfurt am Mein, Germany.

Sequencing data were manually edited, aligned and analysed using Sequencher® version 5.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA).

Results and Discussion

Virulence analyses. Virulence analyses (2001–2010) showed that the 398 analyzed isolates belonged to 67 different pathotypes. The number of pathotypes recorded ranged from 33 in 2001 to 5 in 2007. The most frequent pathotypes detected varied by year, but overall pathotypes 15.14.10 and 15.14.11 were the most frequently recorded. One pathotype (15.15.15) was named "super pathotype" and detected frequently in 2001, 2003, 2004, 2008, and 2010, and belongs to the four most frequently recorded pathotypes, esp. in the years 2003, 2008 and 2010. At the isolate level, 73.4% the virulence variation was represented by eleven *P. cubensis* pathotypes (Lebeda et al., 2013a).

Eleven unique isolates, originating from *Cucurbita melo*, *C. pepo*, *C. maxima C. moschata* and *Citrullus lanatus*, sampled in 2009 and 2010, were determined as 7 different pathotypes (Lebeda et al., 2013a). Two of them (4.15.0 and 15.6.0) were unique in the whole period of study (Lebeda et al., 2012, 2013a,b).

Over time, clearly *P. cubensis* populations have been evolving toward higher levels of virulence, with substantial changes when compared to the period 2001-2009 (Lebeda et al., 2010). Since 2009, the pathogen population has changed dramatically, and new pathotypes are now able to establish serious infections on *Cucurbita* spp. and *C. lanatus* (Lebeda et al., 2011, 2012, 2013a,b), which was not observed between 2001 and 2008 (Lebeda et al., 2013a). These data are also supported by most recent molecular analyses.

Molecular analyses. A total of 385 fragments were detected for thirteen selective primer combinations, with 304 segregating (78.9%) and 187 polymorphic fragments (48.6%) (polymorphism at 1% and 5% level). The genetic relationship based on AFLP patterns is presented in the PCoA plot (Fig. 1), where co-clustering of isolates sampled during year 2009 is apparent, apart from group of isolates collected during 1995-2004. Nevertheless, no clustering of samples according to its geographical origin nor pathotype code was observed.

Preliminary analyses of sequencing data revealed differences and changes in the genetic background of *P. cubensis* isolates sampled *before* and *after* 2009. While all isolates sampled *before* 2009 exhibited the pre-epidemic genotype of the subspecies of *Clade II*, probably indigenous to East Asia, *Clade I* (*P. cubensis sensu stricto*) was observed among isolates sampled from 2009 onwards. In addition, over 60 % of the post-2009 samples from *Clade II* had two heterozygous positions in their nrITS sequence, providing hints for sexual reproduction (Fig. 2).

The investigation of the genetic structure of *P. cubensis* populations from a worldwide collection showed rather large genetic variation within specific geographic regions, across a wider range of hosts, or during different time points during the growing season (Quesada-Ocampo et al., 2012). Also in our recent study both molecular markers clearly proved dramatic changes in genetic structure of *P. cubensis* populations sampled over a large period of time that are in agreement with our previous phytopathological observations (Lebeda et al., 2010, 2011, 2013a,b). Thanks to the involvement of a subset of isolates characterized by Runge et al. (2011) in our analysis, we know that all *before* 2009 isolates are

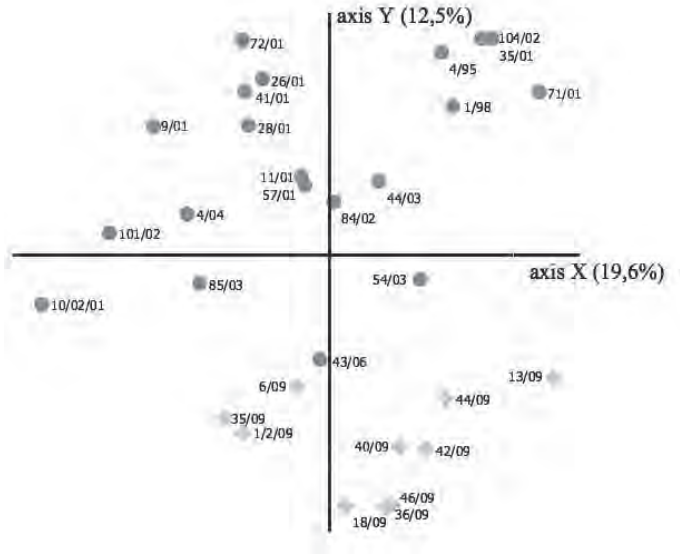

Fig. 1. Principal coordinate analysis (PCoA) of the pair-wise Jaccard's similarity matrix of 30 P. cubensis strains. The two principal coordinates accounted for 32.1% of the total variation.

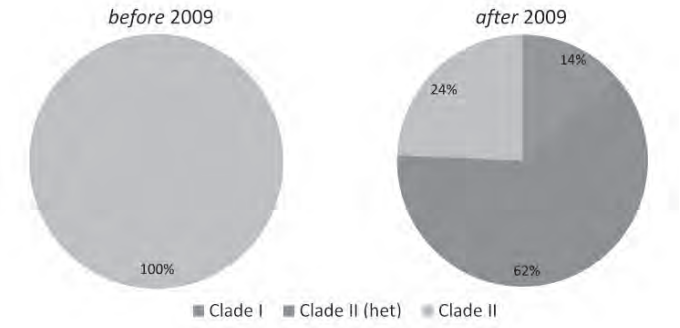

Fig. 2. Changes in population structure of P. cubensis isolates sampled before and after 2009. Isolates with heterozygous SNP´s in ITS region are designated as Clade II (het).

similar to *Clade II* and that the changes in population genetic structure are related to the occurrence of new genetic material identical to *Clade I* or to the offspring coming from hybridization between *Clade I x Clade II* isolates. All isolated sampled *before* 2009 (*Clade II*) were observed on *Cucumis* species, while broader host range is related to a subset of *after* 2009 (*Clade I*) isolates. According to Cohen et al. (2013) there are two *P. cubensis* mating types: A1 with host preferences to *Cucumis* species, and A2 preferring other cucurbit hosts. Thus we can hypothesize that these clades and mating types are similar and that invading A1 isolate could caused the 1980s epidemics and prevailed in the population structure up 2009. Due to the indices to possible hybridization, it seems probable that genetically divergent isolate carrying the A2 mating type locus has recently appeared in the South-Eastern Europe and the Czech Republic, and caused a major reshuffling among the strains occurring in this region. Also the results obtained by ISSR and SRAP molecular markers indicate on migration and/or frequent sexual reproduction of the pathogen in Israel. Moreover the selected markers can be suggested for monitoring genetic diversity within *P. cubensis* isolates in further studies (Polat et al., 2014). Thanks to indices pointing to possible occurrence of sexual reproduction it seems possible that at least a minor part of pathogen population is surviving in the Czech Republic by the means of oospores. An overwintering of *P. cubensis* in the Czech Republic is also suggested by dramatic shift of *P. cubensis* population virulence and the subsequent continuation of a similar virulence structure (Lebeda et al., 2013a,b). Detailed description of sequencing results and comprehensive data interpretation will be published elsewhere (Kitner et al., 2014, under review).

Acknowledgements

The work of AL, MK and BS were supported by the Internal Grant Agency of the Palacký University in Olomouc (IGA_PrF_2013_001 and Prf-2014-001), project MSM 6198959215 (Ministry of Education, Youth and Sports) and the project QH 71254 (Czech Ministry of Agriculture). MT and RS are supported by LOEWE, Landesinitiative zur Entwicklung wissenschaftlich-ökonmischer Exzellenz, a funding program of the Government of Hessen, Germany, in the framework of the IPF – Integrative Fungal Research Cluster and the BiK-F – Biodiversity and Climate Research Centre.

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Virulence Structure (Pathotypes, Races) of Cucurbit Powdery Mildew Populations in the Czech Republic in the Years 2010–2012

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Additional index words. Host-pathogen interaction, *Cucumis melo,* Cucurbitaceae, *Golovinomyces orontii, Podosphaera xanthii,* virulence variability, differential set, new determination approach

Abstract. Virulence structure (pathotypes, races) of cucurbit powdery mildew (CPM) species, *Golovinomyces orontii s.l.* (*Go*) and *Podosphaera xanthii* (*Px*) were studied on a set of 115 CPM isolates (71 *Px*, 44 *Go*). Isolates originated from different host species of Cucurbitaceae, throughout various locations of the Czech Republic from 2010-2012. Previous work screened five *Px* isolates from different cucurbit hosts and pathogen populations from one German (GER) location (Erfurt, 2012). For the screening, there was used a set of 6 CPM pathotype differentials from Cucurbitaceae and 21 CPM race differentials of *Cucumis melo* (Lebeda et al., 2008). In total, 6 different pathotypes (25, 27, 31, 47, 59 and 63) among Czech CPM isolates were detected and four of them (27, 31, 59, 63) were also found among GER *Px* isolates. Differences in frequency of individual pathotypes were noted between both CPM species and also among surveyed years. Altogether, 106 different races (40 *Go,* 66 *Px*) in Czech CPM populations and 5 various races among GER *Px* isolates (not found in Czech screened CPM populations) were determined. Differences in response to differential genotypes of *C. melo* were found within individual CPM species, between both CPM pathogens and as well as among the studied years. Only five *Px* and four *Go* races were detected repeatedly (two-times) in screened Czech CPM population. During the three-years of study, highly virulent pathotypes and races of both pathogens prevailed. The recent results also confirmed our previous studies showing that Czech CPM populations are very heterogeneous in terms of their virulence, and that these isolates differ significantly from other countries.

Recently, two genus (*Golovinomyces, Podosphaera*) and three species (*Golovinomyces orontii, G. cucurbitacearum, Podosphaera xanthii*) were reported as causal disease agents on cucurbits across Central Europe (Braun and Takamatsu, 2012). *Golovinomyces* is probably a complex of two species (*G. orontii, G. cucurbitacearum*) that are very closely related and differ in some morphological features of anamorph stages (Braun and Takamatsu, 2012). In the current manuscript, we used *Golovinomyces orontii s.l.* (*Go*) and *Podosphaera xanthii* (*Pf*) for cucurbit powdery mildew (CPM) causal agents occurred in the Czech Republic (CR). This two species differ in host range, environmental requirements and geographic distribution (Křístková et al., 2009). They are also highly variable in their pathogenicity and virulence as indicated by the existence of a large number of different pathotypes and races (Lebeda et al., 2011, 2012; McCreight, 2006, McCreight et al., 2012) and by variability in response to fungicides (Sedláková and Lebeda, 2008). Altogether, 7 pathotypes (4 *Go*, 3 *Px*) were worldwide described (Lebeda et al., 2011). Races of *Gc* and *Px* have, to date, been reported only on melon; two races of *Go* and about 31 races of *Px* have been worldwide identified on melons (Lebeda et al., 2011; McCreight et al., 2012). Recent results suggest that even more races exist (Lebeda et al., 2011; McCreight, 2006, McCreight et al., 2012). According our previous studies from CR (Křístková et al., 2004; Lebeda and Sedláková, 2004, 2006), Czech CPM populations are highly variable in their pathogenicity, and are unique and markedly different compared to those of some western and southern European countries and other parts of the world. 20 pathotypes (13 *Go*, 7 *Px*) and 90 *Go* races and 61 *Px* were identified in CR from 2000 to 2009 (Lebeda et al., 2011, Sedláková et al., unpubl. results). The majority of Czech CPM pathotypes (12 *Go*, 6 *Px*) and races (all *Go* races and 57 *Px*) have been detected only in CR (Lebeda et al., 2011, Sedláková et al., unpubl. results). Various independent systems of CPM pathotype and race determinations and denominations have been used worldwide. Lebeda et al. (2008, 2011) critically reviewed the current state, gaps, and perspectives in our understanding of pathogenicity variation in these two CPM pathogens at the pathotype and race levels. They proposed two sets of differential cucurbit genotypes for the identification of CPM pathotypes and races, and an objective, efficient, uniform and comprehensive coded system for meaningful, concise designation of CPM pathotypes (sextet code) and races (septet code). The preliminary study of a set of 18 CPM isolates using a new proposed race-differential set according to Lebeda et al. (2008) has been presented by Lebeda et al. (2012) at Cucurbitaceae 2012 (Adana, Turkey).

Here, we present a case study using a large set of Czech CPM isolates (115 isolates from 2010 to 2012) for application of the above approach for characterization and denomination of CPM pathotypes and races. The data described herein reveal novel features regarding the pathogenicity and host plant resistance variability among plant pathogens.

Acknowledgements. This research was supported by the following grants: MSM 6198959215, QH 71229 and Internal Grant Agency (PrF_2013_003, Prf_2014_001).

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Materials and Methods

Plant materials. Seeds of various genotypes of sets for pathotype (6 differentials of Cucurbitaceae family, Table 1) and race determination (21 differentials of *C. melo*, Table 2) were sown in Perlite and grown in an environmentally growth chamber. Seedlings were transplanted at the cotyledon stage and grown in a CPM-free greenhouse (for details see Lebeda and Sedláková, 2010).

Pathogen isolation, multiplication and maintenance. CPM samples were microscopically examined before isolation; those determined to be a mixture of *Px* and *Go* were excluded. Conidia of pure cultures were transferred by tapping onto primary leaves of highly susceptible cucumber (*Cucumis sativus*) 'Stela F_1'. 115 Czech CPM isolates (71 *Px*, 44 *Go*) isolated from different host species of family Cucurbitaceae from various locations of CR from 2010–2012 were used for the study. There have been also screened five *Px* isolates from different cucurbit hosts and pathogen populations from one German (GER) location (Erfurt, 2012). Isolates were cultured on leaf discs in plastic boxes [one isolate per box; 24 °C/18 °C (day/night)] for 12 h. Multiplication and maintenance of isolates was performed as previously described (Lebeda and Sedláková, 2010).

Determination and denomination of pathogenic variability. All CPM isolates described above were screened for pathogenic variability (pathotypes, races) by a leaf-disc method (Bertrand et al., 1992; Lebeda and Sedláková, 2010). For pathotype determination, there was used a set of 6 CPM pathotype differentials from family Cucurbitaceae proposed by Lebeda et al. (2008) and races were detected using the set of 21 *C. melo*–CPM race differentials proposed also by Lebeda et al. (2008) (Tables 1 and 2). Each genotype was represented by three leaf discs (15 mm diam.) from true leaves (2 to 3-leaf stage) in three replicates (one replicate per plant). Discs were inoculated by tapping a primary leaf of cucumber 'Stela F_1' covered with 3- to 4-day-old, sporulating mycelia and incubated under the conditions described above. CPM infection of each disc was evaluated 6 to 14 days post-inoculation using a 0–4 scale (Lebeda, 1984). Data were used to calculate a degree of infection (DI) value for each genotype that was classified as resistant ($0 \leq DI \leq 1$), or susceptible ($1 < DI \leq 4$). Pathotype identifications of all screened CPM isolates were determined using sextet system proposed by Lebeda et al. (2008). Races were detected using the triplet-septet system according to the same authors (Lebeda et al., 2008).

Table 1. Proposed CPM pathotype differentials by number. (according to Lebeda et al., 2008).

No.	Taxon	Differential	Original source	Country of origin
1	*Cucumis sativus*	Marketer 430	–	Czech Republic
2	*Cucumis melo*	Védrantais	–	France
3	*Cucumis melo*	PMR 45	USDA	USA
4	*Cucurbita pepo*	Kveta[x]	–	Czech Republic
5	*Cucurbita maxima*	Goliáš	–	Czech Republic
6	*Citrullus lanatus*	Sugar Baby	–	the Netherlands

[x] 'Kveta' was proposed by Lebeda et al. (2008) to replace 'Diamant F1' in the standard set of CPM pathotype differentials.

Table 2. Proposed CPM race differentials by triplet group and number. All are *Cucumis melo* (according to Lebeda et al., 2008, 2012).

Group No.	Differential: Designation	Other designation(s)	Original source	Country of origin
1.1	Iran H	–	–	Iran
1.2	Védrantais	–	–	France
1.3	PI 179901	Teti	–	India
1.4	PI 234607	Sweet Melon	–	South Africa
1.5	ARHBJ	AR Hale's Best Jumbo	USDA	USA
1.6	PMR 45	–	USDA	USA
1.7	PMR 6	–	USDA	USA
2.1	WMR 29	–	USDA	USA
2.2	Edisto 47	Ames 8578	Koelz 2563	USA
2.3	PI 414723	LJ 90234	PI 371795	India
2.4	PMR 5	–	USDA	USA
2.5	PI 124112	Koelz 2564	–	India
2.6	MR-1	–	PI 124111	USA
2.7	PI 124111	–	–	India
3.1	PI 313970	VIR 5682	–	India
3.2	Noy Yizre´el	–	–	Israel
3.3	PI 236355	–	–	England
3.4	Negro	–	–	Spain
3.5	Amarillo	–	–	Spain
3.6	Nantais Oblong	–	–	France
3.7	Solartur[z]	–	–	Czech Republic

[z] Not included in the proposed set (Lebeda et al., 2008) and could be replaced by another genotype after discussion.

Results and Discussion

A high variability in virulence (mainly at the level of races) was found in the surveyed CPM populations (2010 through 2012) by using a new approach for characterization and denomination of CPM pathotypes and races proposed by Lebeda et al. (2008). Using this method, we identified a variation in virulence (pathotypes and races) between both CPM species as well as among individual years during the three-year period of study.

In total, 6 different pathotypes (25, 27, 31, 47, 59, and 63) were identified in Czech CPM surveyed populations. (Table 3, detailed data from individual years are not presented here). Except pathotype 25, which was detected only at *Go,* all the others were found in both CPM species. Nevertheless, the frequency of occurrence of individual pathotypes varied between both CPM species as well as among surveyed years. In Czech CPM populations, there were prevailed highly virulent pathotypes. This phenomenon corresponds with the situation in the previous years (2000–2009) in Czech CPM populations (Lebeda and Sedláková, 2004, 2006, 2010; Lebeda et al., 2007, 2011; Sedláková et al., unpubl. results) and verified the shift in virulence in Czech CPM populations toward the prevalence of highly virulent strains. Among five German *Px* isolates isolated from one location (Erfurt), we identified four different pathotypes (27, 31, 59, 63), with pathotype 31 detected twice. In the case of German isolates, it's surprising the we identified a large number pathotypes in one location. Unfortunately there isn't available any information about variation in virulence (at the level of pathotypes and races) in German CPM populations from the previous years. This is the first report about virulence (at the level of pathotypes) from Germany, more detailed study at this field would be necessary in the future.

At present, limited information exists regarding CPM populations worldwide, yet it is know that the majority of these isolates are related only to *Px.* Because there is no unified system

Table 3. CPM pathotype sextet codes[x] identified from 2010 through 2012 using a new proposed system by Lebeda et al. (2008). See Table 1 for identification of the pathotype differentials.

Differential number / Differential value								Country/No. of isolates		
								Czech Republic		Germany
1	2	3	4	5	6	Sextet	Total no.	CPM species[y]		
1	2	4	8	16	32	code	of isolates	*Go*	*Px*	*Px*
1	0	0	8	16	0	25	1	1	–	–
1	2	0	8	16	0	27	14	6	7	1
1	2	4	8	16	0	31	39	13	24	2
1	2	4	8	0	32	47	2	1	1	–
1	2	0	8	16	32	59	14	3	10	1
1	2	4	8	16	32	63	50	20	29	1
							120	44	71	5

[x]CPM pathotype sextet codes are based on compatability scores summed across pathotype differentials.
[y]*Go–Golovinomyces orontii, Px–Podosphaera xanthii.*

describing the determination and denomination of pathotypes worldwide, the work described herein represents a foundation for further comparative studies in this area. Indeed, previous work has shown that recently detected CPM pathotypes in Czech CPM population from 2010 to 2012 differ from pathotypes of both species detected in France (Bertrand et al., 1992) and from *Px* pathotypes reported from Spain (del Pino et al., 2002). And they verified also our previous results from our long-lasting study of this phenomenon and showed that Czech CPM populations are very heterogeneous, unique in their virulence (at the level of pathotype) and vary from CPM populations from other countries from Europe and in the world (Bertrand et al., 1992; Lebeda et al., 2011; del Pino et al., 2002).

Altogether, 106 different races (40 *Go,* 66 *Px*) in Czech CPM populations and 5 various races among GER *Px* isolates (not found in Czech screened CPM populations) were defined using a new approach for characterization and denomination of CPM races previously proposed by Lebeda et al. (2008). Only five *Px* (19.4.125, 55.15.125, 55.39.125) and four *Go* races (23.0.125, 55.63.119, 55.31.127, 103.63.71) were detected repeatedly (i.e., more than twice) in screened Czech CPM population. During the three-year period of study, highly virulent races of both pathogens prevailed, with Czech CPM populations exhibiting a temporal shift to higher virulence. The race diversity of among these 115 CPM isolates verified previous observations of great racial diversity of both CPM pathogens in Czech Republic (Lebeda et al. 2007, 2011; Lebeda and Sedláková 2006), (Křístková al., 2004; Lebeda a Sedláková, 2004, 2006, 2010; Lebeda et al., 2007, 2011; Sedláková, pers. commun.) and confirmed that Czech CPM populations are unique, highly variable and different in race structure from other countries (Bardin et al., 1997, 1999; Hosoya et al., 2000; McCreight , 2006; McCreight and Coffey, 2011; McCreight et al., 2012; Miazzi et al., 2011; del Pino et al., 2002). Differences in response to 21 *C. melo* differentials were found within individual CPM species, between both CPM pathogens and as well as among the studied years (Fig. 1, detailed data from individual years are not presented here). None of the races of both pathogens infected all 21 differentials. Ten differentials were susceptible to the majority of CPM isolates of both species (75% to 100% of susceptible reactions): 'Iran H' (1.1), 'Védrantais' (1.2), 'PI 179901' (1.3), 'ARHBJ' (1.5), 'PMR 45' (1.6), 'PI 414723' (2.3), 'PI 313970' (3.1.), 'PI 236355' (3.3), 'Nantais Oblong' (3.6), and 'Solartur' (3.7). On other two differentials: 'WMR 29' (2.1) and 'Edisto 47' (2.2), there were

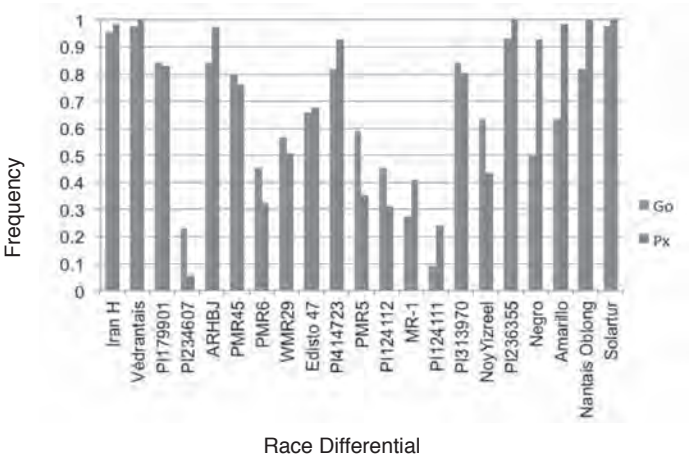

Fig. 1. Frequency of occurrence of susceptible reactions of *C. melo* race differentials in reaction to the set of Czech CPM isolates (*Golovinomyces orontii, Podosphaera xanthii*) originated from 2010 to 2012.

observed similar reaction of screened isolates of both pathogens when more than a half of CPM isolates (50% to 66%) expressed profuse sporulation there. However, there were noted differences in reaction between both CPM species to some differentials (see below). Whereas more than 90% of *Px* isolates infected two differentials: 'Negro' (3.4), 'Amarillo' (3.5), less *Go* isolates (only 50% to 65%) showed the similar reaction pattern. On other two differentials: 'NoyYizreel' (3.2) and 'PMR 5' (2.4), there were opposite situation between both pathogens when 59% to 64% *Go* and 35% to 44% *Px* isolates expressed profuse sporulation there. Both CPM species differ also in reaction to 'PMR 6' (1.7) and 'PI 124112' (2.5) even though within individual species the reaction was very similar (46% *Go* isolates infected these two differentials as compared to 31% to 32% *Px*). Differentials MR-1 (2.6) and 'PI 124111' (2.7) that are closely related, were distinctly different from each other in response to both pathogens. Frequency of occurrence of compatible reactions on 'PI 124111' (2.7) ranged from 9% to 24% and on 'MR-1' (2.6) was higher (27% to 41%). 'PI 234607' (1.4) was resistant to 94% *Px* and 77% *Go.* There was no obvious relationship between source species and race identity for either CPM species. Cucumber

and squashes, which are commonly grown in CR have not been reported to exhibit CPM–race specific interactions. Melon is a minor crop in the Czech Republic limited to home gardens, yet the Czech CPM populations exhibited many virulence factors on the 21 melon differentials.

Our results presented herein revealed the diversity and classification previously described by our group (Lebeda et al., 2012). In total, the data described show that the new set of 21 differentials and septet code could characterize more completely the race variation among CPM populations. Results regarding to the determined CPM pathotypes using a new approach proposed by Lebeda et al. (2008) are the first published application of a new methods.

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Evidence for Cucurbit Powdery Mildew Pathogen Races Based on Watermelon Differentials

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Additional index words. *Podosphaera xanthii, Citrullus lanatus* var. *lanatus, Cucumis melo*

Abstract. Powdery mildew (PM) caused by *Podosphaera xanthii* occurs in open fields and greenhouses and can severely limit cucurbit production. Presently seven races of *P. xanthii* have been identified using melon (*Cucumis melo*) differentials. Physiological races of this pathogen have not been classified for other cucurbits because of the lack of differentials and fully resistant germplasm. We have developed several highly resistant watermelon (*Citrullus lanatus* var. *lanatus*) germplasm lines by pure line selection from plant introductions (PI). We evaluated 11 PM isolates collected from various cucurbit plants on cotyledons of five watermelon germplasm lines, cultivar Mickey Lee, melon differentials PMR 45 and Iran H and squash cultivar EPSN. All isolates grew and produced abundant conidia on Iran H, and EPSN. A watermelon germplasm line derived from PI 269677 (USVL677-PMS) was susceptible to all 11 isolates. None of the PM isolates grew on the four highly resistant USVL germplasm lines. Of the 11 PM isolates that grew on USVL677-PMS, only three grew on Mickey Lee. Of these three that grew on Mickey Lee, two were melon race 1 and one a melon race 2. Based on the reactions of Mickey Lee and USVL677-PMS to PM isolates, we can identify the presence of at least two races using two watermelon differentials.

Powdery mildew caused by *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*), is a foliar pathogen and can be a severe limiting factor in cucurbit production in open fields and greenhouses. Powdery mildew can result in reduced vigor of the seedlings and in some instances death of the seedlings (Jarvis, 1992). The pathogen can infect cotyledons, stem, leaves and fruit (Keinath and Dubose, 2004; Kousik et al., 2011; Cohen et al., 2004). Powdery mildew has also been reported to significantly reduce the yield of watermelon (Keinath and Dubose, 2004; McGrath, 2010). In addition, powdery mildew can also predispose the plant to other foliar diseases (McGrath, 2010). In recent years the occurrence of powdery mildew on watermelons has increased significantly based on the number of reports from various states within the United States.

Powdery mildew has been reported on most cucurbits (Hammett, 1977; McGrath and Thomas, 1996; Cohen et al., 2004). A detailed description of the cucurbit powdery mildew pathogen and race classification system has been provided by Cohen et al. (2004). Seven races of *P. xanthii* have been identified using melon (*Cucumis melo* L.) differentials (Cohen et al., 2004; McGrath and Thomas, 1996; Pitrat et al., 1998). A new race (called race S) has been detected on melons in Georgia in recent years (McGrath, 2010). In another study the potential for existence of 28 putative races based on an extended set of melon cultivars and PI was also suggested (McCreight, 2006). Physiological races of this pathogen have not been clearly classified for other cucurbits because of the lack of differentials and fully resistant germplasm (Cohen et al, 2004).

Recently, watermelon varieties and pollenizers with resistance intermediate resistance to powdery mildew have been released by seed companies. Therefore, it will be important to determine if races can be identified based on watermelon differentials. So far the race classification based on melon differentials has been used for evaluating watermelon germplasm (Davis et al., 2007; Tetteh et al., 2010). The term Race 1W has been used if the powdery mildew isolate can infect watermelon leaves and the melon differential Iran H, or Vedrantais but not PMR-45. The term race 2W has been used if the powdery mildew isolate can infect watermelon leaves and the melon differential Iran H, Vedrantais and PMR-45. For the past few years we have been developing advanced watermelon (*Citrullus lanatus* var. *lanatus*) lines with high levels of resistance to powdery mildew by pure line selection from plant introductions (PI). In the present study we used some of these resistant lines and a highly susceptible watermelon line derived from PI 269677 to determine if a race structure can be identified based on watermelon differentials.

Materials and Methods

Powdery mildew isolates were collected from various cucurbit crops from across the eastern United States (Florida, Georgia, New York, and South Carolina). Cucurbit crops from which the isolates were collected include: watermelon, squash, melon, bottle gourd, and pumpkin. To determine if the isolates belonged to the genus *Podosphaera*, conidia from all isolates were treated with 3% potassium hydroxide solution (3 % KOH) on a microscopic glass slide. Treated conidia were examined for the presence of fibrosin bodies (McGrath and Thomas, 1996; Kousik et al., 2011). In addition the ITS region of several isolates used in this study were previously sequenced to confirm that the powdery mildew isolates from cucurbit crops were *P. xanthii* (Kousik et al., 2008, 2011). All of the powdery mildew isolates were routinely maintained

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on cotyledons of the squash variety Early Prolific Straight Neck (EPSN) in petri dishes. The isolates were aseptically transferred to fresh EPSN cotyledons on a monthly basis.

Plants of potential watermelon differentials were germinated in 50 cell jiffy pots in a laboratory that was not being used for any research and had minimal human activity. Watermelon lines used were derived from advanced selections PI 482362, PI 482288, PI 560010, PI 560005, PI 494531, PI 560006 (all *C. lanatus* var *lanatus*) and PI 189225 (*C. lanatus* var. *citroides*). Cotyledons from an advanced watermelon line (USVL677-PMS) derived from PI 269677, and was specifically selected for susceptibility to powdery mildew. The commercial watermelon variety Mickey Lee released in 1986 in Florida was also included in the study. The melon differentials PMR-45 (race 1 resistant and race 2 susceptible) and Iran H (susceptible to both race 1 and 2) were also included to confirm the melon race designation (McGrath and Thomas, 1996). In addition the melon race designations for these isolates were determined previously on the melon differentials (McGrath and Thomas, 1996). Cotyledons of the squash variety EPSN were included as a positive susceptible control to monitor powdery mildew inoculation and development.

Cotyledons from the individual powdery mildew resistant and susceptible lines were harvested and placed on a blue blotter paper disc on water agar petri plates. Four to five cotyledons were placed in each 10 cm (diameter) petri dish by inserting the petiole into the water agar (Fig. 1). The blotter paper prevented the direct contact of cotyledons with the agar and helped reduce other fungal contamination. The cotyledons were sprayed with a conidial suspension in water with 0.02% Tween 20 of each individual powdery mildew isolate. The plates were placed on laboratory benches (25 ± 2 °C) that were illuminated with fluorescent lights on a 12 h hour cycle. All isolates had two replications for each watermelon differential and the experiment was repeated twice.

Fourteen days after inoculation the cotyledons were observed under a stereo-binocular microscope and rated on a 0–10 scale of increasing powdery mildew severity; 0 = no powdery mildew mycelium or conidia observed and 10 = entire cotyledon covered with powdery mildew conidia.

Fig. 1. Cotyledons excised from advanced powdery mildew resistant and susceptible watermelon germplasm and placed on blotter paper in a water agar plate. The one cotyledon at the bottom of the petriplate showing abundant powdery mildew development was from the susceptible watermelon germplasm line USVL677-PMS. The remaining four cotyledons with no powdery mildew are the resistant watermelon germplasm lines.

Results and Discussion

All isolates grew and produced abundant conidia on cotyledons of the squash variety EPSN that were included as a positive check in the study. Similarly all the isolates also produced abundant conidia on the melon differential Iran H.

The watermelon germplasm line derived from PI 269677 (USVL677-PMS) was highly susceptible to all the isolates tested regardless of the host the isolate was collected from. Abundant conidia were produced by all 11 isolates on USVL677-PMS (Fig. 1). None of the PM isolates grew on the highly resistant USVL germplasm lines that were derived from various powdery mildew resistant PI suggesting the broad nature of resistance of these germplasm lines. Of the 11 PM isolates that grew on USVL677-PMS, only three grew on the watermelon variety Mickey Lee. The remaining isolates did not produce mycelial growth or conidia on Mickey Lee cotyledons. Of these three that grew on Mickey Lee cotyledons, two were melon race 1 (1W) and one a melon race 2 (2W) based on their reaction to the melon (*C. melo*) differentials Iran H and PMR-45.

Based on the reactions of Mickey Lee and USVL677-PMS to the eleven PM isolates, we can simply identify the presence of at least two races on watermelon. All the isolates that grow on USVL677-PMS and not on Mickey Lee can be considered as one race and those that grow on both Mickey Lee and USVL677-PMS as the second race. These powdery mildew races on watermelon that we described may be melon race 1 or 2 based on melon differentials. The classification of race 1W or race 2W in previous literature (Davis et al., 2007; Tetteh et al., 2010) was based on melon powdery mildew race differentials and the ability to infect watermelon. If the powdery mildew isolate did not infect watermelon the designation 'W' would not be added. However, in our study all the isolates we evaluated produced abundant conidia on the watermelon line USVL677-PMS. PI 269677 from which USVL677-PMS was developed for increased susceptibility was also susceptible to powdery mildew race 1W and race 2W in previous studies (Davis et al., 2007; Tetteh et al., 2010). Thus USVL677-PMS can be considered as the susceptible watermelon differential for powdery mildew race classification. Various systems of race designations have been described for the cucurbit powdery mildew pathogen (Lebeda et al., 2012; McGrath and Thomas, 2006; MCCreight, 2006).

Because of the lack of highly resistant differentials, races were not previously classified for watermelon or other cucurbits. However, our study indicates the presence of at least two races based on a line derived from one highly susceptible genotype and an old commercial watermelon cultivar Mickey Lee. It is also likely that with the development and release of new cucurbit powdery mildew resistant watermelon varieties and pollenizers new races may emerge. Therefore, to extend the life of the newly released resistant sources, an integrated approach including the use of conventional (Keinath and Dubose, 2004; Keinath et al., 2010) and organic fungicides (McGrath, 2005, 2010) as well as other strategies to manage powdery mildew must be adopted.

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Fruit Age and Development of Phytophthora Fruit Rot on Resistant and Susceptible Watermelon Lines

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Abstract. Phytophthora fruit rot caused by *Phytophthora capsici* is an emerging disease in most watermelon producing regions of Southeast U.S. and has resulted in severe losses to watermelon growers especially in GA, SC and NC. We recently released four germplasm lines (USVL203-PFR, USVL020-PFR, USVL782-PFR and USVL489-PFR) with high levels of resistance to Phytophthora fruit rot for use in breeding programs. To evaluate these lines further, plants of each of these along with fruit rot susceptible cultivars were planted at weekly intervals for five consecutive weeks. Flowers were routinely inspected and pollinated to produce fruit of differing ages. Fruit from all plants of varying ages were harvested on the same day and inoculated with a 5-mm agar plug from an actively growing colony of *P. capsici.* Inoculated fruit were maintained in a room with >95% RH for five days after which data on disease development (lesion diameter, pathogen growth, & sporulation intensity) was recorded for each fruit. Fruit of susceptible checks (Sugar Baby and PI 536464) were susceptible at all ages, and the resistant germplasm lines were resistant at all fruit ages. Significantly lower amounts or no *P. capsici* DNA was detected in fruit tissue of the resistant germplasm lines compared to the susceptible lines using qPCR at all fruit ages. Our results suggest that, resistance or susceptibility to Phytophthora fruit rot in watermelon is not correlated with fruit age.

Phytophthora capsici is a serious and devastating pathogen on many vegetable crops in the families' Solanaceae, Cucurbitaceae, Fabaceae and has been reported to cause disease on plants belonging to around 20 other families (Hausbeck and Lamour, 2004; Granke et al., 2012). Phytophthora fruit rot of watermelons was first reported in 1940s from Colarado in the United States (Wiant and Tucker, 1940). The disease is now prevalent in most of the watermelon growing areas in the south and north eastern states which produce over 50 % (77,000 acres) of the U.S. watermelons valued at $274 million (USDA NASS, 2010; McGrath, 1996; Gevens et al., 2008; Kousik et al. 2011). Phytophthora fruit rot is also considered an important research priority by the National Watermelon Association (NWA) and the emergence of this pathogen also was responsible for the start of the International *Phytophthora capsici* conference which has been held in Florida every other year since 2007.

Phytophthora fruit rot can be a pre- or postharvest problem and if the disease occurs early during fruit formation it could result in total loss in cucurbit crops.

For example, between 2003 and 2008 and again in 2013 many watermelon farms did not harvest the crop in Georgia, South Carolina and North Carolina due to severe fruit rot (preharvest). In some instances fruits rotted (postharvest) after shipping, resulting in rejection of loads and loss of revenue (Jester and Holmes, 2003; Kousik et al., 2011).

Various strategies are recommended for managing *P. capsici* and these include a combination of several control methods such as cultural practices that ensure well-drained soils, crop rotation, soil solarization, reducing splash dispersal of soil, and application of fungicides (Babadoost, 2004; Hausbeck and Lamour 2004; McGrath, 1996; Kousik et al., 2011; Granke et al., 2012). Several commercial fungicides that are effective in managing Phytophthora fruit rot of watermelon have been identified (Kousik et al., 2011). However, the prevalence of *P. capsici* isolates insensitive to fungicides such as mefenoxam and cyazofamid also has been well documented (Keinath, 2007; Kousik and Keinath, 2008; Hausbeck and Lamour, 2004; Granke et al., 2012). Furthermore, the application of fungicides is not very effective when disease pressure is high (McGrath, 1994; Kousik et al., 2011; Granke et al., 2012). Therefore, alternative strategies such as host resistance for managing Phytophthora fruit rot are needed, but are not yet available in commercial watermelon varieties.

Host plant resistance can be considered the cornerstone of an integrated disease management program (IPM), and watermelon cultivars with resistance to *P. capsici* would be extremely useful in management of Phytophthora fruit rot. It has been extremely challenging to identify and develop resistance to Phytophthora fruit rot in cucurbits. Gevens et al. (2006) identified cucumber varieties that limit development of *P. capsici,* however, none of the varieties tested had complete fruit rot resistance. More importantly, resistance in cucumber was related to the developmental stage of the fruit, increasing with increasing age and size of the fruit (Ando et al., 2009; Gevens et al., 2006). We identified several watermelon plant introductions (PI) with varying levels of resistance to Phytophthora fruit rot (Kousik et al., 2012) and by pure line selection developed highly resistant germplasm from

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these PI (Kousik et al., 2014). However in these studies for identification of sources of resistance all the evaluations were done when the fruit were mature. The present study was conducted to determine if resistance to Phytophthora fruit rot holds up at different development stages of the fruit.

Materials and Methods

Seeds: The original seeds of U.S. watermelon PI belonging to the core collection were obtained from PGRCU, Griffin, GA, USA (http://www.ars-grin.gov/cgi-bin/npgs/html/desc.pl?151021). We recently developed and released four Phytophthora fruit rot resistant watermelon germplasm (Kousik et al., 2014) using pure line selection procedure from the most resistant PI that were identified (Kousik et al., 2012). The four germplasm lines used in this study were USVL020-PFR, USVL203-PFR, USVL489-PFR and USVL782-PFR and these were derived from PI 560020, PI 595203, PI 186489 and PI 306782 respectively. For evaluating these lines we included a highly susceptible line from the PI collection, PI 536464 as a comparison. The commercial watermelon variety Sugar Baby was also used as a susceptible control and seeds were purchased from Willhite seeds (Willhite seeds, Pooleville, TX, USA).

Pathogen. *Phytophthora capsici* isolate RCZ-11 which is highly aggressive on watermelon fruit and causes severe rot was used as the source of inoculum. The isolate was collected in 2003 from Zucchini (*Cucurbita pepo*) plants in South Carolina and belongs to mating type A2 and was kindly provided by Dr. A.P. Keinath, Clemson University. The isolate was routinely maintained on V8 juice agar amended with antibiotics (PARP) as described by others (Keinath, 2007; Quesada-Ocampo et al., 2009) and was routinely re-isolated from watermelon fruit to maintain its virulence.

Field planting and harvesting. The study was conducted for three summers (2011, 2012 and 2013). In 2011 four germplasm lines (USVL020-PFR, USVL203-PFR, USVL489-PFR and USVL782-PFR) and two susceptible checks (Sugar Baby and PI 536464) were evaluated. In 2012 and 2013 only two of the resistant germplasm lines (USVL020-PFR, USVL203-PFR) and the two checks were evaluated. Plants of each of the lines were seeded in 50-cell Jiffy trays (Jiffy Products of America, Norwalk, OH) filled with Metro Mix (Sun Gro Horticulture, Bellevue, WA) and allowed to germinate and grow in a greenhouse for four weeks. To harvest fruit of different ages, watermelon transplants were grown by seeding once every week for a total of five consecutive weeks in each of the three years. Four week old plants were transplanted every week for a total of five consecutive weeks on to 96 cm wide raised beds covered with white plastic mulch. Standard watermelon production practices with respect to irrigation and weed management were followed (Sanders, 2006; Kemble, 2010). Female flowers were pollinated and each developing fruit was tagged by monitoring the plots regularly. When most of the fruit from the first planting were mature, all the fruit were harvested and assessed for resistance to Phytophthora fruit rot as described below.

Fruit rot assessment. To handle the large number of fruit of varying sizes and ages to be evaluated, a walk-in-humidity chamber (4 × 3 × 3.7 m height) with wire shelves was used as described before (Kousik et al., 2013, 2014). Tagged fruit at various growth stages from the different germplasm lines, and susceptible checks were placed in a completely randomized design on the wire shelves. Fruit were surface disinfested with 10% sodium hypochlorite prior to inoculation (Gevens et al., 2006; Granke et al., 2012a). Each fruit was inoculated by placing a 5-mm agar plug from a 4-day-old actively growing isolate of *P. capsici* in the middle as described before (Kousik et al., 2012). After inoculation, high relative humidity (>95%) was maintained in the room using a humidifier and the temperature was maintained at 26 ± 2 °C. The room was continuously illuminated with fluorescent lights. Five days after inoculation the lesion diameter, diameter of the area with visible pathogen growth and sporulation was measured (pathogen growth diameter). The intensity of sporulation within the lesion was recorded on a 0–5 scale, where 0 = no visible sporulation, and 5 = abundant sporangia, very dense and covering most (>85%) of the lesion area as described before (Kousik et al., 2012). The length and width of each fruit was also measured to determine the area of each fruit covered by lesion (%) as fruit size varied greatly due to fruit of different ages.

After completing the disease ratings, fruit tissue (10 mm length × 7 mm diameter) from fruit of all ages were collected from either side of the agar plug using a 7-mm cork-borer for use in qPCR analysis to quantify *P. capsici* DNA in the fruit tissue as described before (Kousik et al., 2012).

Results and Discussion

Phytophthora Fruit rot developed very rapidly on the two susceptible checks (Sugar Baby and PI 536464) in all the three years and by five days severe rot was observed on susceptible fruit of all ages. Sporulation was very intense on fruit of PI 536464 at all fruit ages (Fig. 1). Similar results were observed for Sugar Baby (Fig. 2). Fruit of PI 536464 were highly susceptible and in some instances completely collapsed due to fruit rot. In contrast the fruit of the four resistant germplasm lines were resistant

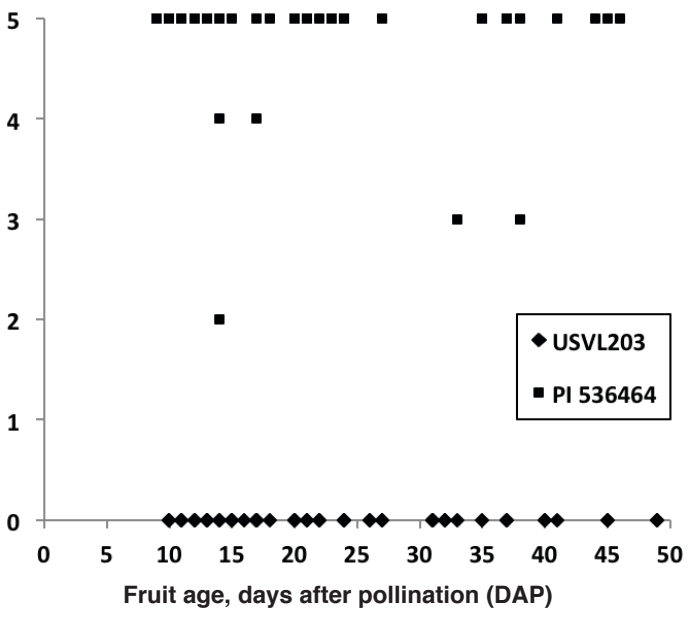

Fig. 1. Sporulation intensity on a 0-5 scale on fruit of the Phytophthora fruit rot resistant germplasm line USVL203-PFR (♦) and a susceptible line PI 536464 (■). Intense sporulation was observed on fruit of PI 536464 at all ages with most fruit having heavy sporulation that were rated as 5 on the 0–5 scale. However, fruit of USVL203-PFR were resistant at all ages.

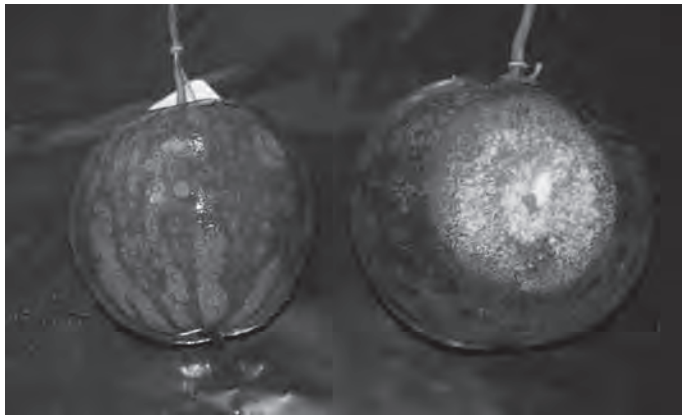

Fig. 2. Intense sporulation and mycelial growth of the fruit rot causing pathogen *Phytophthora capsici* on fruit of susceptible variety Sugar Baby (dark fruit) on right and no lesion or sporulation on fruit of the resistant germplasm line USVL020-PFR.

at all ages. No sporulation was observed on fruit of the four resistant germplasm lines (Fig. 1). Significantly lower amounts to no *P. capsici* DNA was detected in fruit tissue of the resistant germplasm lines compared to the susceptible lines using qPCR at all fruit ages (*data not shown*). Resistance or susceptibility to Phytophthora fruit rot was not correlated with the age of the fruit. Similar results were observed on the susceptible and resistant lines in all three years.

In studies with cucumbers it was observed that infection generally occurred on the blossom end in the field which was found to be more susceptible (Ando et al., 2009). However, we observed no difference in the level of infection when watermelon fruit were inoculated at the peduncle, middle or the blossom end with respect to lesion diameter or sporulation intensity (Kousik et al., *unpublished*) on resistant or susceptible accessions. Therefore in these studies we inoculated at the middle of the fruit. In addition, it was easier to measure the disease parameters in the middle of the fruit with the advantage that the agar plug did not slide and fall off despite the high relative humidity in the room. Watermelon fruit of a susceptible cultivar Crimson Sweet in the study by Ando et al. (2006) were susceptible at all developmental stages, similar to observations in our studies on Sugar Baby and PI 536464.

From a disease management point of view our data also suggests that fungicide application may be needed throughout the fruit development stages to manage fruit rot on susceptible varieties. Alternatively it also suggests that once fruit rot resistant varieties are developed, it is likely that they may hold up their resistance at all ages.

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Identification and Genetic Mapping of a Dominant Gene for Resistance to Powdery Mildew in Squash (*Cucurbita pepo*)

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Additional index words. Squash (*Cucurbita pepo*), powdery mildew (PM), Resistance gene, Molecular markers

Abstract. Powdery mildew (PM) is one of the most destructive pathogens that badly reduce the production of squash (*Cucurbita pepo*) all over the world. PM infection can significantly reduce squash yield up to 40 %. Developing resistant squash cultivars is the best approach to reduce the losses. So far, many PM resistant genes have been described in squash, but none have been mapped. This study was carried out to identify molecular makers linked to PM resistance in the squash inbred line 'BS6' which showed high level PM resistance in our multiple year screening studies. Inheritance analysis in F1, F2 and BC1 populations suggested that the resistance to PM race 2F in BS6 was conferred by a single dominant gene, which was designated as *Pm-1*. From bulked segregant analysis, two SSR markers *SSR237* and *SSR14* were identified flanking Pm-1 at at a genetic distance of 5.2 and 8.7 cM, respectively. The molecular makers identified here are useful in in marker-assisted selection in squash breeding for PM resistance, and for further cloning of the resistance gene.

Powdery mildew (PM) is a common and serious disease of squash (*Cucurbita pepo*) worldwide. Over-wintering chasmothecia produce ascospores that then develop into whitish colonies on leaves, leaf petioles, and stems, which leads to premature loss of foliage and, subsequently, to loss of fruit quality and yield (Glawe, 2008). PM is caused by two fungal species, *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea* Schlech ex Fr. Poll.) and *Golovinomyces cichoracearum* (syn. *Erysiphe cichoracearum* DC ex Merat.). *P. xanthii* has been identified as the primary cause of PM in cucurbits of China (Wang et al., 2006). Fungicides and resistant varieties are the major means of PM control. But fungicides are costly, environment unfriendly and also can be rendered ineffective through emergence of insensitive races of the pathogen (McGrath, 2001). Resistant cultivars provide a more economical and environmentally safe control strategy, but few squash cultivars in China are highly resistant to PM.

Disease resistance breeding has traditionally been done by phenotypic selection. The efficiency of phenotypic selection is reduced by variability in the pathogen, infection, and disease development (Cohen et al., 2004). Molecular markers tightly linked to PM resistance genes can eliminate these drawbacks to enable more efficient breeding strategies in squash improvement (Eileen et al., 2010; Rebecca and James, 2001). Development of molecular markers linked to the squash PM-resistant genes will facilitate marker-assisted selection (MAS) and map-based gene cloning of PM-resistant genes. However, although a number of PM resistant genes have been described, none of them have been molecularly mapped (Cohen, et al., 2003).

Here, we report inheritance of PM resistance in squash inbred line BS6 to a unique race of *P. xanthii* and identification of two SSR markers linked to this dominant PM resistance gene in BS6. These SSR markers will be helpful for breeders to develop new PM-resistant squash by MAS, and for further PM-resistant gene cloning.

Materials and Methods

Plant materials. Two squash elite inbred lines, PM resistant BS6 (P1) and susceptible BS11 (P2) (Fig. 1), and their F_1 (30 plants), F_2 (911 plants), and reciprocal backcross-1 progeny (BC1P1, 60 plants; BC1P2, plants) were used for investigation of the inheritance and genetic mapping of PM resistance in this study.

Powdery mildew source, inoculation, and evaluation. PM was collected at National Engineering Research Center for Vegetables (BVRC, Beijing), and determined to be *P. xanthii* based on the presence of fibrosin bodies by microscopic observation. The inoculum used in screening test was obtained from a monosporic culture and maintained on young plants of squash cv. Hubafu in an isolated greenhouse. The PM isolate was characterized before the genetic study based on the reactions of 13 commonly used race differentials (Table 1; McCreight, 2006). The differential genotypes were arranged in a randomized complete-block design with three replications and 15 plants for each differential per replication. PM race characterization, inoculation, disease index (DI) calculation and genetic studies were done as described by Liu et al. (2010).

Acknowledgements. The work was supported by grants from the National Natural Science Foundation of China (Project 31101547), the Beijing Municipal Science and Technology Project (2131100003113012), the Beijing Academy of Agriculture and Forestry Sciences international Cooperation Finance of China (CGJHZ2013-), the Beijing Municipal Bureau of Finance of China (czzx008-08), and the Special Fund for Agro-scientific Research in the Public Interest (201303112).

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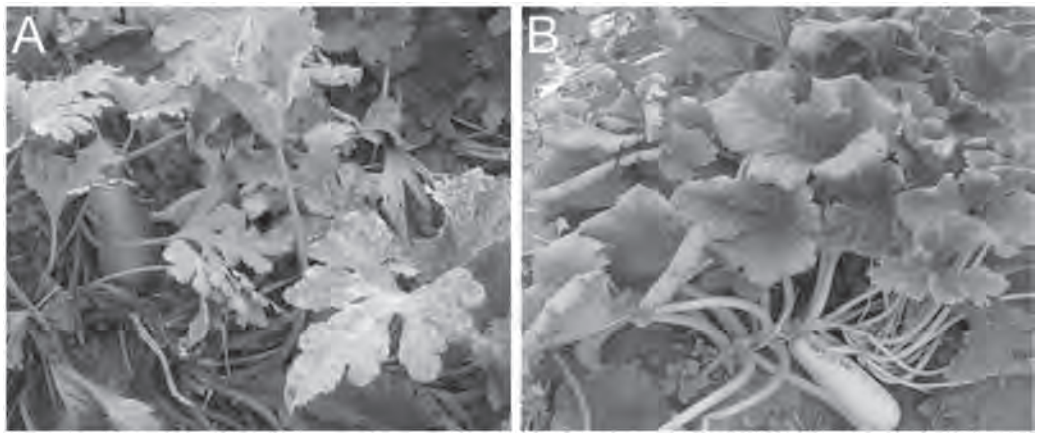

Fig. 1. Greenhouse performance of squash inbred line BS11 (**A**) and BS6 (**B**) after inoculation with powdery mildew race 2F.

Table 1. Reaction of melon cucurbit powdery mildew race differentials to *P. xanthii* isolate in a controlled inoculation test.

Differential lines	Disease index	Phenotype
Iran H	9.5	S
Ve´drantais	8.3	S
Top Mark	8.6	S
Nantais Oblong	9.2	S
PMR 45	8.1	S
PMR 5	0	R
PMR 6	0.2	R
WMR 29	0	R
Edisto 47	0.1	R
PI 414723	0.1	R
MR 1	0.3	R
PI 124111	0.8	R
PI 124112	0.7	R

DNA extraction and molecular marker analysis. Squash genomic DNA was extracted from young leaves using a CTAB method (Murray and Thompson 1980). For bulked segregant analysis, two resistant and two susceptible DNA bulks were generated from the F_2 population and each composed of ten plants. Microsatellite (SSR) markers (total 743) were screened for polymorphism between the parental lines and the resistant and susceptible bulks. The resulting polymorphic markers were then used to genotype the F_2 population. PCR amplifications were carried out in 15 µL reaction containing 30 ng DNA, 3.0 pmol of each primer, 2 mM dNTPs, and 1 unit of *Taq* DNA polymerase (Takara, China) in 1 Taq buffer. The PCR program included an initial denaturing at 95 °C for 3 minutes followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 35 s at 72 °C, followed by a final extension at 72 °C for 5 min. The products were separated on 8% non-denaturing polyacrylamide gels and visualized using silver staining (Sambrook and Russell 2001).

Genetic mapping. The linkage map was constructed with the software package MAPMAKER/EXP, Version 3.0 (Lander et al., 1987). The recombinant frequencies between the PM resistant locus and SSR markers were calculated through two-point tests and linkage map was constructed by three-point or multiple-point tests with a LOD threshold of 3.0. The recombination values were converted into centiMorgans (cM) using the Kosambi mapping function.

Table 2. Disease reactions of BS6, BS11, and their F_1 and segregating generations to *Podosphaera xanthii* race 2F.

Lines or populations	Resistant	Susceptible	Expected ratio	χ^2	*P*
BS6 (P1)	30		All resistant		
BS11 (P2)		30	All susceptible		
F_1	30		All resistant		
F_2	698	213	3:1	1.31	0.25
BC_1P1 (F1×BS6)	60		All resistant		
BC_1P2 (F1×BS11)	34	26	1:1	1.07	0.30

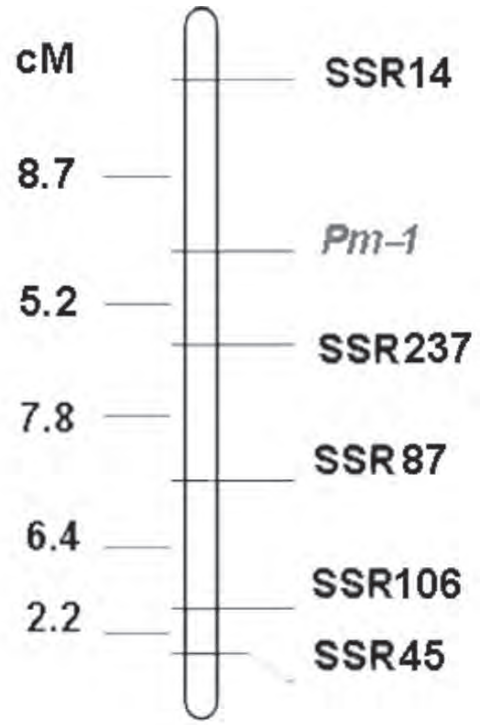

Fig. 2 Microsatellite markers linked with powdery mildew resistance locus *Pm-1* conferred by squash inbred line BS6. Map distance on the left is in centimorgans (cM).

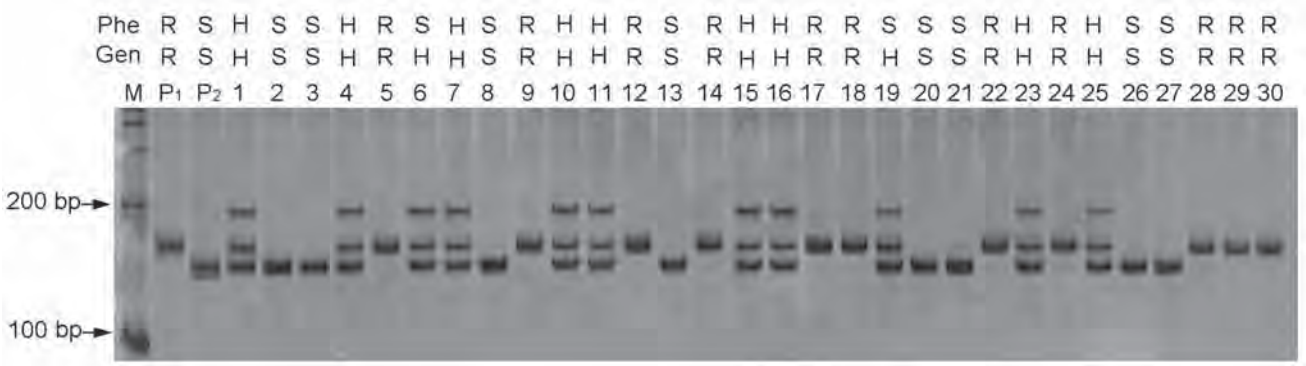

Fig. 3. Electrophoresis patterns of polymerase chain reaction-amplified with primer SSR237. Genomic DNA of F_2 lines (30 samples) was used as a template DNA; M = 100-bp ladder; P_1= BS6 (resistant), P_2 = BS11 (susceptible); Phe, Phenotype; Gen, Genotype. Individuals 6 and 19 are recombinants between *Pm-1* and SSR237.

Results and Discussion

Evaluation of the powdery mildew race in squash by melon differentials. The isolate of PM used in this research was collected from squash leaves, and identified based on the reaction pattern of 13 melon differentials (Table 1). Resistance of PMR 5, PMR 6, WMR 29, Edisto 47, PI 414723, MR-1, PI 124111, PI 124112 and susceptibility of Iran H, Ve´drantais, Top Mark, Nantais Oblong, and PMR 45 demonstrated that the powdery mildew race was *P. xanthii* race 2F (McCreight, 2006), suggesting the pathogens are the same for PM in melon and squash, which confirmed the results that *P. xanthii* race 2F was the predominant race in cucurbits in Beijing (Wang et al., 2006).

Genetic analysis for powdery mildew resistance. The parental line BS6 and F_1 were resistant after inoculation race2F of *P. xanthii* (DI < 1), suggesting that PM resistance of BS6 was controlled by a single, dominant gene (Table 2). The F_2 populations segregated 698 resistant to 213 susceptible fitting 3:1 ratio ($\chi^2 = 1.31$, $P = 0.25$). The backcross progeny plants were all resistant when BS6 was the recurrent parent, whereas segregation of resistant to susceptible plants in BC1P2 was in agreement with 1:1 ratio ($\chi^2 = 1.07$, $P = 0.30$). The results suggested that PM resistance in BS6 was consistent with a single dominant gene, which will be designated as *Pm-1*.

Identification of the SSR markers linked to the Pm-1 gene. Among 743 SSR primer pairs screened, 68 (9.2%) were polymorphic between the two parental lines, of which five also exhibited polymorphisms between the resistant and susceptible bulks. These five markers were applied to 911 F_2 plants resulting in a linkage map for *Pm-1*. The genetic linkage distances of *Pm-1* with SSR237 and SSR14 were 5.2 and 8.7 cM, respectively (Fig. 2). The PM phenotypes of F_2 plants were highly consistent with the genotype of the marker SSR237 (Fig. 3) indicating that this marker may be very useful in marker-assisted selection of PM resistant squash plants and future cloning of this resistant gene.

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Pseudoperonospora cubensis Sporangia Trapping As a Tool for Early Detection and Epidemiological Studies of Downy Mildew in Michigan

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Additional index words. Pickling cucumber, *Cucumis sativus*, Burkard volumetric spore sampler

Abstract. Pickling cucumbers are worth $28.7 million to Michigan farmers and $240.7 million to the state's pickle industry. Michigan cucurbit production totals $85.1 million in value; the state ranks first in the United States for pickling cucumbers and squash, and sixth for pumpkin and fresh market cucumber. Historically, fungicides were not required to protect these crops from downy mildew (*Pseudoperonospora cubensis*), but the pathogen has been a recurring problem in Michigan since 2005 with initial infections potentially occurring early in the growing season and disease pressure lasting until harvest. Chemical control is costly although required when *Ps. cubensis* is present and the weather favorable. Significant resources can be saved by delaying the initiation of the fungicide program when the risk of disease is low. Monitoring pathogen influx into the Michigan growing region each season and testing new fungicides have been part of Michigan State University's research and extension program since 2005. Burkard volumetric spore samplers were established in key cucumber growing regions in Michigan yearly. Spore reels were processed and daily sporangia counts uploaded to a webpage (www.veggies.msu.edu) throughout the growing season; visits to the website indicate that this is a tool used by growers to determine when fungicides should be applied. Fluctuations in hourly sporangial counts throughout the day were observed, with peak concentrations occurring the in the morning and early afternoon. Total 4-hour airborne sporangial concentrations were positively correlated with average air temperature and negatively correlated with both average RH and total leaf wetness in the same for 4-hour period.

Historically, the *Pseudoperonospora cubensis* downy mildew pathogen has not been a concern of cucurbit growers in Michigan due to genetic resistance bred into the cucurbit crops. The first downy mildew outbreak in 2005 occurred suddenly and was so widespread and severe, that many growers were unable to react quickly enough to protect their large acreages of cucumber crops, which are highly susceptible to the pathogen (Savory et al., 2011). Costs of fungicides alone for downy mildew in cucumbers can reach $1,482/ha.

Downy mildew causes catastrophic losses in a brief period of time; unprotected foliage becomes completely blighted within 19 days of infection (Fig. 1A–B). Once the foliage dies, the fruits stop developing, quality decreases, sun scald occurs, and secondary rots develop (Fig. 1E). Repeated fungicide applications are needed to protect the cucumber crop and their cost reduces the already low profit margin of these crops. In order to prevent yield losses, plants must have a protective barrier of fungicide present on the leaves when downy mildew sporangia are deposited on them (Fig. 1F). Growers may need to apply up to 12 applications to protect their crop. Since 2005 new and experimental fungicides have been developed to manage this disease. These newer chemistries rely on site-specific modes of action, which have a higher likelihood of inducing fungicide resistance in the pathogen. Fungicides need to be evaluated yearly to determine their efficacy at managing the local downy mildew populations.

The downy mildew pathogen is only able to survive in growing regions that do not experience frost/freeze events or in greenhouses that carry over cucumber production during the winter (Ojiambo and Holmes, 2011). Lemon-shaped spores (sporangia) are produced in large numbers on the undersides of diseased cucurbit leaves and are released into the atmosphere where they travel via wind currents (Ojiambo and Holmes, 2011; Granke and Hausbeck, 2011). In Michigan, spores enter fields annually and may come from greenhouses or southern states (Ojiambo and Holmes, 2011; Quesada-Ocampo et al., 2012).

In Michigan, downy mildew has been a yearly threat since 2005. Growers wish to delay the initiation of fungicide applications in their fields to reduce costs and the risk of fungicide insensitivity. Spore traps have been established in Michigan's production regions since 2005 to alert growers to sporangia of *Ps. cubensis* in the air, and evaluate the relationship between spore concentrations, environmental conditions, and disease severity.

Materials and Methods

Volumetric spore samplers (Burkard Manufacturing Co., Ltd.) (Fig. 1C) with a sampling airflow rate of 10 liters/min were placed at various sites in Michigan counties (commercial and research farms) from 2006 to 2013. The number of spore samplers varied, depending on the year, from two (2012–2013) up to eight (2010) (Fig 2). The sites represented key points of entry to the state and/

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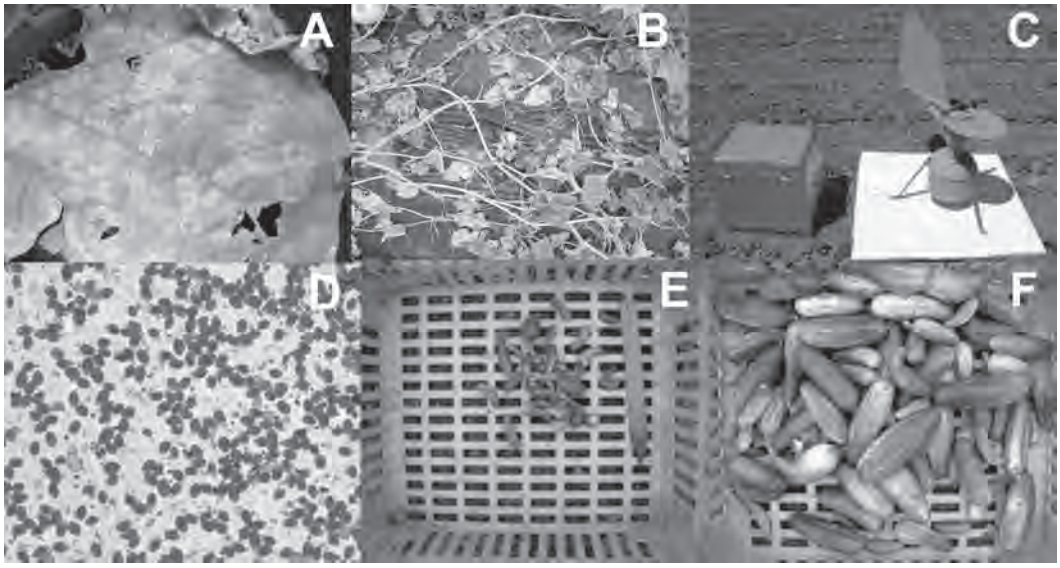

Fig. 1. (**A**) Downy mildew infection of an untreated cucumber leaf. (**B**) Blighting and death of leaves of untreated cucumber plants. (**C**) Spore trap placed in a field of cucumber seedlings. (**D**) Cucurbit downy mildew spores collected from the air and embedded onto a spore trap film (magnified 100×). Yield comparison of (**E**) untreated cucumbers, and (**F**) cucumbers treated with effective downy mildew fungicides.

or significant cucumber production areas. Samplers were placed with the orifice approximately 0.5 m above the ground in or near cucumber fields. The traps continuously sampled the air and spores were impacted onto Melinex tapes (Burkard Manufacturing Co. Ltd) coated with an adhesive mixture (Granke and Hausbeck, 2011; Granke et al., 2014). Tapes were removed weekly and collected downy mildew sporangia were taken to the laboratory for identification and quantification (Fig. 1D). *Ps. cubensis* sporangia were identified by morphological characteristics at x400 magnification. Spore counts were uploaded as available to the 'For Growers' webpage (www.veggies.msu.edu) during the growing season.

From 2008-11, hourly environmental data were collected using a Watchdog model 450 data logger, and a tipping bucket rain gauge (Spectrum Technologies) (Granke and Hausbeck, 2011; Granke et al., 2014). All statistical analyses were performed using SAS v9.1–9.3 (SAS Institute Inc., Cary, NC); data were transformed when necessary to stabilize the variance (Granke and Hausbeck, 2011; Granke et al., 2014). Variables were considered significant when the $P \leq 0.05$.

Field trials conducted in 2006-13 evaluated almost 150 new, registered and experimental products, fungicide programs, fungicide delivery mechanisms, or application intervals for their ability to control cucurbit downy mildew (Foster et al., 2010; Hausbeck and Cortright, 2009; Hausbeck and Glaspie, 2010; Hausbeck et al., 2010, Hausbeck and Cortright, 2012). Fungicide delivery mechanisms included foliar, drench or applied through the drip, and application intervals evaluated differences in control at 5- to 7-day intervals and 10-day intervals. Field trials were conducted at research farms and on site with grower cooperators and plants were grown according to standard cultural practices.

Results

Airborne sporangia were detected as soon as May and throughout September; concentrations increased during this period. In each year, spore samplers detected atmospheric concentrations of spores prior to the observation of downy mildew field symptoms in the county (Fig. 3). Hourly sporangial counts fluctuated, and peak concentrations were observed in the morning and early afternoon. Airborne sporangial concentrations were positively

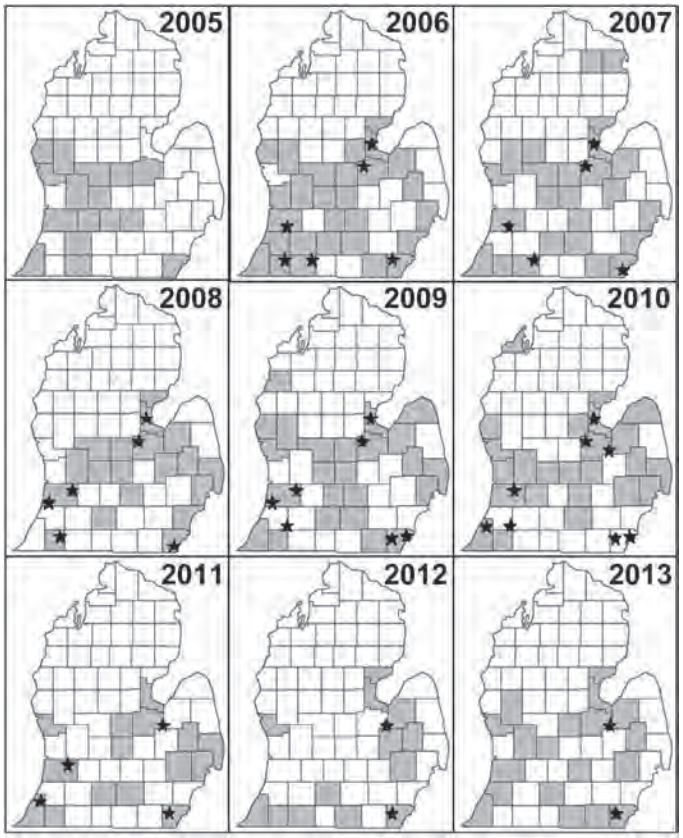

Fig. 2. Confirmed downy mildew reports by county (shaded areas) and locations of spore trapping sites (black stars) in Michigan from 2005 through 2013.

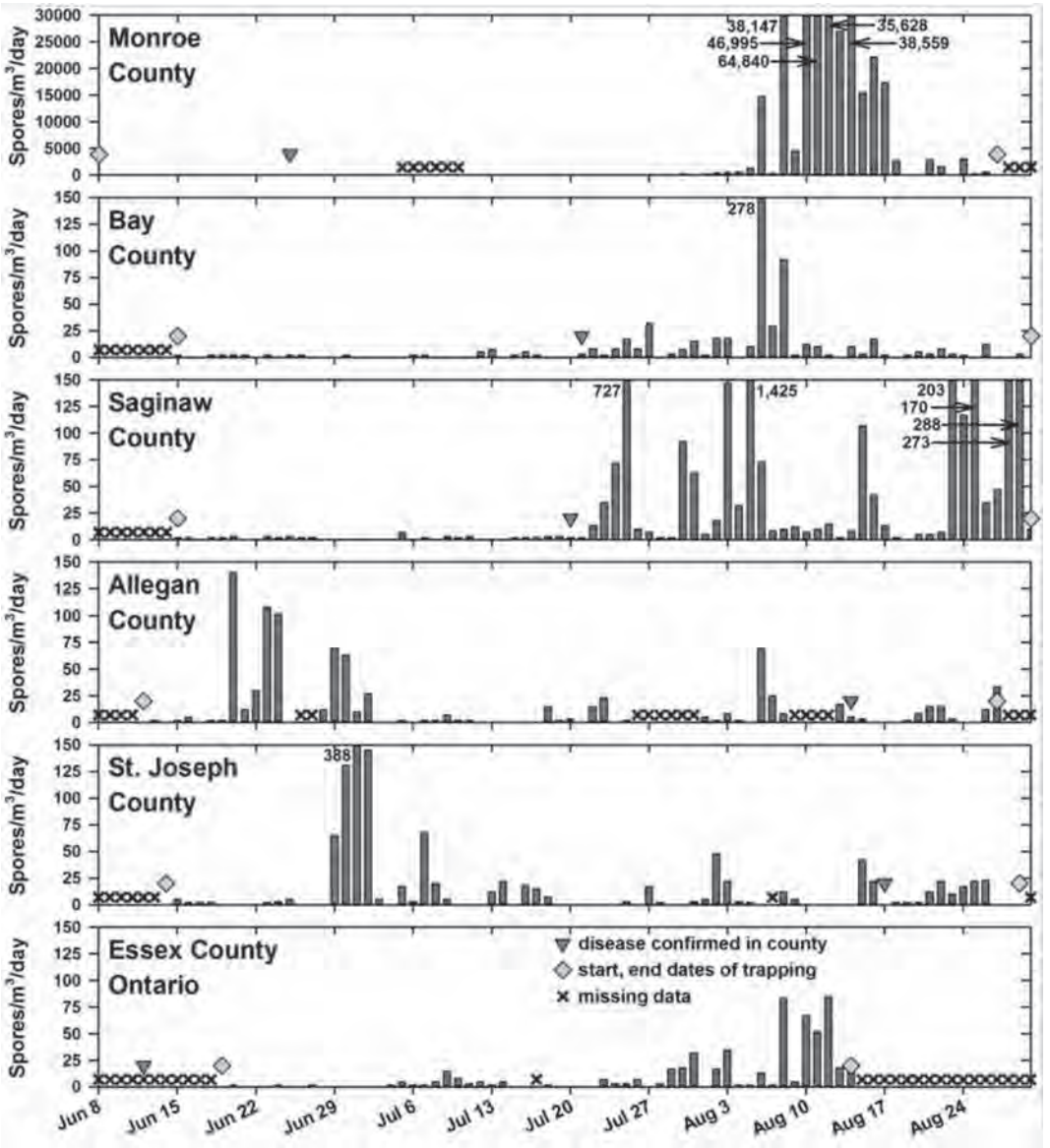

Fig. 3. Daily counts of Pseudoperonospora cubensis sporangia collected by volumetric spore traps located in five sites in Michigan and one site in Ontario from 8 June through 30 August, 2007.

correlated with temperature and negatively correlated with relative humidity and leaf wetness and peaked daily between 0800 and 1300 hours (2008-2009) [Granke and Hausbeck, 2011]. This research showed that sporangia are likely to be airborne above the crop canopy during periods of high temperatures and low relative humidity and leaf wetness. Temperature and leaf wetness were positively associated with disease occurrence, while solar radiation was negatively associated with it (2011–2012) (Granke et al., 2014).

Fungicide efficacy evaluations determined that fluopicolide (Presidio 4SC) can be applied via drip irrigation and provide effective downy mildew control. In 2011 and 2012, two experimental unregistered fungicides were determined to be effective against downy mildew when applied either as a spray or through the drip irrigation. Field trials focusing on different spray schedules found 5- to 7-day intervals to be more effective than 10-day intervals. One of the effective treatment programs in Michigan has included Presidio + chlorothalonil (Bravo), Presidio + Bravo alternated with propamocarb hydrochloride and carbamate monohydrochloride (Previcur Flex) + Bravo, and Previcur Flex + Bravo alternated with ametoctradin/dimethomorph (Zampro) + mancozeb (Manzate) [unpublished data].

Monthly visits to the 'For Growers' page (www.veggies.msu.edu) featuring downy mildew news and recommendations typically peaked in July and was viewed 3,441 and 3,251 times in 2010 and 2011, respectively (the next most popular page was visited 1,175 times).

Discussion

Michigan grew more than 18,211 ha of cucurbits in 2013, valued at over $103 million and ranked in the top five states in

the United States for the production of pickling cucumber (#1), squash (#2), fresh market cucumber (#4) and pumpkin (#5) (Anonymous, 2014). Since 2005, Michigan growers have battled downy mildew, incited by the water mold, *Ps. cubensis*. Since then, downy mildew costs Michigan growers and processors as much as $6.4 million annually in scheduled fungicide applications. The cost of frequent fungicide applications has weakened the Michigan cucurbit industry and narrowed or eliminated the profit margin for growers.

Spore sampling and current information on disease in surrounding counties can help growers determine when preventive fungicide sprays should start. Our data have shown periods of high airborne sporangial concentrations do not occur until disease has been confirmed in the county, which can occur weeks or months after spores are first detected. Growers rely on these spore counts, and incidence of disease in surrounding counties to determine when fungicides should be applied. MSU research has shown that some fungicides provided the same level of control when applied as drip as when applied as a foliar spray with less than 2% foliar disease symptoms and no crop defoliation. Applying fungicides through the drip irrigation systems in low and high tunnels would be invaluable to the cucurbit fresh market industry to limit disease developing from early downy mildew inoculum. The drip irrigation application was thought to be effective for soilborne diseases only; it was surprising that this application method also offered control of a foliar disease.

Over the years, our efforts have included monitoring airborne sporangial concentrations and environmental conditions suitable for infection, and testing and outreach in regards to effective fungicide programs for downy mildew management.

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Population Structure of *Pseudoperonospora cubensis* in Michigan and Canada

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Abstract. Cucurbit downy mildew (CDM), caused by the oomycete pathogen *Pseudoperonospora cubensis,* is a devastating disease that affects cucurbit species worldwide. In the United States (U.S.), approximately half a million acres of susceptible crops are planted annually. Michigan, the number one producer of pickling cucumbers in the U.S., grows approximately 46 thousand acres of susceptible cucurbits. Since 2005, growers have relied on fungicide sprays to manage CDM and pathogen resistance is increasingly common. Each growing season, this pathogen is dispersed from its overwintering sites by wind currents. Previous studies identified similar population structure among *P. cubensis* populations within the northern U.S. and Canada. To more closely evaluate the regional population structure of *P. cubensis*, sporangia from CDM lesions were collected from cucurbit foliage grown in Michigan and Canada field locations in 2011. Population structure and genetic diversity among the 100 isolates were evaluated using five single-copy simple sequence repeat (SSR) markers to assess differences between Michigan and Canadian isolates. Michigan and Canada had no differences in population structure at the macro level; however, individual Michigan counties had significant differences in population structure were compared against Canada.

Cucurbit downy mildew (CDM), caused by the oomycete plant pathogen *Pseudoperonospora cubensis,* is a foliar disease that can reduce harvestable yields by up to 100% (Holdsworth, et al., 2014; Call, et al., 2012). Michigan, the number one producer of pickling cucumbers, produces over 45,000 acres of susceptible crops each year. While *P. cubensis* affects many cucurbit species, cucumbers are the most susceptible and economically important host (Widrlechner, 2003).

P. cubensis is a wind-dispersed pathogen, producing abundant asexual sporangia. The pathogen does not overwinter in debris or the soil, and travels to fields each year from overwintering sites. In Michigan, spores can overwinter on greenhouse-grown cucurbits or enter the state through neighboring regions where cucurbit species are grown (Ojiambo and Holmes, 2011; Quesada-Ocampo et al., 2012; Granke et al., 2014). Sporangia can spread up to 1,000 km, though viability of the sporangia are limited by environmental conditions (Ojiambo and Holmes, 2007; Kanetis et al., 2010).

Prior to 2004, *P. cubensis* was primarily managed through host resistance introduced in the 1960s (Savory et al., 2011; Holmes et al., 2006). However in 2004, a major outbreak of CDM was reported in North Carolina (Holmes and Thomas, 2009) that quickly spread to surrounding states in the following months, and by 2005, major outbreaks were detected in Michigan (Granke and Hausbeck, 2011). Current management strategies rely on calendar-based fungicide applications every 5–7 days depending on the presence of the disease (Granke et al., 2014). In MI, the estimated cost to maintain this regime is $6 million annually (Granke et al., 2014; Savory et al., 2011). The efficacy of these applications has decreased with fungicide use due to the appearance of fungicide-resistant populations in several regions (Lebeda and Cohen, 2011; Urban and Lebeda, 2007; Moss, 1987; Wang et al., 2014; Zhang et al., 2008.

Population studies have evaluated changes in population structure in local and worldwide populations at the genetic and phenotypic level (Quesada-Ocampo et al., 2012; Lebeda et al., 2013; Sarris et al., 2009). In a global analysis of *P. cubensis* structure by Quesada-Ocampo et al. (2012), six genetic clusters were detected. Approximately 50% of the isolates evaluated had membership in a single cluster, while the remaining isolates were admixed (membership in more than one cluster). The Michigan isolates evaluated had significant genetic differentiation from fifteen of the other states evaluated including South and North Carolina, and Indiana, but was not different from Canadian populations (Quesada-Ocampo et al., 2012). The lack of regional differences could, in part, be due to limited samplings from Michigan and Canada. The objective of this study was to evaluate regional differences in cucurbit downy mildew population structure in Michigan and Canada cucumber fields during a single growing season.

Materials and Methods

Plant material and isolations. Twenty-five symptomatic cucumber leaves were collected from two previously described

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field sites in Michigan (Granke et al., 2014) and five fields in Canada. Samples were returned to the lab for CDM identification. The presence of *P. cubensis* was confirmed using morphological characteristics on symptomatic leaves (Zitter et al., 1996). Sporangia were removed from individual lesions by pipetting 200 µl of sterile ddH_2O water onto a lesion, gently dislodging the sporangia with the end of the pipette tip, and collecting the liquid. Sporangia collected from individual lesions are hereafter referred to as isolates. A total of 200 isolates were collected from Canada (125) and Michigan (75).

Molecular analyses. An aliquot of each individual sample was added to a single well of a 96 well plate and heated to 96 °C for 15 min to promote cell lysis as described by Calmin et al (2007). PCR was performed on each sample using the previously published *P. cubensis* primers for the beta tubulin gene for species confirmation (Quesada-Ocampo et al., 2012). Reactions were performed in 20 µl total volume and contained 1 µl DNA, and 0.40 µl GoTaq (Promega Corporation Madison, Wisconsin), 1.25 µl 25µM $MgCl_2$, 0.50 µl dNTPs, 0.04 µl of BSA, 1.25 µl of DMSO, 5.0 µl 5x GoTaq buffer, and 1.00 µl each of forward and reverse primers (Integrated DNA Technologies, Inc.), with 8.56 µl ddH_2O. PCR reactions were performed in a programmable thermal cycler (Eppendorf, Westbury, NY) using the program: initial denaturation, 94 °C (3 minutes) followed by 40 cycles at 94 °C (30s), 60 °C (30s) and 72 °C (1 minute), and a final extension step of 10 minutes at 72 °C. PCR products were analyzed by electrophoresis in 4% (wt/vol) agarose gel in 1x Tris-borate-EDTA buffer, stained with ethidium bromide (5 µg/ml) for visualization and compared to a 100-bp ladder (Invitrogen Life Technologies Burlington, ON Canada) to determine amplicon sizes. Samples that contained a band of the expected size were retained; all other samples were removed from analyses. Fifty primers, developed from single copy simple sequence repeats (SSRs) from the genome sequence of *P. cubensis*, were evaluated against a subset of eight individuals from the population to identify polymorphic markers. SSR markers identified as polymorphic in the population were used for population structure analyses. The sequenced genotype, MSU-1, (Tian et al., 2011) was included as a positive control.

Population structure. Population structure was evaluated in the software STRUCTURE (v 2.3.4) using the identified polymorphic markers (Pritchard et al., 2000). The number of genetic clusters was determined using the methods described by Evanno, et al. (2005). In brief, the log likelihood was compared for three replicates at each estimated number of genetic clusters (k) for k 1–15, using the admixture model with correlated allele frequencies and 500,000 MCMC chains with a burnin of 300,000 The population was sorted by country and field using the Population Sorting Tool (PST) in R (J.J. Morrice, R Development Core Team, 2012).

Results

One hundred isolates (51 from Michigan and 49 from Canada) were successfully amplified and used for molecular analyses. Most markers evaluated were unspecific or did not amplify in the subpopulation evaluated. Five markers were polymorphic across the entire population. For each plate, band size of each sample was normalized to the band size produced by the control isolate, MSU-1. The software STRUCTURE identified 2 genetic clusters (Ln = –1196.8, lambda = 1.153). Differences in clusters ranged from moderate to very great according to the population differentiation guidelines suggested by Hartl and Clark (2007). Ninety-six isolates belonged to a discrete cluster (membership was ≥ 0.60). Eight individuals with < 0.60 membership were classified as admixed, indicating that they were comprised of both clusters. Differentiation between the two populations was moderate (0.751). The isolates collected from Allegan County were represented by individuals from the purple and yellow clusters, similar to Canada (Fig. 1). Isolates from Berrien County were represented by the purple cluster only. Significant differentiation was detected between Allegan and Berrien counties, and Allegan and Canada. The average polymorphism information content (PIC) was moderately high (0.6209) within the population with both Michigan (0.6236) and Canada (0.55) having moderately high PIC values. Within MI, there was variation in PIC values between counties. Allegan County had a higher PIC value (0.6366) compared to Berrien County (0.49) Gene diversity was moderately high for Michigan (0.6743) and Canada (0.5912).

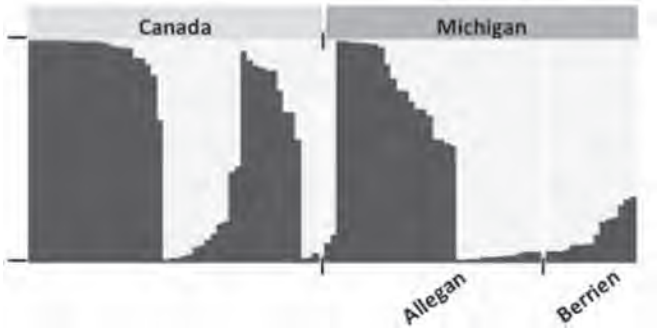

Fig. 1. Population structure of *Pseudoperonospora cubensis* isolates collected from cucurbit fields in MI (Allegan and Berrien counties) and Canada.

Discussion

Pseudoperonospora cubensis is a wind-dispersed, foliar pathogen capable of causing disease on cucurbits. The pathogen is characterized by prolific asexual reproduction, which allows it to quickly spread through fields during the growing season. Overwintering sites (greenhouses and southern states) can serve as inoculum sources for more northern states, where the pathogen could not normally survive.

Previous studies have shown that population structure is present in *P. cubensis* populations, populations can change annually, and Michigan and Canada have similar genetic structure (Quesada-Ocampo et al., 2012; Sarris et al., 2009). In this study, genetic variation was evident within the population. Two genetic clusters were detected, and were present in both countries at each site evaluated. The overall PIC in the population was moderately high (0.6209) suggesting the pathogen is highly heterozygous. Approximately 10% of the isolates were admixed, compared with the 50% detected by Quesada-Ocampo et al. (2012). This is likely due to differences in sampling and markers used for isolate genotyping. Quesada-Ocampo et al. (2012) evaluated isolates from multiple host species, countries, and years, while this study focused on a single host and one year of sampling in two countries.

In this study we compared the population structure of CDM between pickling producing regions of the Michigan and Canada. Michigan and Canada had no differences in population structure at the macro level. However, when individual Michigan counties

were compared against Canada, there were significant differences in population structure.

This work provides a more detailed comparison of regional cucurbit downy mildew populations between Michigan and Canada, and independently confirms previous findings with a different set of markers. Population structure was identified and changes in population structure varied by location. Future studies should evaluate more fields with higher resolution markers to better understand CDM population differences in Michigan and Canadian cucurbit fields.

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Molecularly Tagged Genes and Quantitative Trait Loci in Cucumber

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Additional index words. *Cucumis sativus*, molecular breeding, marker-assisted selection, gene cloning

Abstract. Since the release of the cucumber draft genomes, significant progress has been made in molecular mapping or cloning of economically important genes and quantitative trait loci (QTLs) in cucumber, which provides the foundation for marker-assisted selection in cucumber breeding. In this paper, I summarized molecularly tagged genes and QTLs for simply inherited traits (total 21), host resistances against the downy mildew, *Fusarium* wilt, gummy stem blight, powdery mildew, target leaf spot, CYSDV, and ZYMV (54 genes/QTLs), growth and development-related traits (128 genes/QTLs) such as leaf size, shape, color, hypocotyl length, plant architecture (lateral branch number, height, compact, internode length, trichomes, growth habit), traits related with flowers (flowering time and node positions, pistillate flower numbers, sex determination) and fruits (size, shape, number, weight, skin color, flesh color, spine color/density/number, bitterness), as well as seed-related traits (number, size and weight). The 203 genes/QTLs were placed on a scaffold-based cucumber physical map using the Gy14 draft genome assembly as the reference. These molecularly mapped genes and QTLs were located mainly in chromosomes 1, 5, and 6 with only 4 in Chromosome 7. Compared with field crops, available mutants and characterized genes in cucumber are very limited, but molecular mapping and cloning of more cucumber genes and QTLs is expected in the near future which will greatly facilitate marker-assisted cucumber breeding.

Cucumber, *Cucumis sativus* L. ($2n = 2x = 14$) is a diploid species with relatively small genome size (367 Mb), short life cycle (2-3 months from seed to seed) and no inbreeding depression, making it an advantageous crop in genetic and genomics studies. With the release of the cucumber draft genome assemblies and rapid development of cucumber genomics infrastructure such as molecular markers and high density genetic maps (e.g., Huang et al, 2009; Qi et al, 2013; Yang et al, 2012, 2013), significant progress has been made in molecular mapping, gene cloning and QTL mapping for horticulturally important traits in this specialty crop. Among the approximately 145 genes documented in the 2010 cucumber gene list (Call and Wehner, 2011), many have been tagged with molecular markers. Some traits, especially resistances to the downy mildew or powdery mildew pathogens were investigated in multiple QTL mapping studies. On the other hand, many newly mapped genes/QTLs were not listed in the 2010 cucumber gene catalogue. In this paper, recent literature was reviewed, and the cucumber gene list was updated including their physical locations in the cucumber genome. This work could serve as the start point for more systematic characterization and documentation of cucumber genes.

Materials and Methods

The literature on molecular tagging, gene cloning or QTL mapping in cucumber was reviewed. For most microsatellite markers that are linked with target genes or QTLs, their physical locations in the 9930 or Gy14 cucumber draft genome assemblies are known (Huang et al, 2009; Qi et al, 2013; Yang et al, 2012, 2013). For other types of markers, their physical locations in the Gy14 draft genome scaffolds were inferred with BLASTn sequence alignment or *in silico* PCR according to Cavagnaro et al. (2010). A Gy14-scaffold based physical map was drawn with the MapChart program (V2.2) (www.wageningenur.nl/en/show/Mapchart.htm) using their locations in the Gy14 assembly (Version 1.0) as reference. Due to page limit, only references directly reporting molecular markers associated with target traits were cited; literature identifying RAPD or AFLP markers that could not be aligned with the Gy14 or 9930 genome scaffold assemblies was not included. The various factors (marker types, population sizes, genetic backgrounds etc.) may affect the accuracy of the marker locations on a genetic map; therefore, the inferred scaffold locations for the genes or QTLs were, in many cases, only approximations.

Results and Discussion

Simply inherited genes. Molecular mapping and cloning of 21 simply inherited cucumber genes have been reported (Table 1). Consistent with classic genetic analysis, the *F* and *de* genes are linked, and several genes controlling epidermal features *D*, *Fr*, *H*, *Tu*, *u* and *ss* form a cluster in chromosome 5 (Fig. 1). Among the 21 genes, the two sex determining genes *m* (1-aminocyclopropane-1-carboxylic acid synthase-2, ACS2) (Boualem et al, 2009; Li et al, 2009) and *F* (ACS1g) (Trebitsh et al, 1997) and the tuberculate fruit gene *Tu* (C2H2 zinc finger transcription factor) (Yang et al, 2014) have been cloned. A β-carotene hydroxylase gene is believed to be a candidate for the orange flesh color locus *or* (Qi, 2013), and an R2R3-tye MYB transcription factor co-localizes with the

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Table 1. Molecular mapped simply inherited genes in cucumber.

Gene	Description	Chr	Gy14 sca*
B	black spine	4	s2901–s0919
bi-1	bitterfree foliage	6	s3611–s3487
bi-3	bitterfree fruits	5	s2951
Bt	bitter fruits	5	s1153
cp	compact	4	s2920
D	Dull fruit skin	5	s2633
de	Determinate growth	6	s1001–s0927
F	Femaleness	6	s0927
Fr	ribbed fruit	5	s2633–s0789
gl-2	glabrous-2	2	s4100–s1132
H	netted fruit	5	s2633–s0789
ll	littleleaf	6	s0998
m	bisexual flower	1	s2698
nlb	no lateral branch	1	s1357–s0923
or	orange flesh	3	s0931
Psm	paternal mitochon sorting	3	s3810
R	Orange mature fruit color	4	s2901–s0919
Tu	tuberculate fruit (warty)	5	s2633
u	uniform imm. fruit color	5	s2633–s0789
v1	Virescent leaf	6	s0998–s3904
w	white immature fruit color	3	s2995–s3356

*Cavagnaro et al. (2010); Yang et al. (2012, 2013).

black spine gene *B* which has pleiotropic effect on orange mature fruit color (gene *R*) (Li et al, 2013). Six genes, *bi-1*, *Bt*, *cp*, *D*, *ll* and *u* have been fine mapped and should be cloned soon.

Genes or QTL for disease resistances. Molecular markers for resistance genes or QTLs against six pathogens are available (Table 2). Genes for target leaf spot (*cca*), zucchini yellow mosaic virus (*zym*) and scab (*Ccu*) are simply inherited which are located in chromosomes 6, 2 and 5 respectively (Fig. 1). *Ccu* is located in a region containing a NB-LRR cluster (Kang et al, 2011); a vacuolar protein sorting-associated protein 4-like (VPS4-like) gene seems to be a candidate for the *zym* gene (Amano, 2013). The gene for *Fusarium* wilt (*Foc*) has not been mapped, but it seems to be co-localized with *Ccu* in chromosome 2 (Kang et al, 2011). Two closely linked QTL conditioning *Fusarium* wilt resistance from a different source were mapped in chromosome 6 (de Milliano, 2012).

The majority of mapped disease resistance QTLs is for downy mildew (DM) and powdery mildew (PM) (Table 2) which are distributed in all seven chromosomes. Despite the different sources of DM and PM resistances and the methods used for phenotyping, it seems that major-effect DM and PM QTLs are located in chromosomes 4 or 5 (Table 2, Fig. 1). A *mlo* type resistance gene homolog seems to be a good candidate for the major-effect PM resistance QTL *pm5.3* (*pm-h*) (Cai R., personal communication). One interesting result from these QTL mapping studies is co-localization of DM and PM QTLs, which is consistent with

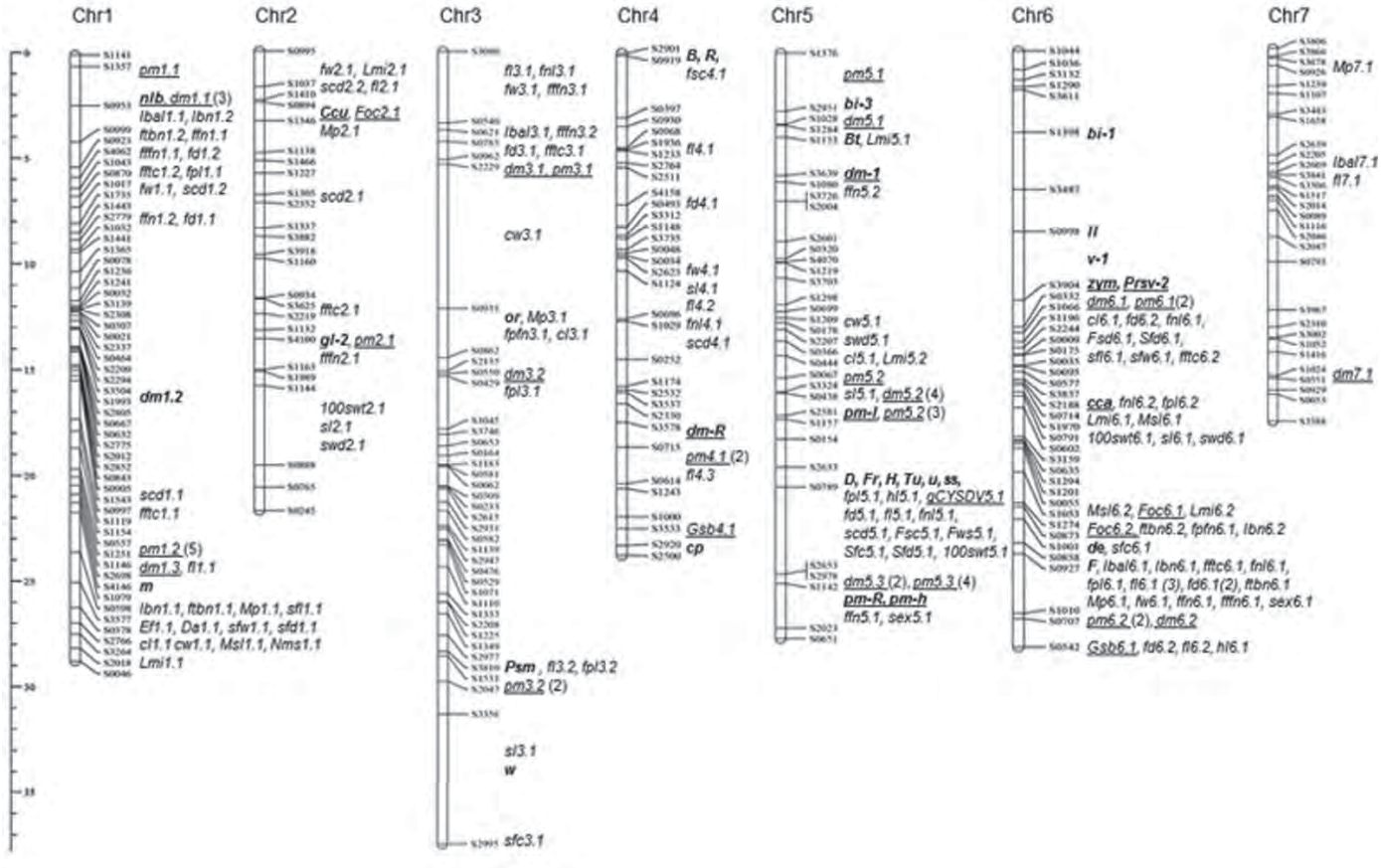

Fig. 1. Distribution of molecularly tagged cucumber genes and QTLs in the cucumber genome represented by the Gy14 draft genome assembly (Version 1.0) (Yang et al, 2012, 2013). The map was drawn with the location of each Gy14 scaffold in the assembly as the coordinate. Rule to the left is the physical length of Gy14 assembly in Mbp. Simply inherited genes and disease resistance genes/QTLs are boldface typed or underlined, respectively. Other loci are for growth and development-related horticultural traits.

Table 2. Molecular tagged genes and QTL for disease resistance in cucumber.

Gene/QTL[a]	Chr	Gy14 scaffolds[b]	Gene/QTL[a]	Chr	Gy14 scaffolds[b]
Target leaf spot			Powdery mildew		
cca	6	s0998–s2188	*pm1.1***	1	s1179–s1357
Scab			*pm1.1***	1	s3577–s0378
Ccu	2	s0894	*pm1.1****	1	s2779–s1543
Downy mildew			*pm1.2**	1	s1543
dm-1	5	s2951–s2601	*pm1.2***	1	s1119–s2018
dm1.1	1	s0953–s0923	*pm1.2***	1	s1543–s0557
*dm1.1***	1	s1357–s0999	*pm2.1**	2	s2219–s4100
*dm1.1****	1	s0953–s0999	*pm3.1**	3	s2208–s3356
dm1.2	1	s1032–s1543	*pm3.1**	3	s0962–s2229
dm1.3	1	s557–s1251	*pm3.1***	3	s3356
dm3.1	3	s0540–s2229	*pm4.1***	4	s1233–s2500
dm3.2	3	s0429	*pm4.1***	4	s2330–s2920
dm5.1	5	s2951–s1153	*pm5.1**	5	s2609–s0067
*dm5.1**	5	s1080–s0444	*pm5.1***	5	s3324–s0154
*dm5.1***	5	s0067	*pm5.1***	5	s1376–s1028
dm5.2	5	s0699–s2633	*pm5.2****	5	s1142–s2653
*dm5.2***	5	s0067–s2633	*pm5.2****	5	s2633–s2207
dm5.3	5	s2653–s2023	*pm5.2****	5	s2653
*dm5.3***	5	s2633–s0789	*pm5.3***	5	s2653–s2023
dm6.1	6	s3904	*pm5.3***	5	s2653
*dm6.1**	6	s0542	*pm6.1*	6	s0998–s3904
dm7.1	7	s1024–s0053	*pm6.1**	6	s0927
dm-R	4	s3578	*pm6.1***	6	s3611–s2188
Fusarium wilt			*pm6.2****	6	s0927–s0542
*Foc6.1**	6	s1053	*pm-h*	5	s2978
*Foc6.2****	6	s0873	*pm-l*	5	s2581
Gummy stem blight			*pm-R*	5	s2653
Gsb4.1	4	s1000–s2500	ZYMV		
Gsb6.1	6	s0927–0542	*zym*	6	s3904
CYSDV					
qCYSDV5.1****	5	s2633–s2653			

[a]Phenotypic variations explained by the QTL: * <10%; ** 10-25%; *** >25%
[b]Gy14 draft genome scaffolds (Cavagnaro et al. 2010; Yang et al. 2012)

observations by cucumber breeders that PM resistant cucumbers are often also resistant to DM. The DM resistance gene *dm-1* originated from PI 197087, which was overcome by a new strain since 2004 in the United States, seems to be consistent with the DM QTL *dm5.1* detected in PI 197088 (Yoshioka et al, 2014), but additional evidence is needed to confirm this.

Two QTLs for gummy stem blight (GSB) resistance were of *C. hystrix* origin and were mapped in chromosomes 4 and 6, respectively (Lou, 2013). For virus resistance, a major-effort QTL for cucurbit yellow stunning disorder virus (CYSDV) was mapped in chromosome 5 between the QTL for leaf (*pm-l*) and hypocotyl resistances (*pm-h*) against powdery mildew (de Ruiter et al, 2008).

Genes or QTL for growth and development-related traits. Among the 128 QTLs for growth and development related traits (Table 3), 21 were for flowers (flowering time, flower position, number, and ratios); 65 for fruits (fruit size/shape/weight, epidermal features), 11 for seeds (size and weight), and 31 for plant vegetative growth (nodes, branches, leaves, hypocotyl etc.). For the majority of these traits, more than two QTLs were detected for each. For some traits, QTLs for the same trait were mapped to the same chromosome location in different studies (Fig. 1) suggesting they may belong to the same QTL. Therefore, the actual number of QTLs for the traits summarized herein may be fewer than reported in original studies. In Fig. 1, some QTLs that were obviously belonging to the same locus were consolidated.

*Distribution of genes/QTL in chromosomes***.** A scaffold-based cucumber physical map was developed using Gy14 draft genome assembly as the reference, and all 203 genes/QTLs were placed on the resulting map (Fig. 1). It is obvious that the distribution of these gene/QTL loci is nonrandom, which were 41, 14, 24, 16, 47, 57, and 4, respectively from chromosomes 1 to 7. Distribution within the chromosomes was also not even. In particular, a significant amount of QTLs were detected in three regions where the *m*, *F* and a cluster of genes controlling epidermal features have been mapped.

Conclusion

The most recent cucumber gene catalogue (Call and Wehner, 2011) documented 145 genes (genes for isozymes not counted), of which ~50 have been tagged with molecular markers (Fig. 1); about two dozen are under investigation; a few have not been studied or need to be re-evaluated, and most of the remaining mu-

Table 3. Summary of QTL underlying traits of growth and development in cucumber.

QTL	Chr	Gy14 sca
100–seed weight		
100swt2.1*	2	s0888–s1144
*100swt5.1**	5	s3703–s0789
*100swt6.1***	6	s0791–s0714
Cotyledon length/width		
cl1.1	1	s1079–s3577
*cl3.1***	3	s0931
cl5.1	5	s3223–s0444
*cl6.1**	6	s3904
cw1.1	1	s1079–s3577
*cw3.1***	3	s2229–s0931
*cw5.1**	5	s1298–s1209
Days to anthesis		
*Da1.1**	1	s1079–s3577
*Ef1.1****	1	s3577
Fruit diameter		
fd1.1	1	s0870–s1443
*fd1.2**	1	s0953–s0923
fd3.1	3	s0540
fd4.1	4	s2511–s1148
fd5.1	5	s2633
fd6.1	6	s0927–s1019
*fd6.1**	6	s0542
fd6.2	6	s3904
First female flower node		
*fffn1.1**	1	s0953–s0999
fffn2.1	2	s4100
fffn3.1	3	s3080
fffn3.2	3	s0540
*fffn6.1***	6	s0927
First flower node		
*ffn1.1***	1	s0953–s0923
ffn1.2	1	s0870–s1443
ffn5.1	5	s2978–s1142
ffn5.2	5	s1080
*ffn6.1***	6	s0927
Fruit flesh thickness		
fftc1.1	1	s2775–s1543
*fftc1.2***	1	s0953
fftc2.1	2	s0934–s2219
fftc3.1	3	s0540–s0621
fftc6.1	6	s0927
fftc6.2	6	s1066–s0095
Fruit length		
*fl1.1**	1	s1251–s1146
fl2.1	2	s1037–s0894
fl3.1	3	s3080
fl3.2	3	s2977–s2047
*fl4.1**	4	s0614
*fl4.1***	4	s0696
*fl4.2**	4	s1936
*fl5.1**	5	s2633–s2653
fl6.1	6	s0927
fl6.1	6	s0927–s1019
*fl6.1**	6	s0927–s0542
fl6.2	6	s0542
fl7.1	7	s1658–s2046
Fruit neck length		
*fnl3.1**	3	s3080
*fnl4.1***	4	s0696
fnl5.1	5	s2633
fnl6.1	6	s0927
*Fnl6.1***	6	s3904–s1970
fnl6.2	6	s2188–s0714
First female flower node		
*Fpfn3.1**	3	s0931
*Fpfn6.1****	6	s1274–s0858
Fruit pedicle length		
fpl1.1	1	s0953
fpl3.1	3	s0429
fpl3.2	3	s3810–s2047
fpl5.1	5	s2633–s0789
fpl6.1	6	s0927
fpl6.2	6	s2188–s3837
Fruit spine color/density		
*Fsc4.1**	4	s0919
Fsc5.1**	5	s2633–s2653
*Fsd6.1**	6	s3904–s1970
First lateral branch node		
ftbn1.1	1	s1079–s0858
*ftbn1.2**	1	s0953–s0999
*ftbn6.1**	6	s0858–s0927
ftbn6.2	6	s1274–s0858
Fruit weight		
fw1.1	1	s0953–s0923
fw2.1	2	s1037–s0894
fw3.1	3	s3080
*fw4.1**	4	s0048–s1124
*fw6.1**	6	s0927–s1019
Fruit wart size		
*Fws5.1****	5	s2633–s2653
Hypocotyl length		
*hl5.1**	5	s2633
*hl6.1***	6	s0542
Lateral branch length		
*lbal1.1**	1	s0953–s0999
lbal3.1	3	s0540
*lbal6.1**	6	s0858–s0927
lbal7.1	7	s3443–s0089
Lateral branch number		
lbn1.1	1	s1079–s0858
*lbn1.2**	1	s0953–s0999
*lbn6.1**	6	s0858–s0927
lbn6.2	6	s1274–s0858
Main stem internode length		
*Lmi1.1***	1	s1079–s3577
Lmi2.1	2	s1037
*Lmi5.1**	5	s1153
Lmi5.2	5	s3223–s0444
*Lmi6.1**	6	s2188–s1970
Lmi6.2	6	s1274
Multiple pistillate flowers		
Mp1.1	1	s1079–s3577
*Mp2.1**	2	s0894
*Mp3.1**	3	s0931
Mp6.1	6	s0858–s0927
*Mp7.1**	7	s3866–s0926
Main stem length		
*Msl1.1***	1	s1079–s3577
Msl6.1	6	s2188–s1970
*Msl6.2**	6	s1201–s1274
# nodes on main stem		
*Nms1.1****	1	s1079–s3577
Seed cavity diameter		
scd1.1	1	s0632–s0905
scd1.2	1	s0953
scd2.1	2	s1227–s1305
scd2.2	2	s1037–s–894
scd4.1	4	s0696
*scd5.1**	5	s2633–s0789
Female/male flower ratio		
sex5.1	5	s0782–s1142
*sex6.1***	6	s0927
Mature fruit color		
*sfc3.1**	3	s2995
*Sfc5.1**	5	s2633–s2653
Sfc6.1	6	s0858
Mature fruit length/diameter		
*Sfd1.1**	1	s1079–s3577
Sfd5.1	5	s2633–s2653
*Sfd6.1**	6	s3904–s1970
Sfl1.1	1	s1079–s3577
*Sfl6.1**	6	s3904–s1970
Mature fruit weight		
sfw1.1	1	s1079–s3577
*Sfw6.1**	6	s3904–s1970
Seed length		
*sl2.1**	2	s0888–s1144
sl3.1	3	s3356
sl4.1	4	s1148–s0696
*sl5.1**	5	s0438
sl6.1	6	s0791–s0714
Seed width		
swd2.1	2	s0888–s1144
*swd5.1**	5	s0178
*swd6.1**	6	s0791–s0714

tants have probably lost. As compared with field crops and some well-studied vegetable crops such as tomato, available mutants and genes cloned in cucumber are very limited. A better assembly of the cucumber draft genome is needed. Efficient functional genomics tools in cucumber are lacking. From cucumber breeding perspective, many of the molecular markers associated with target genes or QTLs are not breeders' friendly, or not closely enough for reliable prediction of the genotypes in marker-assisted selection. Nevertheless, it seems there are ample genetic variations in cucumber natural populations for which systematic evaluation is needed. Mutagenesis to create new variations is being conducted in several groups. More genetic and genomics tools are being developed which will expedite gene discovery, mapping and cloning in cucumber. As such, it is optimistic to expect significant progress in the near future.

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QTL Mapping of Flowering Time and Fruit Shape in Xishuangbana Cucumber (*Cucumis sativus* L. *var. xishuangbannanesis* Qi et Yuan)

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Abstract. The Xishuangbanna cucumber, *Cucumis sativus* L. var. *xishuangbannanesis* Qi et Yuan (XIS) is a semi-wild landrace growing in the tropical southwest China with some unique traits that are useful for cucumber breeding. One such accession is WI7167 that exhibits dark green leaves, short hypocotyl, round fruit shape, orange flesh color in mature fruits, and short-day length requirement for flowering. The genetic basis of these traits in the XIS cucumber is not well understood. In this study, with 138 F_2 plants derived from a cross between WI7167 (XIS cucumber, late flowering, round fruit shape) and WI7200 (cultivated cucumber, early flowering, short columnar fruit), we developed a genetic map with 225 SSR loci covering 775.2 cM in seven linkage groups. Phenotypic data on male and female flowering time, the length and diameter of mature fruits were collected from 136 F_2-derived F_3 families in field trials. QTL analysis identified two major effect QTLs (*fft5.1* and *fft6.1*) for female flowering time, one QTL (*mft6.1*) for male flowering time, three QTLs (*fl3.1*, *fl5.1* and *fl6.1*) for fruit length, and one QTL (*fd1.1*) for fruit diameter. These QTLs explained 9.8% to 67.4% of observed phenotypic variations. The major effect QTL *fft6.1* for female flowering time and *mft6.1* for male flowering time were co-localized in cucumber chromosome 6. Results from this study provide insights into crop evolution of the XIS cucumber.

Cucumber, *Cucumis sativus* L. ($2n = 2x = 14$) is an economically important vegetable crop worldwide. Among the several botanical varieties of *C. sativus*, the semi-wild Xishuangbanna cucumber (XIS hereinafter), *C. sativus* L. var. *xishuangbannesis* Qi et Yuan (Qi 1983) possesses some unique traits that are useful in cucumber breeding. For example, under greenhouse conditions, the XIS accession WI7167 grows vigorously with dark green leaves and good tolerance to low light. It has short hypocotyl and bears round fruits. The mature fruits of WI7167 have orange flesh color which is characteristic of the XIS cucumber because of accumulation of high level of beta carotene (Bo et al. 2011). Due to its semi-wild nature WI7167 requires certain short-days for flowering, and the growth period can last up to nine months. The genetic basis of these traits in the XIS cucumber is not well understood. To facilitate use of the XIS cucumber in cucumber breeding through marker-assisted selection, a QTL mapping study was conducted with a mapping population developed between WI7167 and a cultivated cucumber landrace WI7200. A SSR-based genetic map was developed, and QTL analysis was performed with phenotypic data collected from F_2-derived F_3 families on flowering time (male and female flowers) and mature fruit size (length and width).

Acknowledgement. All authors contributed equally to the work.

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Materials and Methods

Plant materials. One hundred and thirty-eight F_2 plants and F_2-derived F_3 families from the cross between two cucumber lines, WI7167 and WI7200 were used for QTL mapping. WI7167, an XIS inbred, exhibits late flowering and round fruit shape whereas WI7200, a cultivated cucumber has early flowering and short columnar fruit.

Phenotypic data collection and analysis. Parental lines, F_1 and F_2 populations were grown in the Walnut Street Greenhouse of the University of Wisconsin at Madison during 2012 summer season. F_3 families were generated from F_2 individuals through self-pollination and were evaluated in the field (Hancock Agriculture Research Station, Hancock, Wisconsin) in 2013 summer growing season (June to September).

For phenotypic evaluation, at least 15 plants from each F_3 family were grown in the field. F_3 family means were used in QTL analysis. For each plant, female flower time (FFT) and male flower time (MFT) were calculated as days from sowing to the blooming of first female and male flower, respectively. Fruit length (FL) and fruit diameter (FD) of mature fruits (at least 30 days after pollination in the greenhouse) were measured. The number of fruits measured from each plant varied from 1 to 4.

*Linkage map development***.** Polymorphic cucumber or melon SSR markers described in Ren et al. (2009) and Cavagnaro et al. (2010) were used to genotype 138 F_2 plants. DNA extraction, PCR amplification of molecular markers and gel electrophoreses followed Li et al. (2011). For each marker, χ^2 test for goodness-of-fit was performed against the expected 1:2:1 segregation ratio.

Linkage analysis was carried out using JoinMap 4.0 software. Linkage groups were determined with a minimum LOD score of 4.0 and a recombination fraction of 0.3. Genetic distance was calculated with Kosambi mapping function.

The physical locations of all mapped markers in the Gy14 scaffold and draft genome assemblies (Version 1.0) (Yang et al., 2012) were used to verify their genetic map locations. Chromosome assignment (Chr1 to Chr7) of the seven linkage groups followed Yang et al. (2012).

QTL analysis. A whole genome scan was performed to map the QTLs using composite interval mapping (CIM) procedure of R/qtl (Broman, 2003). Genome wide LOD threshold values ($P < 0.05$) for declaring the presence of QTLs was determined using 1,000 permutations. For each QTL, a 2-LOD support interval was calculated and defined by left and right markers. The QTLs were named according to the chromosome locations and trait names (FFT = female flowering time, MFT = male flowering time, FL = fruit length, FD = fruit diameter). For example, *fd1.1* and *fft5.1* designated the first QTL for fruit diameter and female flowering time in cucumber chromosomes 1 and 5, respectively.

Results and Discussion

Phenotypic data analysis. The frequency distribution of F_3 family means for FFT, MFT, FL and FD is illustrated in Fig. 1. While distribution of FL and MFT was skewed toward WI7200, that of FD and FFT showed largely normal distribution. Both

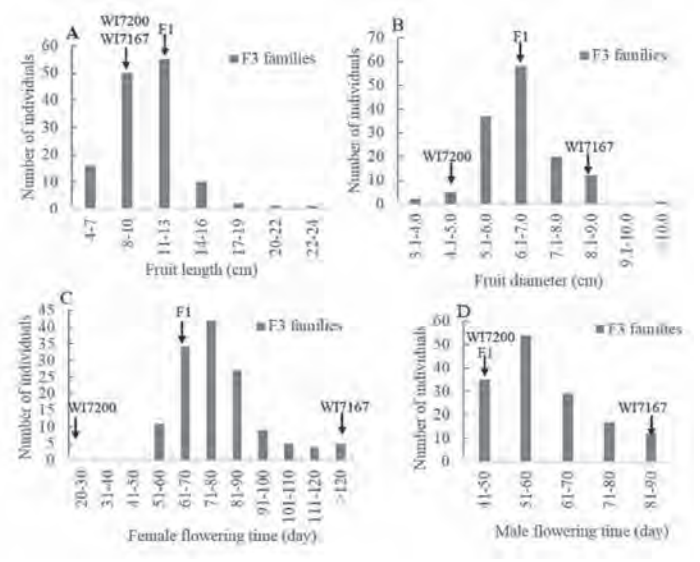

Fig. 1. Frequency distribution of fruit length (FL), fruit diameter (FD), first female flowering time (FFT), and male flowering time (MFT) among F_2 individuals and F_3 families. Arrows indicated corresponding values of WI7200, WI7167 and their F_1.

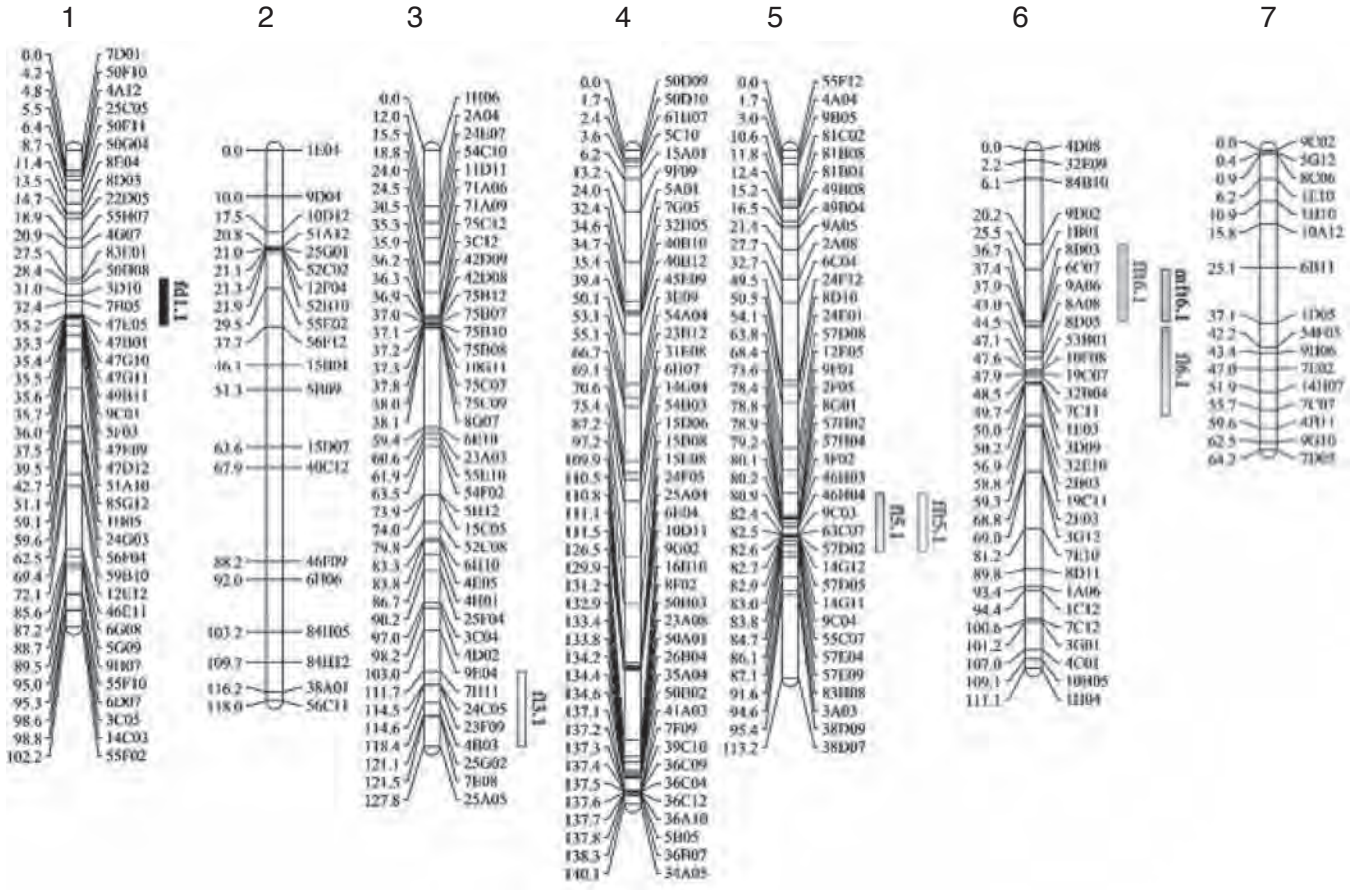

Fig. 2. Genetic linkage map and QTL locations detected in 2013 field experiment. The linkage map was constructed with 138 F_2 individuals derived from the cross between WI7200 and WI7167. Map distance was in centimorgans (cM). The number was shown on top of each map corresponding to chromosome assignment.

Table 1. Information for QTLs for fruit length (FL), diameter (FD), female flowering time (FFT), and male flowering time (MFT)[z].

Traits	QTL	Chr.	Peak (cM)	LOD	R^2 (%)	Add	Dom	2-LOD support interval	
								Left	Right
Fruit length (FL)									
	fl3.1	3	118.4	4.77	9.38	1.06	–0.69	7H11	25A05
	fl5.1	5	80.0	11.17	24.62	–2.08	0.45	9F01	57E04
	fl6.1	6	49.7	5.51	10.97	1.26	–0.10	9A06	32E10
Fruit diameter (FD)									
	fd1.1	1	31.0	5.03	11.08	0.52	–0.25	83E01	47E09
Female flowering time (FFT)									
	fft5.1	5	80.1	9.62	17.16	10.23	–2.21	9F01	57E04
	fft6.1	6	26.0	19.51	41.65	15.86	–3.00	9D02	8B03
Male flowering time (MFT)									
	mft6.1	6.0	26.0	33.36	67.42	11.59	–2.36	1B01	8B03

[z] Chr. = chromosome; R^2 = phenotypic variations explained by specific QTL of total variances.

parental lines had similar fruit length, but the F_1 exhibited clear heterosis suggesting possible additive effects of QTLs from each parent. WI7200 flowered very early, and no F_3 family had FFT earlier or close to WI7200 (based on family means). For anthesis dates of first male flowers, WI7200 and F_1 had very similar flowering time and the distribution skewed toward WI7200. The distributions may suggest that early flowering is dominant over late flowering, and MFT might be controlled by a single gene or major-effect QTL in this population.

Linkage map construction. Among 3,800 SSR markers screened between WI7200 and WI7167, 277 (7.3 %) were polymorphic which were used to construct the linkage map with 138 F_2 plants. Eventually 230 SSR markers were placed on the linkage map (Fig. 2). This genetic map covered 775.2 cM in seven linkage groups with an average marker interval of 3.4 cM. This map had almost complete coverage of the cucumber genome when compared with the high-resolution map of cucumber by Yang et al. (2012). Therefore, this genetic map was suitable for subsequent QTL mapping.

QTL analysis. Phenotype data from F_3 families were used in QTL analysis. The LOD threshold to declare significance of QTL was determined with 1,000 permutation tests (P = 0.05) which was 4.3. Seven QTLs were detected for FL (three), FD (one), FFT (two) and MFT (one) that could explain 9.4-67.4 % phenotypic variations. The locations of the 7 QTLs in the cucumber genome are shown in Fig. 2, details of each QTL including map location, LOD value, percentages of total phenotypic variances explained (R^2), additive effect, and 2-LOD support interval are provided in Table 1. Among the three FL QTLs (*fl3.1*, *fl5.1* and *fl6.1*), *fl5.1* had negative additive effect (reduction of fruit length) whereas other two had positive additive effects (increasing fruit length). Only one QTL was detected for fruit diameter (*fl1.1*) with R^2 = 11.1%. The two FFT QTLs, *fft5.1* and *fft6.1* accounted for 17.2% and 41.7% observed phenotypic variances; respectively suggesting *fft6.1* is a major-effect QTL. Interestingly, the only major-effect QTL for male flowering time (*mft6.1*, R^2 = 67.4 %) was co-localized with *fft6.1* (Fig. 2) implying a major QTL at this location that may control both female and male flowering time. The location of this flowering time major QTL seems to be different from what found in previous QTL mapping studies (Yuan *et al.*, 2008; Miao et al., 2011) that merits further investigation.

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Seed Yield and Quality of Watermelon Genotypes Having Snack Food Potential

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Additional index words. watermelon, *Citrullus lanatus*, seed yield, seed quality, fruit quality, snack food.

Abstract. Snack foods should be healthy, easy to eat and satisfying. With these aims in mind 32 watermelon genotypes were evaluated for their potential as a nutritious snack food. Total yield of fruit and fruit characteristics as average fruit size (weight, length, width, rind thickness) and seed size, protein content and taste were examined. Kar 365 produced the highest seed yield and the seed was the largest of all accessions evaluated. Kar 369 genotype which had the highest seed size and the amount of protein content had been identified to have lowest number of seed per fruit.

Healthy snack foods should be nutritious, low in fat and sugar as well as being quick to eat, portable and satisfying. In Turkey, 550 000 tons of snack food was produced with an annual consumption of 6 to 6.5 kg per capita (Anonymous, 2013). The highest consumption of snack is in Marmara region of Turkey.

Watermelon is a nutritious snack food and can be eaten fresh or the seeds can be consumed dry. To promote healthy snack foods, there is a need to develop large seeded watermelon cultivars that have high protein levels.

Watermelon is the second most produced vegetable after tomato in Turkey (FAO, 2012). There is also considerable genetic variation within the species to select for characteristics important for snack foods. The National Seed Gene Bank at the Republic of Turkey Ministry of Food Agriculture and Livestock Aegean Agricultural Research Institute (AARI), contains 2223 acessions in the *Cucurbitaceae* family, 16 percent of this genetic material constitutes the watermelon seeds (Sari et al., 2008).

In the assessment of seeds as a snack, the most attention matters are high seed size, high 1000 seed weight, low fat ratio, high protein and high amount of other nutrients.

In this study, 32 watermelon genotypes which have high potential for snack were selected meticulously from the genetic resources of Cukurova University, Faculty of Agriculture, Department of Horticulture and investigated for fruit and seed quality.

Materials and Methods

Field experiment of this study was carried out in Research and Application area of Cukurova University, Adana. Also laboratory analysis was carried out at the Department of Horticulture in Cukurova University and Palanci Food Technologies Research and Development Company in Istanbul.

In this study 32 watermelon genetic resources (Kar 23, Kar 38, Kar 40, Kar 43, Kar 46, Kar 50, Kar 67, Kar 75, Kar 96, Kar 111, Kar 127, Kar 130, Kar 131, Kar 132, Kar 145, Kar 157, Kar 159, Kar 175, Kar 176, Kar 185, Kar 186, Kar 197, Kar 209, Kar 213, Kar 279, Kar 296, Kar 310, Kar 350, Kar 356, Kar 361, Kar 365 and Kar 369) collected from different regions of Turkey by Sari et al (2007) were used as plant material and we added 3 genotype from China, Cyprus and Nigeria.

Ninety six seeds which belong to each genotype, were sowed in multipots in Atlas Seedling Company in Adana, Turkey on February 28, 2012. Seedlings were planted in low tunnels spacing of 2 x 0.5 m between rows and plants, on April 18, 2012. Trial was set up as a randomized complete block with 4 repetitions and each plot consisted of 15 plants of each watermelon genotype.

Fruits were harvested on July 31, 2012. Shortly after harvest, number of fruits and fruit weight were determined. Seeds were extracted by hand. Dried seeds were packed and put in cold storage.

In study, total yield, average fruit weight, fruit length, fruit width, fruit rind thickness, amount of total soluble solids (TSSC, by refractometer),total seed yield, seed size, seed ground color, number of seeds per fruit, seed weight per fruit, number of seeds in a one gram, seed width, seed length, seed thickness, ease of cracking of the seed, taste test of seeds, protein content of seeds were examined.

Measurements were made in three fruit taken randomly from each plot for fruit characteristics and also in twenty seeds taken randomly from each plot for seed characteristics.

Foss Tecator Application Sub Note: The Determination of Nitrogen According to Kjeldahl in Oilseeds Asn3105 was taken as reference in the protein analysis.

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The data obtained in the study were analyzed by Costat Package Programme and Tukey's test was used for the comparison of the mean.

Results and Discussion

Considerable fruit and seed variation were seen among the genotypes (Table 1, 2, 3).

Total yield of fruit ranged from 1.16 and 3.44 kg/plant, however, no significant differences were found among the genotypes. Yield variability is common in watermelon trials and thus studies with large plots and many replicates are needed determine yield differences (Sari et al., 2007, Pakyurek and Yanmaz, 2008). Fruit yields were lower than found in other watermelon trials (Sari et al., 2007, Pakyurek and Yanmaz, 2008). Lower yields may have been caused by the genotypes used and trial conditions.

Average fruit weight was found between 1.29 and 3.96 kg. The highest average fruit weight was obtained from Kar 186 and Kar 350 genotypes. The lowest average fruit weight was obtained from Kar 40 genotype. Cordova et al. (1995) categorized watermelons according to fruit size as fruits less than 4 kg, between 4-6 kg and between 8-12 kg to be small, medium and giant, respectively. Sari et al. (2007) found that average fruit weight ranged between 1.885 and 8.033 kg. Pakyurek and Yanmaz (2008), in thirteen watermelon genotype which were suitable for snack consumption, average fruit weight identified to range between 1 and 4 kg. Regarding the fruit weight of genotypes used in this study, all genotypes were classified as small. Fruit of snack watermelon can not be widened too much due to numerous and large seeds inside the fruit. Similar results have been reported by Pakyurek and Yanmaz (2008).

Average fruit length was found to range between 13.30 and 29.93 cm. While longest average fruit length was obtained from Kar 350 genotype, smallest average fruit length was obtained from Kar 40 and Kar 46 genotypes. Sari et al. (2007) determined in their study that fruit length to range between 15.4 and 35.0 cm. Pakyurek and Yanmaz (2008)in thirteen watermelon genotype which were suitable for snack consumption, identified fruit lenght to range between 16.80 and 25.95 cm.

Average fruit width were found to range between 13.34 and 20.60 cm. The highest average fruit width was obtained from Kar 186 genotype. The lowest average fruit width was obtained from Kar 40 genotype. Sari et al. (2007) determined in their study, that fruit diameter was between 13.8 and 24.8 cm. Pakyurek and Yanmaz (2008), in thirteen watermelon genotype which were suitable for snack consumption, fruit width identified to range between 12.90 and 20 cm. Karipcin (2009) determined that highest average fruit diameter was 23.59 and 23.98 cm respectively and lowest average fruit diameter was 12.53 and 4.52 cm respectively by years.

Table 1. Fruit yield and fruit characteristics of different watermelon genotypes.

Genotype code	Total yield of fruit (kg/plant)	Average fruit weight (kg)	Average fruit length (cm)	Average fruit width (cm)	Average fruit rind thickness (cm)	TSSC (%)
Kar 23	1.82	2.79 ab	26.01 ab	15.01 abc	1.49 ab	5.85 c-f
Kar 38	2.28	1.64 ab	14.53 ef	14.50 bc	1.48 ab	8.44 a-d
Kar 40	1.16	1.29 b	13.30 f	13.34 c	1.59 ab	4.49 f
Kar 43	2.43	2.25 ab	16.82 c-f	16.01 abc	1.55 ab	7.15 b-f
Kar 46	1.29	2.07 ab	15.66 f	15.78 abc	0.99 ab	9.89 ab
Kar 50	2.15	2.18 ab	16.68 c-f	15.93 abc	1.32 ab	8.68 a-d
Kar 67	2.08	2.33 ab	16.77 c-f	16.69 abc	0.87 b	7.48 a-f
Kar 75	2.58	2.57 ab	16.41 def	16.81 abc	1.19 ab	9.92 ab
Kar 96	3.05	3.08 ab	16.45 def	18.93 abc	1.68 ab	7.58 a-f
Kar 111	2.13	2.88 ab	16.90 c-f	17.95 abc	1.60 ab	7.23 b-f
Kar 127	3.23	3.47 ab	18.53 c-f	18.70 abc	1.74 ab	8.13 a-e
Kar 130	2.98	3.29 ab	18.82 c-f	17.93 abc	1.53 ab	8.67 a-d
Kar 131	2.01	2.45 ab	16.94 c-f	16.83 abc	1.45 ab	7.31 b-f
Kar 132	2.71	3.34 ab	19.15 c-f	17.84 abc	1.74 ab	6.18 c-f
Kar 145	2.84	3.26 ab	19.23 c-f	18.58 abc	1.43 ab	6.11 c-f
Kar 157	3.03	2.96 ab	22.83 bcd	16.64 abc	1.87 a	8.72 abc
Kar 159	2.91	2.62 ab	19.05 c-f	17.04 abc	1.21 ab	8.43 a-d
Kar 175	2.75	2.94 ab	18.02 c-f	17.84 abc	1.29 ab	9.80 ab
Kar 176	2.77	3.33 ab	17.68 c-f	19.08 ab	1.78 a	7.28 b-f
Kar 185	3.22	2.68 ab	18.42 c-f	16.72 abc	1.38 ab	10.83 a
Kar 186	3.30	3.96 a	18.65 c-f	20.60 a	1.53 ab	7.32 b-f
Kar 197	2.59	3.15 ab	17.40 c-f	18.29 abc	1.41 ab	8.41 a-d
Kar 209	1.81	3.05 ab	23.20 bc	16.62 abc	1.80 a	8.20 a-e
Kar 213	2.88	2.70 ab	16.58 c-f	17.38 abc	1.84 a	5.78 c-f
Kar 279	2.45	2.33 ab	16.60 c-f	16.20 abc	1.45 ab	7.14 b-f
Kar 296	3.17	2.94 ab	17.73 c-f	17.34 abc	1.30 ab	9.09 abc
Kar 310	2.68	3.18 ab	17.58 c-f	18.38 abc	1.70 ab	6.19 c-f
Kar 350	2.94	3.88 a	29.93 a	16.93 abc	1.60 ab	7.80 a-f
Kar 356	2.12	2.05 ab	20.89 b-e	13.90 bc	1.50 ab	6.02 c-f
Kar 361	2.92	3.42 ab	19.01 c-f	19.02 ab	1.80 a	8.25 a-e
Kar 365	3.44	1.91 ab	14.57 ef	15.60 abc	1.60 ab	5.00 ef
Kar 369	2.31	2.54 ab	17.22 c-f	16.23 abc	1.16 ab	5.34 def
D	(5%)	(1%)	(1%)	(1%)	(1%)	(1%)
	(nonsignificant)	2.48	6.66	5.64	0.89	3.36

Average fruit rind thickness were found between 0.87 and 1.87 cm. While highest average fruit rind thickness was obtained from Kar 157, 176, 209, 213 and 361 genotypes, lowest average fruit rind thickness was obtained from Kar 67 genotype. According to quantitative values which developed for measurable characters in watermelon morphological characterization; fruit rind thickness determined as thin (≤10 mm), medium (10–15 mm) and thick (≥15 mm) (Sari et al., 2007). Sari et al. (2007) reported in their study that fruit rind thickness was ranged between 8.4 and 20.2 mm. Karipcin (2009) determined the highest fruit rind thickness as 18.00 and 21.30 mm and lowest fruit rind thickness as 3.22 and 8.20 mm by years.

The amount of total soluble solids (TSSC) were found between 4.49 and 10.83 %. The highest amount of total soluble solids was obtained from Kar 185 (10.83%). The lowest amount of total soluble solids was obtained from Kar 40 genotype (4.49 %). Sari et al. (2007), reported in their study that the amount of total soluble solids (TSSC) to range between 4.2 % and 12.0 %. Karipcin (2009) determined the highest amount of total soluble solids (TSSC) as 10.24 % and 10.28 % and lowest TSSC as 3.09 % and 3.47% by years.

Total seed yield ranged between 21.54 and 81.00 g. While highest total seed yield was obtained from Kar 365 genotype, lowest total seed yield was obtained from Kar 46, Kar 197 and Kar 185 genotypes.

Kar 365 and Kar 369 genotypes produced the largest seeds. Thirty-two of watermelon genotype which used in trial, two of them (6%) were determined giant seedy, one of them (3%) was determined small seedy and remaing 29 of them (91%) were determined big seedy.

Seed ground color were determined to vary from cream to black color. 38 % (12 genotypes) of seeds used in study had cream color, 34 % (11 genotypes) of seeds were black, 22 % (7 genotypes) of seeds were brown and 6 % (2 genotypes) of seeds were red brown. Pakyurek and Yanmaz (2008) reported in their study regarding seed ground color of testa determined light brown and had patches at margin.

The number of seeds per fruit were found between 98 and 453. While highest number of seeds per fruit was obtained from Kar 159, lowest number of seeds per fruit was obtained from Kar 369. Pakyurek and Yanmaz (2008) identified number of seeds per fruit to range between 250 and 648. Karipcin (2009) determined the highest number of seeds per fruit as 637.34 and 723.33 seeds and lowest number of seeds per fruit as 259.48 and 293.78 by years.

Table 2. Seed yield and some seed characteristics of different watermelon genotypes.

Genotype code	Total seed yield (g/plant)	Seed size	Seed ground color	Number of seeds per fruit	Seed weight per fruit (g)	Number of seeds in a one gram
Kar 23	27.36 bc	Big	Cream	167 b-e	26.66 b-e	6.25 c-g
Kar 38	41.85 abc	Big	Black	180 b-e	26.78 b-e	6.75 b-f
Kar 40	27.15 bc	Big	Black	108 de	15.58 e	7.00 b-e
Kar 43	57.43 abc	Big	Brown	348 abc	45.44 a-d	8.00 b
Kar 46	21.54 c	Big	Black	141 cde	20.04 de	7.00 b-e
Kar 50	46.42 abc	Big	Brown	242 b-e	31.35 b-e	7.50 bcd
Kar 67	36.23 abc	Big	Red Brown	191 b-e	26.55 b-e	7.50 bcd
Kar 75	50.37 abc	Big	Brown	202 b-e	31.42 b-e	6.25 c-g
Kar 96	36.61 abc	Big	Red Brown	110 de	21.73 cde	5.00 gh
Kar 111	43.85 abc	Big	Cream	314 a-d	43.85 b-e	7.75 bc
Kar 127	28.29 bc	Big	Brown	185 b-e	25.47 cde	7.25 b-e
Kar 130	48.07 abc	Big	Cream	215 b-e	36.83 b-e	6.00 d-g
Kar 131	33.20 bc	Big	Black	207 b-e	30.20 b-e	6.75 b-f
Kar 132	59.06 abc	Big	Cream	355 ab	54.44 ab	6.50 b-g
Kar 145	60.19 abc	Big	Cream	295 a-e	49.37 abc	6.00 d-g
Kar 157	31.64 bc	Big	Cream	192 b-e	25.33 cde	7.50 bcd
Kar 159	30.00 bc	Small	Black	453 a	30.00 b-e	15.50 a
Kar 175	37.79 abc	Big	Black	155 b-e	26.19 b-e	6.00 d-g
Kar 176	30.33 bc	Big	Cream	142 cde	26.86 b-e	5.25 fgh
Kar 185	24.29 c	Big	Black	144 cde	16.99 de	8.00 b
Kar 186	34.25 bc	Big	Brown	147 b-e	29.25 b-e	5.00 gh
Kar 197	23.04 c	Big	Brown	132 de	23.04 cde	6.00 d-g
Kar 209	58.75 abc	Big	Cream	175 b-e	26.11 b-e	7.00 b-e
Kar 213	48.85 abc	Big	Brown	158 b-e	29.02 b-e	5.75 efg
Kar 279	41.25 abc	Big	Black	214 b-e	33.08 b-e	7.00 b-e
Kar 296	45.35 abc	Big	Black	196 b-e	30.58 b-e	6.75 b-f
Kar 310	46.00 abc	Big	Black	293 a-e	39.67 b-e	7.25 b-e
Kar 350	72.50 ab	Big	Cream	297 a-e	72.50 a	5.00 gh
Kar 356	52.56 abc	Big	Cream	198 b-e	38.75 b-e	5.25 fgh
Kar 361	47.94 abc	Big	Black	206 b-e	32.63 b-e	6.50 b-g
Kar 365	81.00 a	Giant	Cream	148 b-e	45.00 a-d	4.00 h
Kar 369	35.55 abc	Giant	Cream	98 e	29.24 b-e	4.00 h
D (1%)	46.45	–	–	208.72	28.56	1.72

Seed weight per fruit were found between 15.58 and 72.50 g. The highest seed weight per fruit was obtained from Kar 350. The lowest seed weight per fruit was obtained from Kar 40.

The number of seeds in a one gram were found between 4.00 and 15.50. While highest number of seeds in a one gram was obtained from Kar 159, lowest number of seeds in a one gram was obtained from Kar 369 and Kar 365 genotypes.

Seed width was found between 5.85 and 11.76 mm. While longest seed width was found in Kar 365 and Kar 369 genotypes, smallest seed width was found in Kar 159 genotype. Razavi et al. (2006) identified seed width in their study to range between 8.4 and 10.7 mm. Altuntaş (2008) found watermelon seed width as 8.00 mm. Paksoy et al. (2010) determined watermelon seed width as 6.8 mm. Also our study showed that watermelon seed width change depend on genotype and this results are compatable to Razavi et al. (2006), Altuntas (2008) and Paksoy et al. (2010).

Seed length is an important parameter for cracking the seed. The longer the seed, the easier it is to crack. Seed length was found between 9.72 and 18.85 mm. While longest seed length was obtained from Kar 369 genotype, shortest seed length was obtained from Kar 159 genotype. Razavi et al. (2006) identified in their study that seed length of watermelon to range between 13.45 and 18.97 mm. Altuntas (2008) reported average seed length of watermelon as 13.28 mm. Paksoy et al. (2010) identified in their study that average seed length of watermelon as 10.8 mm.

Seed thickness was found between 1.97 and 3.47 mm. While thickest seed was obtained from Kar 365 genotype, thinest seed was obtained from Kar 159 genotype. Razavi et al. (2006), identified seed thickness of watermelon range between 2.91 and 3.10 mm. While Altuntas (2008) found seed thickness to be 2.64 mm. Paksoy et al. (2010) identified in their study as 2.3 mm. Our research results have shown combatibility with concerned studies.

Kar 185 and Kar 197 genotypes were the easiest to crack by 5 points. The most difficult crack seeds were Kar 50, Kar 176, Kar 350, Kar 356, Kar 365 and Kar 369 genotypes by 1 point. Yanmaz et al. (2010) determined that test of crackle ease of seed in fresh seed was more hard than roasted seed. For this reason they suggested who will work on this issue that test of crackle ease of seed should made roasted seed.

In taste test of seeds, Kar 23, Kar 67, Kar 132 and Kar 197 genotypes received the highest point. Kar 369 genotype received the lowest point by 2 point. Fruhwirth and Hermetter (2007) stated

Table 3. Seed characteristics of different watermelon genotypes.

Genotype code	Seed width (mm)	Seed length (mm)	Seed thickness (mm)	Ease of cracking the seed (5 = very easy, 1 = very difficult)	Taste test of seeds (5 = excellent, 1 = worst)	Protein content of seed (% dry weight)
Kar 23	8.42 d-i	15.03 d-i	2.48 g-l	3	5	15.79 c
Kar 38	7.85 i-l	13.79 h-o	2.46 h-l	3	3	16.54 abc
Kar 40	8.50 c-h	14.44 f-l	2.80 cd	2	4	17.17 abc
Kar 43	7.41 lm	12.66 o	2.40 jkl	4	4	17.46 abc
Kar 46	7.79 i-m	13.04mno	2.32 klm	3	3	17.27 abc
Kar 50	7.70 klm	13.46 k-o	2.30 lm	1	4	18.75 abc
Kar 67	8.03 g-l	13.65 j-o	2.15 mn	4	5	17.47 abc
Kar 75	8.59 c-g	13.73 i-o	2.49 g-l	4	4	17.63 abc
Kar 96	9.56 b	15.27 c-g	2.83 cd	3	4	15.55 c
Kar 111	7.17 m	12.89 no	2.55 e-k	4	3	16.07 bc
Kar 127	8.07 f-k	13.22 l-o	2.41 i-l	2	4	16.49 abc
Kar 130	8.91 cde	14.33 g-m	2.65 d-i	4	3	18.44 abc
Kar 131	8.09 f-k	13.73 l-o	2.50 g-l	4	3	17.03 abc
Kar 132	8.10 f-k	13.18 l-o	2.75 c-f	4	5	17.43 abc
Kar 145	8.69 c-f	14.40 f-l	2.85 cd	2	3	16.93 abc
Kar 157	7.91 h-l	14.21 g-m	2.54 f-k	2	3	15.98 c
Kar 159	5.85 n	9.72 p	1.97 n	3	3	20.10 ab
Kar 175	8.37 e-j	14.76 e-k	3.11 b	4	4	17.02 abc
Kar 176	9.08 bc	15.05 d-h	2.78 cde	1	3	15.94 c
Kar 185	7.53 klm	13.35 l-o	2.59 d-j	5	4	16.50 abc
Kar 186	9.06 bcd	15.71 c-f	2.78 c-f	2	4	15.03 c
Kar 197	9.01 b-e	15.98 cde	2.92 bc	5	5	17.66 abc
Kar 209	8.12 f-k	14.95 d-j	2.48 g-l	3	4	17.13 abc
Kar 213	8.95 b-e	14.13 g-n	2.72 c-g	3	4	15.76 c
Kar 279	7.82 i-l	13.81 h-o	2.44 h-l	2	3	16.86 abc
Kar 296	8.06 f-k	14.19 g-n	2.43 h-l	4	4	16.36 abc
Kar 310	7.78 j-m	13.18 l-o	2.33 klm	2	3	15.95 c
Kar 350	8.42 d-i	16.49 bc	2.77 c-f	1	3	*
Kar 356	8.40 e-j	16.23 cd	2.66 d-h	1	3	16.15 abc
Kar 361	7.98 g-l	13.56 k-o	2.49 g-l	3	3	16.18 abc
Kar 365	11.51 a	17.80 ab	3.47 a	1	–	15.80 c
Kar 369	11.76 a	18.85 a	2.80 cd	1	2	20.19 a
D (1%)	0.64	1.31	0.24	–	–	4.07

* Analysis could not be performed because of inadequate amount of sample.

that pumpkin seeds were roasted for formation of characteristic flavor and aroma. In future studies excess amounts of the seeds produced and after roasting will benefit to perform tests.

Protein content of seed were found between 15.03% and 20.19%. The highest protein content of seed was obtained from Kar 369 genotype. The lowest protein content of seed were obtained from Kar 186, Kar 96, Kar 213, Kar 23, Kar 365, Kar 176, Kar 310 and Kar 157 genotypes respectively. Olaofe (1994) was found protein content of watermelon seed between 23.7% and 30.68%, El-Adawy and Taha (2001) were found protein content of watermelon seed as 50.10%. Genotype, environmental conditions and cultivation techniques may change protein content of seeds.

As a conclusion, when total yield of fruit criteria scaned, there was no significant difference between genotypes. However all the other fruit and seed parameters were statistically important according to genotypes. Many parameters were measured to evaluate watermelon genotypes as a potential snack food. In terms of yield and seed size, Kar 365 outperformed all the other genotypes. However, the seed was very difficult to crack. Another large seeded genotype (Kar 369) was also difficult to crack and rated low by the taste panel. However if also other genotype which is entered to classification of large seedy make roast and boiled trials, it might used as snack. Also consumer preferences have importance for evaluation as snack. In terms of easy of cracking and good flavor, Kar 67, Kar 132, Kar 185 and Kar 197 all rated easy to crack and tasted good.

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Fine Genetic Mapping of *Zucchini Yellow Mosaic Virus* Resistance Gene *zym* in Cucumber

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Additional index words. *Cucumis sativus*, *Zucchini yellow mosaic virus*, ZYMV, recessive resistance gene, marker assisted breeding, MAB

Abstract. Marker assisted breeding (MAB) is a useful tool for development of *Zucchini yellow mosaic virus* (ZYMV) resistant cucumber cultivars since the resistance is often inherited recessively in cucumber. Resistant to ZYMV has been evaluated in F_2 or BC_1 cucumber families from a cross between susceptible 'CS-PMR1' and resistant 'A192-18' cucumber inbred lines, we confirmed that ZYMV resistance was conferred by a single recessive locus ($zym^{A192-18}$). The linkage map included 125 simple sequence repeat (SSR) markers that segregated into 7 linkage groups (chromosomes). The $zym^{A192-18}$ locus was mapped on chromosome 6 at genetic distances from 2 closely linked SSR markers of 0.9 and 1.3 cM. For fine genetic mapping, we identified new molecular markers cosegregating with the $zym^{A192-18}$ locus in the $F_{2:3}$ population. Screening of a mapping population of 2,429 F_2 plants allowed us to narrow down the $zym^{A192-18}$ locus to a <50 kb genomic region flanked by two SSR markers, which included six predicted genes. Sequence analysis of the candidate genes' coding regions revealed that the vacuolar protein sorting-associated protein 4-like (VPS4-like) gene had two SNPs between the parental lines. Based on SNPs of the VPS-4-like gene, we developed $zym^{A192-18}$-linked DNA markers and found that genotypes associated with these markers were correlated with the ZYMV resistance phenotype in cucumber inbred lines. These results may be valuable for MAB for ZYMV resistance in cucumber.

Zucchini yellow mosaic virus (ZYMV) is an economically important potyvirus that infects cucumber. During summer to early autumn, cucumber plantings are particularly vulnerable due to increased vector populations and some plants are suddenly wilted by superinfection of ZYMV and other species virus such as *Cucumber mosaic virus* (CMV) (Gal-On 2007). Since no chemical substances have yet been found to effectively control the virus, breeding for ZYMV resistance is the most desirable approach for virus disease control. Recent reports have shown that some Japanese type cucumber lines have resistance to ZYMV and can be inherited as a recessive allele at a single locus (*zym*) (Svoboda et al. 2013, Amano et al. 2011), suggesting marker assisted breeding (MAB) has been shown as powerful tool to develop of ZYMV resistant cultivars in cucumber.

Several DNA markers linked to the *zym* gene have been developed (Park et al. 2004). However, these DNA markers may not be polymorphic between some of ZYMV resistant and susceptible plants. To develop new DNA markers, we constructed genetic linkage map to identify SSR markers linked to the *zym*. Furthermore, we proposed that initiation of eukaryotic translation factors (eIF4E and eIF4G), which were identified as recessive plant virus resistance gene, were not linked to *zym* locus (Amano et al. 2011). Therefore we then carried out fine genetic mapping and identified the most likely candidate gene for the *zym* locus using the cucumber genome and molecular marker resources.

Material and Methods

A Japanese type cucumber ZYMV resistant inbred line A192-18 and a susceptible line CS-PMR1 (weedy Indian type derived from PI197088-1) were used as parents. To determine the inheritance pattern of the ZMVY resistance trait, F_1, F_2 and backcross populations from the cross A192-18 and CS-PMR1 were produced in the greenhouse and then a total of 128 F_3 families were obtained by self-pollination of each F_2 lines derived ($F_{2:3}$ population) for ZYMV inoculation test and genetic mapping. For fine mapping, an additional 2,301 F_2 plants from the same cross were used.

Viral inoculum was prepared by grinding the infected leaves (1:10 w/v) in 0.05 M phosphate buffer (pH 7.2) with a mortar and pestle. Carborundum-dusted cotyledons of 7- to 8-day-old seedlings were mechanically inoculated by rubbing them with cotton swabs dipped in the inoculum. Two weeks later, disease resistance of each plant was scored using a five-step disease rating scale: 0 (no symptoms), 1 (slight mosaic limited to lower

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leaves), 2 (pronounced mosaic on lower leaves and slight mosaic on upper leaves), 3 (moderate mosaic on upper leaves), or 4 (severe mosaic on all leaves). The disease severity indices (DSIs) of 1.0 or less were considered resistant; greater than 3.0 were considered susceptible.

To construct a framework genetic map of the 128 $F_{2:3}$ population, 311 SSR markers distributed throughout the seven chromosomes (Ren et al. 2009, Fukino et al. 2013) was used for first screening of polymorphic SSR markers. For fine genetic mapping, an additional larger F_2 population (2,301 plants) was grown to identify recombinant plants defined by SSR markers flanking the *zym* locus in the $F_{2:3}$ population. Recombinant F_2 plants were planted to obtain respective F_3 families. At least 25 plants from the respective F_3 families were evaluated for ZYMV resistance using a ZYMV inoculation test. New parental polymorphic SSR markers were subsequently found based on SSR markers on the gene scaffold (Cavagnaro et al. 2010). These new markers were then applied to all recombinant plants to narrow down the *zym* locus flanking region. The PCR amplified products were separated by electrophoresis through 5% (stacking) and 13% (running) non-denaturing polyacrylamide gel in 1×Tris-Glycine buffer according to the method used by Xu at al.(2009).

Results and Discussion

The F_2 population almost segregated into a 1:3 [resistance (R) : susceptible (S)] ratio as the result of the inoculation test whereas the F_1 × A192-18 backcross population segregated into a R:S ratio close to 1:1 and the F_1 × CS-PMR1 backcross population was entirely susceptible. These results confirmed that ZYMV resistance in A192-18 is conferred by a single recessive gene locus (designated as $zym^{A192\text{-}18}$).

Of 311 SSR markers on chromosomes (Chr1–Chr7), 125 (40.2 %) were clearly polymorphic between A192-18 and CS-PMR1. Our cucumber framework genetic map had a total length of 677.6 cM, with an average marker interval of 6.27 cM. The $zym^{A192\text{-}18}$ locus was located on Chr6, flanked by markers at a distance of 0.9 and 1.3 cM, respectively. One SSR marker cosegregated with the $zym^{A192\text{-}18}$ locus. The $zym^{A192\text{-}18}$ locus-linked SSR markers were physically located on the same scaffold using the cucumber genome database (Phytozome v9.1, based on the whole-genome sequence of Gy14). According to genome-wide SSR marker resources of Gy14 (Cavagnaro et al. 2010), 95 SSR markers were chosen for polymorphism screening between the parents. As a result, 10 polymorphic SSR markers were newly identified that cosegregated with the $zym^{A192\text{-}18}$ locus in the framework genetic map. Then recombinant plants were used for fine mapping with the new SSR markers. In fine mapping, we found that the physical distance between flanking the $zym^{A192\text{-}18}$ linked SSR markers was approximately 46-47 kb and six putative genes were identified in this flanking region according to two cucumber genome databases (Phytozome v9.1 and the Cucurbit Genomics Database; Huang et al. 2009). No genes were found in this region encoding either translation initiation factors, suggesting that these results could support our previous report (Amano et al. 2011). We sequenced the candidate genes' coding regions and found that the vacuolar protein sorting-associated protein 4-like (VPS4-like) gene had two SNPs between the parental lines. Based on these SNPs, we developed new molecular marker. We used 48 cucumber inbred lines to investigate the utility of these markers for MAB of the *zym* locus, and found that genotypes associated with both markers correlated with ZYMV resistance in all lines. These findings suggest that our developed molecular markers are additional effective tools for ZYMV resistance breeding in cucumber.

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Biodiversity of Cucurbitaceous Vegetable Crops in India

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Additional index words. biodiversity, cucurbits, minor cucurbits, indigenous cucurbits

Abstract. The Indian gene center has affluent biodiversity in cucurbitaceous vegetables like different kind of melons, gourds, cucumber, chow-chow and pumpkin. In Indian background out of 12 major and 10 minor cucurbits, 12 are said to have been originated in India alone. Cucumber (*Cucumis sativus*) bitter gourd (*Momordica charantia*), ridge gourd (*Luffa acutangula*), sponge gourd (*Luffa cylindrica*), pointed gourd (*Trichosanthes dioica*), snake gourd (*Trichosanthes cucumerina*), ivy gourd (*Cocccinia grandis*), long melon (*Cucumis melo* var. *utilissimus*), round melon (*Praecitrullus fistulosus*), snapmelon (*Cucumis melo* var. *momordica*), sweet gourd (*Momordica cohinchinensis*) of Assam and bitter melon (*Cyclanthera callosus*) are important indigenous cucurbits. Besides this Indian sub-continent is secondary center of origin for watermelon, bottle gourd and muskmelon. The major diversity of above crops are concentrated in Indo-Gangetic plains, North-Eastern region, North-Western Himalayas, Western and Eastern Ghats and sporadically in the tribal belts of Central India. There is need of collection, conservation and their utilization. The National Bureau of Plant Genetic resources, New Delhi has about 7025 germplasm holding of cucurbits collected from India as well as other part of worlds. The PCPGR (Pantnagar Centre for Plant Genetic Resources), under G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India, has germplasm holding of about 4,000 cucurbits includes wild and cultivated species. The collected materials may be characterized for biotic resistance and horticultural traits. The wild gene pool possesses important traits may be utilized for improvement breeding program.

Cucurbitaceae is a family of frost sensitive vine crops. It consists of 2 subfamilies, 8 tribes, 118 genera and 825 species (Jeffrey, 1990) and it is one of the most homogeneous families comprising one of the most genetically diverse groups of plants in this plant kingdom in form of landraces, traditional cultivars, wild edible forms and related non-edible wild and weedy species (Robinson and Decker-Walters, 1997).Being the family of melons and gourds, it contributes the world with a pack of diverse commodities and in particular, it has been a source of traditional food and medicine both to the native and rural folks. India is endowed with myriad genetic variability of cucurbits, comprising of 34 genera and 108 species of which 38 species are endemic. Many cucurbits have their centres of diversity in this country (Pandit and Acharya, 2008). Although most of them originated in Old World, many species originated in the New World and at least seven genera in both hemispheres. There is tremendous genetic diversity within the family, and the range of adaptation for cucurbitaceous species includes tropical and subtropical regions, arid deserts, and temperate regions. The genetic diversity in cucurbits extends to both vegetative and reproductive traits and significant range in the monoploid (*x*) chromosome number including 7 (*Cucumis sativus*), 11 (*Citrullus* spp., *Momordica* spp., *Lagenaria* spp., *Sechium*spp., and *Trichosanthes* spp.), 12 (*Benincasahispida*, *Cocciniacordifolia*, *Cucumis* spp. other than *C. sativus*, and *Praecitrullus fistulosus*), 13 (*Luffa* spp.), and 20 (*Cucurbita* spp.). Cucurbits are consumed in various forms, *i.e*., salad (cucumber, gherkins, long melon), sweet (ash gourd, pointed gourd), pickles (gherkins), desserts (melons) and culinary purpose. Some of them (e.g. bitter gourd) are well known for their unique medicinal properties. In recent years, abortifacient proteins with ribosome-inhibiting properties have been isolated from several cucurbit species, which include momordicin (from *Momordica charantia*), trichosanthin (from *Trichosantheskirilowii*), and beta-trichosanthin (from *Trichosanthes cucumeroides*) (Rai et al., 2008). Economically these crops are tremendously important and are cultivated throughout the world from tropical to subtemperate zones. The Indian gene centre has rich diversity in genetic resources of different cucurbits (Table 1). Use of native genetic diversity for breeding new varieties has yielded many new ones as selection from and using indigenous collections at the national level. Out of these varieties Hara Madhu and Kashi Madhu of muskmelon, Kalyanpur Baramasi of bitter gourd, Arka Jeet of watermelon, Pusa Naveen of bottle gourd, Pusa Vishwas of Pumpkin etc are continued to be among the popular varieties due to their higher yield potential and consumer's preference.

Measurement of genetic diversity with derivation and domestication in India

The Indian sub-continent is considered to be the centre of origin for a number of wild and cultivated cucurbits(Chakravarty, 1982). *Cucumis sativus* is reported to have originated in India where its close relatives with same chromosome number (2n = 14) occur. Among all species, *C. sativus* and to a limited extent *C. callosus* are cultivated while *C. anguria, C. hystrix*, *C. prophetarum* and *C. setosus* are wild (Arora and Nayar, 1994). Rich variability is found in foothills of North-West Himalayas and Southern hills for *C. hardwickii*; North-East Himalayas (Meghalaya, Mizoram, Assam) for *C. hystrix*; Rajasthan (Sirohi district) and parts of Gujarat, Maharashtra and Tamil Nadu for *C. prophetarum* and parts of Maharashtra for *C. setosus*. Cucumber is cultivated for at least 3000 years in India. *Precitrullus fistulosus* (round melon) is believed to have originated in India. The genetic variability in fruit morphological traits is concentrated in North-Western India (Western Uttar Pradesh, Haryana and Punjab), but the variability

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Table 1. List of important cucurbitaceous vegetables.

English name	Scientific name	Chromosome no.	Origin
Cucumber	*Cucumis sativus*	14	India
Bitter gourd	*Momordica charentia*	22	Indo-Burma
Ridge gourd	*Luffa acutangula*	26	India
Sponge gourd	*Luffa cylindrica*	26	India
Bottle gourd	*Lagenaria siceraria*	22	Ethiopia
Pumpkin	*Cucurbita moschata*	40	Peru and Mexico
Ash gourd	Benincasa hispida	24	Malaysia (South East Asia)
Snap melon	*Cucumis melo var. momordica*	24	India
Sweet gourd	*Momordica cohinchinensis*	32	Latin America
Snake gourd	*T. anguina*	24	India
Pointed gourd	*Trichosanthes dioica*	22	India
Bitter melon	*Cyclanthera callosus*	24	India
Ivy gourd	(*cocccinia grandis*	24	India
Long melon	*Cucumis melo var. utilissimus*	24	India
Round melon	*Prae citrullus fistulosus*	24	Indo-Burma

is rather limited suggesting its partial or recent domestication. *Luffa* has nine species out of which seven (*L. acutangula, L. cylindrical, L. echinata, L. graveolens, L. tuberose, L. umbellata*) are found in India. The probable centre of origin and primary gene centre of *Luffa* is India. *L.acutangula* (ridge gourd) and *L. cylindrica*(sponge gourd) are grown throughout India in tropical and subtropical climates. *L. acutangula* has three varieties: var*acutangula* is grown in South-Eastern Asia and other tropical areas; var. *amara*, a wild form, is confined to peninsular India, while var. *forskohllii*, another wild form, is confined to Yemen. *L. echinata* grows in natural habitat in Western Himalayas, Central India and Gangetic plains. *L. graveolens* is a wild species distributed in parts of North-Central India, and *L. hermaphrodita* (*satputia*), that bears fruit in clusters, is cultivated in Eastern Uttar Pradesh and Bihar (Sirohi et al., 2005). In Trichosanthes, 41 species have been reported, and 21 of these have originated in India, parts of tropical Asia or Indo-Malayan region. Among these, snake gourd (*T. anguina*) and pointed gourd (*T. dioica*) are cultivated throughout India. The major diversity is distributed in North-Central and North-Eastern India for *T. dioica*; whereas *T. anguina* is distributed in warm humid areas throughout the country but rich diversity is observed in North-Eastern region, West Bengal, Karnataka and Kerala. Out of 45 species of *Momordica* 7 are found in India. Bitter gourd (*M. charantia*), a popular vegetable, is grown all over tropical and sub-tropical regions. It has striking variability in India. Sweet gourd (*M. cochinchinensis*) is popular in Tripura, Assam and West Bengal whereas, Kakrol (*M. dioica*) is cultivated in West Bengal, Assam, parts of Bihar and adjoining areas. There are several other *Momordica* species of minor importance, which include *M. foetida* and *M. grosvenori* which has potential for the extraction of sweet glycoside component. In addition, *Momordica balsamina* occurs in semi dry North Western plains, Northern parts of Eastern and Western Ghats.Ash gourd (*Benincasa hispida*) was domesticated in India during pre-historic times. It is widely grown all over the country in tropical and subtropical regions and possesses large variability for morphological and quality characters (Srivastava, 1993). In *Cucurbita* spp. diversity occurs in the North Easternpart of this country. Ivy gourd (*Coccinia grandis*) is cultivated in warm and humid areas in India and *Coccinia cardifolia* (raw fruits used as vegetables) is growing wild throughout this country (Ram et al., 2002).

Diversification of minor/underutilized species in India

There are several minor/underutilized cucurbits which are grown and consumed in tribal belt in India. These are mainly *Cucumis hystrix, cucumis trigonus, Luffa graveolens, Momordica macrophylla, Momordica subangulata, Trichosanthes cucumerina, Trichosanthes khasiana, Trichosanthes ovata,* and *Trichosanthes truncasa* (Ram and Srivastava, 1999).

Rare and endangered cucurbits in India

Some rare and dying out species of cucurbits which are still available in India are given in Table 2.

Intensification of germplasm

The National Bureau of Plant Genetic Resources (NBPGR), New Delhi, has made concerted efforts to augment the plant genetic resources from indigenous and exotic sources. Through this organization, a number of cultivated and their wild relatives have been introduced from different countries. These include *C. lanatus* (18 lines from USA), *C. melo* (25 lines from France and Japan), *C. sativus* (35 lines from Japan and USA), *Cucumis heptadactylus* from USA, *Cucumis metuliferus* (16 lines from USA), *Cucumis anguria*(94 lines from USA),*Cucurbitapepo*(22 lines from USA), *Cucurbita moschata*(4 from Algeria and Japan,6 from USA), *Luffa cylindrica*(1 from Japan) and *Lagenaria siceraria*(1 from Japan,). Besides cultivated species, some wild species have also been introduced including *Citrullus rehmi, Cucumis aculeatus, Cucumis africanus, Cucumis dipsaceus, Cucumis meeurei, Cucumis myriocarpus, Cucumis pustulatus,*

Table 2. Rare and endangered cucurbits in India.

Species	Biogeographic zones
Corallocarpousgracillipes	Western Ghats
Gomphogynemacrocarpa	East Himalaya
Indogevilleakhasiana	North East India
Luffaumbellate	Western Ghats
Melothriaamplexocaulis	Deccan Peninsula
Momordica sub sp. angulata	Deccan Peninsula, Western Ghats
Trichosantheslepiniana	Deccan Peninsula, Western Ghats
Trichosanthesperrotteliana	Western Ghats

(Sirohi et al., 2005).

Table 3. Introduction commercialized in India.

Crop	Introduced variety	Country of origin
Watermelon	Asahi Yamato	Japan
	Sugar Baby	USA
	New Hampshire Midget	USA
	Improved Shipper	USA
	Dexielce	USA
Cucumber	Japanese Long green	Japan
	Straight Eight	USA
	Poinsette	USA
Summer squash	Australian Green	Australia
	Patty Pan	USA

Cucumis sagittatus, Cucumis zeyheri, Cucurbita ecuadorensis and Cucurbita martinezii (Hore and Sharma, 1990).Several exotic cultivars introduced through NBPGR have directly been adapted for large-scale commercial cultivation in India (Table 3). Characterization and evaluation of cucurbit germplasm is being done by NBPGR and crop based institutes and a number of promising accessions have been identified. Twelve genetic stocks having unique traits, have been registered (Table 4). Over 400 base collections of different cucurbitaceous crops are under long-term conservation in NBPGR. Recently an attempt has been made to augment and collect available germplasm diversity in cucumber and melon under Indo-US-PGR programme from Rajasthan and Madhya Pradesh. After proper evaluation and seed increase, the material will be conserved in Indian Gene Bank at NBPGR and at National Seed Storage Laboratory (NSSL), Fort Collins, USA. NBPGR has initiated assembling germplasm (presently 7025) in 15 different cucurbits (Rai et al., 2005).

The Central Institute of Arid Horticulture (CIAH), Bikaner has identified super lines in drought tolerant kachri, viz. AHK 119, AHK200, in drought tolerant and disease resistant snap melon, viz. AHS 10 and AHS 82 and in watermelon with longer shelf-life Mateera, viz., AHW 19. Tumba (*Citrullus colocynthis*) is a medicinal plant allied to watermelon (Seshadri and Srivastava, 2002).

At IIVR, Varanasi, groundwork evaluation of some of the cucurbits has been made. In bitter gourd, 219 collections have been completed. A significant observation in the evaluation studies was a gynoecious segregate, which has been maintained. Nine collections have shown tolerance to CMV. In bottle gourd 58 collections are available at NBPGR. Two accessions (U10-316 and NIC 1225) were found tolerant to red pumpkin beetle, three (U10-316, IC 92362 and IC 92418) were tolerant to leaf miner and four (U10-316, IC 92362, Laxmipur and Patna-1) were found to be tolerant to downy mildew. In pointed gourd, a parthenocarpic line (PG 105) has been identified at IIVR, Varanasi. Root-gall free accessions have also been located and evaluation is in progress in pointed gourd germplasm against heat tolerance, sun-scorching, mites and leaf miner incidence. In watermelon exotic collections were evaluated for leaf miner incidence and in cucumber against CMV and red pumpkin beetle (Rana et al., 1995).

This organization has identified some useful breeding traits (drought resistance, high rainfall and low temperature resistance, short day responsive, resistance to anthracnose, angular leaf spot, cucumber green mosaic mottle virus, downy mildew, powdery mildew, belly rot, root knot nematode) and their sources among Indian cultivated and wild cucumber (*C. prophetarum, C. callosus, C. hystrix, C. sativus, var. sikkimensis, C.sativus vat. hardwickii*) (Sharma and Hore, 1996).

Diversity, as a tool of improvement

Varietal development. The evaluation of indigenous and exotic germplasm introductions, and their hybridization resulted in the selection of 112 superior varieties of different cucurbits. Several varieties of cucurbits like Asahi Yamato from Japan, Sugar Baby, New Hampshire Midget, Improved Shipper and Dexielce from USA in Watermelon, Japanese Long Green from Japan and Poinsette from USA in Cucumber and Australian Green from Australia and Patty Pan from USA in Summer Squash have been introduced. Of these varieties, Hara Madhu, Arka Rajhans, Pusa Sarbati, Punjab Sunehari, Punjab Rasila and Kashi Madhu of musk melon, Kalyanpur Baramasi, Priya, Coimbatore Long and Pusa Domausami of bitter gourd, Sugar Baby, Arka Manik, Durgapura Meetha and Arka Jeet of watermelon, Pusa Naveen, Pusa Sandesh, PSPL(Pusa Summer Prolific Long), PSPR(Pusa Summer Prolific Round) and Arka Bahar of bottle gourd, PusaVishwas, Arka Chandan and Kashi Harit of pumpkin and in cucumber Japanese Long Green, Himangi, Pusa Uday and Swarna Ageti are among the popular varieties due to their high yield potential, qualitative traits and consumer's preference. As a result of multi-location testing under All India Coordinated Vegetable Improvement Project, 48 improved varieties in 8 major cucurbits have been identified and recommended for cultivation

Table 4. Registered germplasm of cucurbits for unique traits.

Crop	Line	National germplasm Identity No.	Registered trait
Pointed gourd	IIVR PG-105	INGR-03035	Parthenocarpic fruits
Bitter gourd	GY-63	INGR-03037	Gynoecious sex with high yield
Watermelon	RW-187-2	INGR-01037	High yield and yellow coloured flesh
	RW-177-2	INGR-01038	Simple unlobed Leaf mutant
Bottle gourd	Androman-6	INGR-99009	Andromonoecious sex
	PBOG-54	INGR-99022	Segmented leaves
Cucumber	AHC-2	INGR-98017	High yield and long fruit
	AHC-13	INGR-98018	Small fruit, drought and temperature tolerant
*Cucumismelo*var*callosus*	AHK-119	INGR-98013	High yield and drought tolerance
Round melon	HT-10	INGR-99038	Tolerant to downy mildew, root rot and wilt
Snap melon	AHS-10	INGR-98015	High yield and drought tolerance
	AHS-82	INGR-98016	High yield and drought tolerance
	B-159	INGR-07044	Downy mildew resistance

Fig. 1. Parthenocarpic cucumbers.

and release in various agro-climatic regions of the country (Rai et al., 2004 & 2007).

Hybrid development. Hybrids are commercialized in many cucurbits, due to desirable heterosis for yield and other traits. In the case of muskmelon (Pusa Rasaraj, Punjab Hybrid-1, MHY-3), watermelon (Arka Jyoti), cucumber (Pusa Sanyog, AAUC-1, PCUCH-1(Pant cucumber hybrid-1), PCUCH-3(Pant cucumber hybrid-3, bottle gourd (Pusa Manjari, Pusa Meghdoot, Kashi Bahar, Pusa Hybrid-2, NDBH-4) (Narendra bottle gourd hybrid-4), several hybrid cultivars have been developed (Kalloo et al., 2000).

Breeding for resistance. Utilizing some land races and wild relatives most of the resistant varieties in cucurbits have been developed by simple selection viz., in musk melon cv. Arka Rajhans, Punjab Rasila and DMDR-2 and DVRM-1 are resistance against Powdery mildew, downy mildew and cucumber green mottle mosaic virus respectively. Watermelon cv. Arka Manik is resistance to powdery mildew, downy mildew and anthracnose.

Clonal selection. Pointed gourd, Ivy gourd, spine gourd, sweet gourd are vegetatively propagated crops and promising clone of above crops are selcetd todevelopnew varieties. Seedless pointed gourd is selected from a population as clonal selection at IIVR, Varanasi. Pointed gourd clones identified for cultivation includes SwarnaAlaukik, SwarnaRekha, Rajendra Parwal-1, Rajendra Parwal-2, Narendra Parwal-260, Narendra Parwal-307, Narendra Parwal-604, IIVR PG-1, IIVR PG-2, and IIVRPG-105. Clone IIVR IG-1 of ivy gourd has also been identified for cultivation.

Varieties of bitter gourd (MDU-1) and ridge gourd (PKM-1) have been developed through mutation breeding. Similarly, 'Pusa Bedana', a triploid watermelon variety is an example of polyploidy breeding in cucurbits in India (Bose et al., 2002).

Pioneer work on parthenocarpic cucumber in India for protected condition

Polyhouse vegetable breeding programme in cucurbits was initiated at G.B.P.U.A. &T., Pantnagar, India. Large numbers of cucumber gene pool/germplasm lines were collected from different parts of the country and abroad. These accessions were evaluated for the search of Pc (parthenocarpic) and F (gynoecious) gene (Singh et al., 2010).Out of which some genotypes were evaluated for protected conditions (Singh and Padiyar, 2009). Two varieties of parthenocarpic cucumber namely; Two parthenocarpic cucumber lines were selected (Fig.1) and tested in multi-locational trials under polyhouse condition. These genotypes were released by Uttarakhand State Variety Release Committee for commercial poly house cultivation.

Upcoming standpoint

More focus should be given on collection, characterization, evaluation and utilization. Unexploited minor cucurbits should be exploited there is a need to screen them for biotic and abiotic stresses, desirable yield and quality characters. Besides, molecular techniques may be utilized for phylogenetic study of indigenous cucurbits and identification of divers germplasm to avoid the duplication. Awareness should be generated among the scientists and farmers to register the promising and unique germplasm.

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Recurrent Selection for Melonworm Resistance in Tropical Pumpkin

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Additional index words. *Diaphania hyalinata*, *Cucurbita moschata*, squash, quantitative resistance, insect resistance, phenotypic selection, detached leaf test

Abstract. The melonworm, *Diaphania hyalinata,* is a destructive pest of squash and pumpkin in tropical and subtropical areas of the Americas. Two cycles of phenotypic recurrent selection (RS) for resistance to melonworm were carried out in the open-pollinated tropical pumpkin population *Cucurbita moschata* cv. Taína Dorada. In each cycle, 350 to 500 greenhouse-grown seedlings were evaluated in detached leaf tests to estimate foliar damage by melonworm larvae. Entries with the least damage were further tested in the field in Lajas, Puerto Rico. Three trials were then conducted to test the effectiveness of RS. Trials also included two commercial genotypes ('Soler' and 'Verde Luz') and an experimental genotype ('Nigerian Local'). Entries were evaluated for foliar damage, number of eggs and larvae per leaf, and fruit size and yield. Foliar damage was not reduced, and only a slight reduction in the numbers of eggs and larvae per leaf was observed after two RS cycles. Selection for resistance had no effect on yield and average fruit weight. More precise estimation of foliar damage and number of eggs or larvae per leaf may be required to improve response to RS. A selection index combining leaf damage and number of eggs or larvae may be more effective than selection for leaf damage alone. The RS, based on replicated families, may also improve response to selection for melonworm resistance.

In Puerto Rico and other parts of the American tropics and subtropics cucurbit production can be severely limited by the melonworm *Diaphania hyalinata* (L.) (Lepidoptera:Pyralidae) (Medina-Gaud et al., 1989; Guillaume and Boissot, 2001; Cruz and Segarra, 1992). This crop defoliator is also among the most important pests of cucurbits in Florida. According to Webb (1994), melonworm causes more damage to *Cucurbita* species in Florida than does the related pickleworm (*D. nitidalis*). While other members of the cucurbit family are also hosts to the melonworm, the insect prefers to feed on *Cucurbita* species. In a Florida study, Peña et al. (1987) found that *C. pepo* (summer squash and zucchini) was the principal host for pickleworm, whereas tropical pumpkin (*C. moschata*) was the principal host for melonworm.

Genetic differences in resistance to pickleworm or melonworm have been reported. Brett et al. (1961) noted higher galacturonic levels acids in *C. moschata* 'Butternut 23', the most resistant of the cultivars investigated. Dilbeck and Canerday (1968) noted that some *C. pepo* cultivars were more susceptible to pickleworm than *C. moschata* cv. Butternut or *C. maxima* cv. Hubbard. Elsey (1985) found *C. moschata* cultivars to be more resistant to both pickleworm and melonworm than *C. pepo* cv. Table Queen. He noted that the principal mechanism of resistance was oviposition nonpreference. Glabrous mutants exist in both melon and cucumber that are resistant to oviposition by pickleworm and melonworm females (Elsey and Wann, 1982). Elsey (1981) did not observe differences in the survival and development (antibiosis) of pickleworm larvae on excised foliage, flowers and fruit of various cucurbits with the exception of bottlegourd (*Lagenaria siceraria*). Wehner and Kennedy (1983) and Wehner et al. (1985), working with pickleworm in cucumber, suggested that an effective genetic control would prevent oviposition by adult females and/or feeding by larvae, but that antibiosis to larvae would be an easier trait to evaluate. In Guadeloupe, Fench West Indies, Guillaume and Boissot (2001) looked for resistance to melonworm in 15 melon (*C. melo*) genotypes and two wild *Cucumis* species in two field trials and laboratory detached leaf tests. Both antibiosis (expressed as longer duration and higher mortality of immature stages) and antixenosis (nonpreference) were found to play a role in resistance.

In 2003 and 2004 in Puerto Rico, Pérez-Arocho (2011) carried out a field screening of approximately 300 accessions of *Cucurbita moschata* and *C. argyrosperma* in an unsuccessful attempt to identify sources of qualitative resistance to melonworm. This result led us to pursue an alternative approach: breeding for quantitative resistance via phenotypic recurrent selection.

Materials and Methods

Detached-Leaf Tests. Larvae used in detached-leaf tests were obtained from eggs laid by laboratory-reared adults following a protocol similar to that of Elsey (1985), with some modifications. Pumpkin leaves with melonworm larvae were collected from the field and placed in water reservoirs in cages with crumpled paper towels. Fresh leaves were added to the cages as needed. As the mature larvae moved onto towels to pupate, pupa were collected and placed in petri dishes in cages with pumpkin seedlings. Emerging adults were allowed to oviposit on these plants and the 2nd instar larvae were collected for use in detached leaf (no-choice) tests.

A total of 500 seedlings of the susceptible open-pollinated cultivar Taína Dorada (TD) were evaluated in detached-leaf tests.

Acknowledgements. This research was funded by USDA award numbers 2006-38422-17106 and 2005-34135-15883 and Puerto Rico Experiment Station Hatch funds.

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Tests were carried out on groups of 100 seedlings at a time. At about 20 days after planting, the third and the fourth true leaves were excised from each plant (2 reps/plant). A 6 cm x 4 cm area of tissue was cut from each leaf and placed in a 14 cm diameter petri dish lined with filter paper, to which 2.5 ml of water was added. Each leaf was infested with three 2nd instar larvae using a paintbrush or fine point tweezers. Percentage of leaf damage was estimated at 24 and 48 h using a 6 cm × 4 cm clear plastic template divided into 24 squares measuring 1 cm × 1cm. Percentage of damage was estimated for each square and averaged over the 24 squares.

Field Evaluation and Intercrossing. On the basis of the detached leaf test, 206 of the most resistant plants were transplanted to an isolated crossing block at Lajas, Puerto Rico. Melonworms were present in the field in high numbers within two weeks following transplanting. All plants were eliminated prior to flowering except 50 plants with the least amount of foliar damage, for a final selection intensity of 10% (50 of 500 plants tested). Selected plants were allowed to intermate and their seed bulked to form TD-Cycle 1.

In the 2nd cycle of recurrent selection, 350 seedlings from the TD-Cycle 1 bulk were subjected to the detached leaf test. One hundred thirty-three plants were transplanted to the field in Lajas and 35 plants were selected before anthesis (10% selection intensity). Selection and intermating was as in Cycle 1. Harvested seed was bulked to produce TD-Cycle 2.

Evaluation of Two Cycles of Recurrent Selection. Three field trials were conducted at two locations (Lajas and Isabela). In addition to the base population (TD) and two cycles of selection of TD (TD-Cycle 1 and TD-Cycle 2), three additional genotypes were tested: 'Soler' (the local standard), 'Nigerian Local' (a susceptible genotype), and 'Verde Luz' (an open pollinated, silverleaf-resistant selection from 'Soler'). In Trial 1 (Lajas), populations were planted in single-row plots 14.4 m long. In Isabela, populations were planted in two-row plots 7.2 m (Trial 2) or 13.5 m long (Trial 3). Plots in Isabela were separated by a distance of 7.2 m, with a row of TD running down the center of this area. At 8 weeks after planting, both individual plots and the spreader rows of TD were infested using melonworm-infested leaves collected from another field in order to obtain a more uniform distribution of insects throughout the experiment. The purpose of Trial 3 was to have a larger plot size to obtain more reliable yield data compared to the smaller plots of Trials 1 and 2. Spacing between plants varied, depending on plant architecture (viney or semi-bush). Plant density was 2,964 plants per hectare for 'Soler', 'Nigerian Local' and 'Verde Luz' (viney types) and 5,928 plants per hectare for TD, TD-Cycle 1 and TD-Cycle 2 (semi-bush types). Raised beds with drip irrigation and silver plastic mulch were used in Lajas. Drip irrigation alone was used in the two Isabela trials.

Damage due to melonworm was rated on a scale of 1 to 5 (0 = no visible damage; 1 = >0 to 20% defoliation; 2 = >20 to 40% defoliation; 3 = >40 to 60% defoliation; 4 = >60 to 80% defoliation; 5 = more than 80% defoliation). In Trial 1 (Lajas), data for foliar damage was collected during a period of eight weeks (July to September 2007); in Trial 2 (Isabela) data was collected during a period of nine weeks (October and November 2007). Egg and larva counts were taken in a sample of leaves from each plot using the third leaf from the growing point. In Trial 1 (Lajas), 4 leaf samples per plot were collected at 6 weeks after planting. At 7, 8, and 9 weeks 8 leaves per plot were sampled, followed by 25 leaves per plot at 10 weeks. In Trial 2 (Isabela), sampling was carried out 8 to 10 weeks after planting. Fifteen leaves per plot were sampled for larval counts and 31 leaves per plot were collected for egg counts. Within a plot, samples were collected in a systematic "zig zag" manner. Egg counts were done using a 1X stereoscope. Larval counts were done by eye.

Foliar damage and egg and larva counts were not taken in Trial 3, and no yield data was collected in Trial 1. In Trials 2 and 3, fruits in each plot were harvested, weighed and counted.

Results

During the first cycle of selection (population TD) the damage among the 500 plants ranged from 0% to 96% in the detached leaf test at 24 hours and 0% to 100% at 48 hours. About 58% of the seedlings were eliminated based on the detached leaf test. In the second cycle of selection (population TD Cycle-1) damage was 0% to 46% at 24 hours and 2 to 100% at 48 hours. About 62% of the seedlings were culled based on the detached leaf test. In both cycles of selection many plants had a similar percentage of damage, and therefore selection among those plants was not possible.

The selected plants were transplanted in the field at Lajas under conditions of natural infestation of melonworm. Plants had approximately 5 true leaves when transplanted to the field. In each cycle of selection, the presence of melonworm larva was noted within two weeks of transplanting, and well before anthesis. Plants were eliminated as soon as it was clear that they were highly susceptible to melonworm. All selections were made before open female flowers were present in the field. Thus, inter-mating occurred only among the 10% of plants most resistant to melon.

Evaluation of Two Cycles of Recurrent Selection. The natural population of melonworm was too low during Trial 1 (Lajas) and no foliar damage was observed among the tested populations (data not shown). In Trial 2 (Isabela), damage due to melonworm was minimal in weeks 1 to 5 (data not shown), but more than 80% of the leaves were damaged by 11 weeks after planting (Table 1). Within a particular week, there were no differences among TD, TD-Cycle 1 and TD-Cycle 2, indicating that recurrent selection was not effective for reducing foliar damage.

In both Trial 1 (Lajas) and Trial 2 (Isabela), there was a consistent, although non-significant, trend of fewer eggs on leaves of TD-Cycle 2 compared to TD (Table 2). A similar trend was not observed for number of larvae except at 10 weeks after planting (Table 3). In general, 'Verde Luz' and 'Nigerian Local' had the lowest number of both eggs and larvae although again, these differences were usually not significant (Tables 2 and 3).

Table 1. Foliar damage by melonworm in tropical pumpkin populations evaluated in Isabela, Puerto Rico (Trial 2). Taína Dorada (TD) was subjected to two cycles of recurrent selection.

Week	TD	TD-Cycle 1	TD-Cycle 2	Soler	Verde Luz	Nigerian Local
6	0.0 a	0.0 a	1.0 b	0.0 a	0.0 a	0.0 a
7	1.0 b	1.0 b	1.0 b	1.0 b	0.0 a	0.0 a
8	2.5 a	2.0 a	3.0 a	2.0 a	2.0 a	0.0 a
9	3.0 a	2.5 a	3.5 a	2.5 a	2.0 a	0.0 a
10	4.8 b	4.0 b	3.8 b	3.2 b	3.8 b	0.5 a
11	5.0 a	5.0 a	5.0 a	5.0 a	5.0 a	5.0 a

Damage scale (defoliation): 0 = no visible damage; 1 = >0 to 20%; 2 = >20 to 40%; 3 = >40 to 60%; 4 = >60 to 80%; 5 = more than 80%. Within rows, means followed by the same letter are not significantly different at $\alpha = 0.05$ according to Fishers protected LSD.

Table 2. Number of melonworm eggs per leaf in populations of tropical pumpkin evaluated in Lajas (Trial 1) and Isabela (Trial 2), Puerto Rico. Taína Dorada (TD) was subjected to two cycles of recurrent selection.

	Lajas	Isabela			
Genotypes	8 weeks	7 weeks	8 weeks	9 weeks	10 weeks
TD	2.09 c	0.69 ab	1.15 b	0.32 a	1.00 b
TD-Cycle 1	2.31 c	0.48 a	0.92 ab	0.53 a	0.77 ab
TD-Cycle 2	1.34 b	0.61 ab	0.90 ab	0.26 a	0.54 ab
Nigerian Local	3.53 d	1.16 b	0.16 a	0.13 a	–
Soler	0.84 ab	0.48 a	0.75 ab	0.13 a	0.59 ab
Verde Luz	0.59 a	0.13 a	0.55 ab	0.23 a	0.22 a

Within a column, means followed by the same letter are not different at the $\alpha\alpha$ =0.05 probability level according to Fisher's protected LSD.

Table 3. Number of larvae per leaf in populations of tropical pumpkin evaluated in Lajas (Trial 1) and Isabela (Trial 2), Puerto Rico. Taína Dorada (TD) was subjected to two cycles of recurrent selection.

	Lajas	Isabela			
Genotypes	8 weeks	7 weeks	8 weeks	9 weeks	10 weeks
TD	4.00 c	1.30 b	0.50 ab	0.22 ab	1.62 b
TD-Cycle 1	0.50 ab	0.63 ab	0.73 ab	0.28 ab	0.52 ab
TD-Cycle 2	3.00 bc	1.40 b	1.17 b	0.52 b	0.30 a
Nigerian Local	0.00 a	0.27 a	0.00 a	0.00 a	–
Soler	0.75 ab	0.67 ab	0.23 ab	0.18 ab	0.28 a
Verde Luz	0.25 a	0.33 a	0.27 ab	0.38 ab	0.00 a

Within a column, means followed by the same letter are not different at the $\alpha\alpha$ = 0.05 probability level according to Fisher's protected LSD.

In Trial 2 in Isabela, TD, TD-Cycle 1, TD-Cycle 2 and 'Soler' produced the same number of fruits (Table 4). 'Verde Luz' produced the greatest number of fruits. No significant yield differences were observed in the base population (TD) and the two cycles of selection (TD-Cycle 1 and TD-Cycle 2). 'Verde Luz' produced the highest yield. 'Soler' had the largest average fruit weight. 'Verde Luz' consistently produced small fruits (low average fruit weight), whereas the semi-bush types (TD, TD-Cycle 1, TD Cycle 2) tended to produce fruits of intermediate weight. In Trial 3 in Isabela, no yield differences were observed among the base population and the two cycles of selection.

Discussion

The logistics involved in managing the large number of plants and insects needed in the detached leaf test was challenging. Producing sufficient numbers of larvae at one time requires careful planning and good rearing facilities (our facilities were limited). In order to test two leaf samples from each group of 100 plants, 600 2nd instar larvae needed to be available at a given time.

Variability of larval size likely introduced considerable variability into the detached leaf test and limited its usefulness as a selection technique. Within a group of 100 plants an effort was made to infest each leaf with 3 larvae of the same size. However, there was likely a tendency to use larger larvae (more easily seen) on the first leaves infested in a group of a 100 plants, and smaller larvae on the last leaves of the same group. Differences in the age of the plant might also affect insect behavior. Since we planted all 500 seeds at the same time, the age of seedlings from different groups of 100 differed by up to as much as 10 days.

Table 4. Number of fruit, yield, and average fruit weight of melonworm-susceptible tropical pumpkin Taína Dorada (TD) and populations from two cycles of selection for resistance.

Genotype	Number of fruit per hectare	Yield (kg/ha)	Average fruit weight (kg)
Isabela, Puerto Rico Trial 2—small plot trial			
TD	8,339a	25,737 a	3.2 ab
TD-Cycle 1	8,920a	28,084 a	3.2 ab
TD Cycle 2	8,434a	27,449 a	3.4 b
Soler	6,727a	35,368 a	5.3 c
Verde Luz	20,864b	51,320 b	2.5 a
Isabela, Puerto Rico Trial 3—large plot trial			
TD	6,564a	20,947a	3.2a
TD-Cycle 1	9,976a	35,924a	3.6a
TD Cycle 2	6,418a	21,970a	3.4a

Within a column and trial, means followed by the same letter are not different at α=0.05 probability level according to Fisher's protected LSD.

At the field level there were also various challenges. A sufficient population of melonworm must be uniformly distributed throughout the field. In addition, an efficient evaluation technique for foliar damage needs to be used. In general, the limited success of selection for resistance encountered in this research was often due to the difficulties of managing the many aspects of a dynamic population of insects.

The effectiveness of phenotypic recurrent selection for resistance was not as great as we hoped at the start of this research. Heritability of quantitative resistance to melonworm is clearly low. In addition to improving upon the detached leaf tests and techniques used to estimate foliar damage and egg and larva numbers in the field, a recurrent selection scheme using replicated half-sib or S_1 families would likely result in greater improvement in resistance. Selection based on an index combining foliar damage and egg and larvae counts might be more effective than basing selection on foliar damage alone. Larger numbers of leaves will likely need to be sampled in order to obtain better estimates of numbers of egg and larva.

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Comparative Analysis of rDNA Loci in *Citrullus* Species and *Acanthosicyos naudinianus* by Fluorescence *in situ* Hybridization

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Additional index words. rDNA, *Citrullus* species, phylogenetic relationship, fluorescence *in situ* hybridization

Abstract. Number and position of the 5S and 45S ribosomal DNA (rDNA) loci are important species characteristics. Comparative mapping of rDNA repeats by fluorescence *in situ* hybridization (FISH) is very useful in determining phylogenetic relationships between closely related species. In this study, the number and position of 5S and 45S rDNA sites in *Citrullus* species and *Acanthosicyos naudinianus* were reported. The cultivated watermelon (*C. lanatus* var. *lanatus*), *C. colocynthis* and *A. naudinianus* had one 5S rDNA locus and two 45S rDNA loci, while the wild watermelon (*C. lanatus* var. *citroides*), *C. ecirrhosus* and *C. rehmii* exhibited two 5S rDNA loci and one 45S rDNA locus. Among the species examined, *C. colocynthis* appears more closely related to the cultivated watermelon. This result supports the earlier hypothesis that *C. colocynthis* might be the progenitor of the cultivated watermelon.

The Cucurbitaceae is a large and diverse family containing several economically important crop species such as squash/pumpkin (*Cucurbita spp*), melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Robinson and Decker-Walters, 1997). The watermelon ($2n = 2x = 22$) belongs to the *Citrullus* genus. The taxonomy of the genus *Citrullus* is complex and four or five species are usually distinguished: *C. lanatus* (Thunb.) Matsum. & Nakai, *C. colocynthis* (L.) Schrad., *C. eccirrhosus* Cogn., *C. rehmii* de Winter and *C. naudinianus* (Sond.) Hook.f. (De Winter, 1990; Robinson and Decker-Walters, 1997). *C. lanatus* is the most polymorphic species of the genus which could be further divided into three subspecies. The most widely cultivated forms belong to *C. lanatus* subsp. *vulgaris*. The second subspecies *C. lanatus* subsp. *mucosospermus* is a wild or semi-cultivated taxon. The third subspecies, *C. lanatus* subsp. *lanatus*, includes wild annual forms, primitive cultivars and weeds in watermelon fields. The cultivated watermelon and *mucosospermus* types have been designated as *C. lanatus* var. *lanatus* (Thunb.) Mat. & Nak., while wild *C. lanatus* subsp. *lanatus* forms are classified as *C. lanatus* var. *citroides* (Bailey) Mansf (Robinson and Decker-Walters, 1997; Jeffrey, 2001). *C. naudinianus* differs sufficiently from the other *Citrullus* species that Jeffrey (2001) included it in the genus *Acanthosicyos* (*A. naudinianus* (Sond.) C. Jeffrey).

Although various characteristics have been used for studying phylogenetic relationships among different *Citrullus* species, the conclusions are contradictory and the exact origin of domesticated watermelon has not yet been determined (Wasylikowa and van der Veen, 2004). Cytological (Whitaker, 1933) and cross-compatibility (Shimotsuma, 1960) observations favored *C. colocynthis* as the ancestor of *C. lanatus*. However, *C. ecirrhosus* has also been found to be closely related and suggested as probable ancestor of *C. lanatus* based on sequencing analysis of cpDNA regions (Dane et al., 2004). But the analysis of the internal transcribed spacers of the rDNA indicated that annual *C. rehmii* was more closely related to the annual cultivated watermelon than the perennials *C. ecirrhosus* and *C. colocynthis* to the cultivated watermelon (Jarret and Newman, 2000).

Chromosomes often serve as one of the most important aspects of studying the evolution of species. One of the fundamental tools used for elucidating the origin of genome/chromosomal rearrangements between related taxa is the study of large repetitive DNA families (Cerbah et al., 1998; Shan et al., 2003). The two most common used are the ubiquitous tandemly arrayed 5S and 45S ribosomal DNA (rDNA) families. Fluorescent *in situ* hybridization (FISH) is an excellent tool for physical mapping of rDNA (Jiang and Gill, 1994). To date, the position and number of rDNA loci have been determined in more than 1,000 plant species with FISH (Garcia et al., 2012). These studies showed that the number, position and organization (either linked or unlinked) of the 5S and 45S rDNA on the chromosomes were usually characteristic of a given species, genus or group (Garcia et al., 2009) and 5S and 45S rDNA tended to occupy similar chromosomes and positions in closely related species (Cai et al., 2006; Srisuwan et al., 2006; Berjano et al., 2009). Therefore, FISH mapping of rDNA repeats is very useful in determining phylogenetic relationships and the origin and evolution of species.

In *Citrullus* species, previous studies showed that there were two 45S rDNA sites and one 5S rDNA site in the cultivated watermelon genome. The 5S rDNA site was located syntenic to one of the 45S rDNA sites (Ren et al., 2012). Guo et al. (2013) further investigated rDNA patterns in genomes of three watermelon (*C.*

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lanatus) subspecies using FISH and found that the number and location of 5S and 45S rDNA sites in the genomes of modern cultivated (*C. lanatus* subsp. *vulgaris*) and semi-wild watermelon (*C. lanatus* subsp. *mucosospermus*) were identical, but different from those in the distantly related wild watermelon (*C. lanatus* subsp. *lanatus*). Therefore, they proposed evolutionary scenario of *C. lanatus* subsp. *mucosospermus* to *C. lanatus* subsp. *vulgaris*. However, the distribution patterns of 5S and 45S rDNA in other members of the *Citrullus* genus have not been reported. In this study, we investigated the number and distribution of 5S and 45S rDNA loci in other *Citrullus* species and *Acanthosicycos naudinianus* by FISH in order to gain additional information about evolutionary and phylogenetic relationships between the cultivated watermelon and its related species.

Materials and Methods

Plant materials. The tested materials include *Citrullus lanatus* var. *lanatus*, *C. lanatus* var. *citroides*, *C.colocynthis*, *C. ecirrhosus*, *C. rehmii* and *A. naudinianus*.

Chromosome preparation. Root tips were harvested from germinated seeds, pretreated in 0.002M 8-hydroxyquinoline at room temperature for 2 h to shorten chromosomes, and fixed in methanol:glacial acetic acid (3:1). After washing with water, root tips were macerated in 2% cellulose and 1% pectolyase at 37 °C for 2.5 h. Finally, the treated root tips were squashed and dried in flame.

Fluorescence in situ hybridization (FISH). The 5S and 45S rDNA were labeled with digoxigenin-dUTP and biotin-dUTP via nick translation and detected with antidigoxigenin antibody coupled with Rhodamine (Roche) and avidin-conjugated with FITC (Vector Laboratories), respectively. FISH was performed according to published protocols (Jiang et al., 1995).

Results and Discussion

Comparison of chromosome number in Citrullus species and A. naudinianus. Fig.1 a–f showed the results of double-target FISH using the 5S rDNA (red) and 45S rDNA (green) probes to mitotic metaphase chromosomes of *Citrullus species* and *Acanthosicycos naudinianus,* respectively. All *Citrullus* species (Fig. 1 a–b, d–f) showed the same chromosome number 2n = 22 while *Acanthosicycos naudinianus* had 24 chromosomes (Fig. 1c). It is not possible to precisely identify the chromosomal pairs in which the rDNA clusters are located because the chromosomes are relatively small in size and are morphologically similar in these species.

Chromosomal distribution patterns of rDNA loci in Citrullus species and A. naudinianus. Like the genome of cultivated watermelon (*C. lanatus* var. *lanatus*) (Fig. 1a), there were one 5S rDNA locus (red) and two 45S rDNA loci (green) in the genomes of *C. colocynthis* (Fig. 1b) and *A. naudinianus* (Fig. 1c). In *C. colocynthis,* 5S rDNA locus was positioned at the chromosome arm carrying one of the 45S rDNA loci. The 5S and 45S rDNAs on chromosome arms occupied the proximal and distal sites, respectively (Fig. 1b). In *A. naudinianus* (Fig. 1c), 5S rDNA locus was positioned at the chromosome carrying one of the 45S rDNA loci. But the 5S rDNA and 45S rDNA loci weren't located on the same chromosome arm.

In the genomes of *C. ecirrhosus* (Fig. 1e) and *C. rehmii* (Fig. 1f), two 5S rDNA loci (red) and one 45S rDNA locus (green) were observed, which were identical to *C. lanatus* var. *citroides* (Fig. 1c). The 5S loci were located on the two pairs of chromosomes which did not carry 45S rDNA sequences. One 5S rDNA locus localized at the distal end of the short chromosome arm, while the other 5S rDNA locus located intercalary near to the centromere on the short arm (Fig. 1e, 1f).

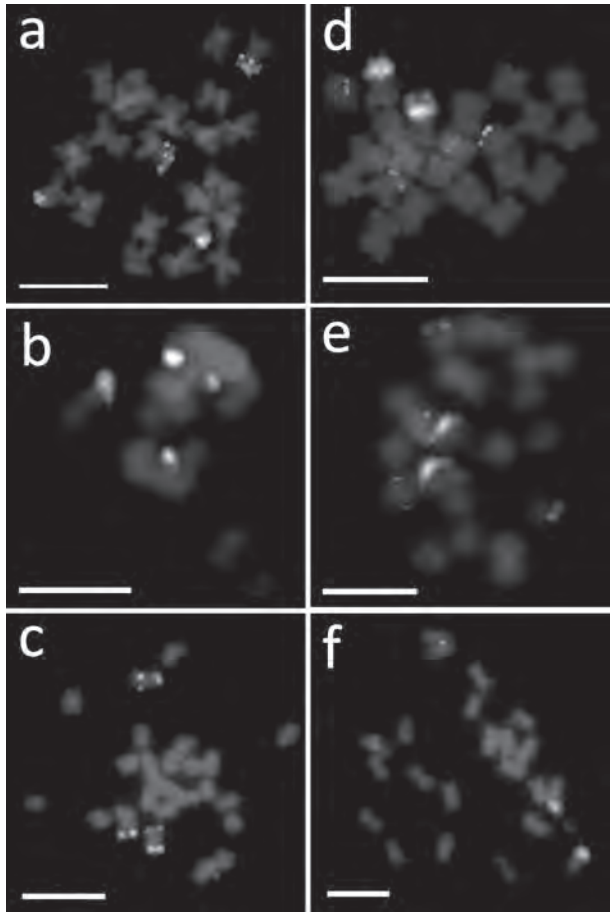

Fig. 1. Chromosomal distribution patterns of 5S (red) and 45S rDNA (green) in *Citrullus* species and *A. naudinianus*. **(a)** *Citrullus lanatus* var. *lanatus*. **(b)** *C. colocynthis*. **(c)** *Acanthosicycos naudinianus*. **(d)** *C. lanatus* var. *citroides*. **(e)** *C. ecirrhosus*. **(f)** *C. rehmii*. Chromosomes were counterstained with DAPI in all images and pseudocolored in blue. Bars = 5 μm.

In contrast to other characteristics such as diploid number and chromosomal morphology that are more conserved, FISH mapping of rDNA genes can be used as an efficient tool to help determine phylogenetic relationships as the most similar rDNA FISH patterns are likely to be in the most closely related taxa (Srisuwan et al., 2006). In some organisms, the most frequent location of rDNA genes on specific chromosomes has been suggested to be the ancestral condition. The present study showed that the number and positions of rDNA sites varied among *Citrullus* species and *A. naudinianus*. Among the species examined, *C. colocynthis* appears more closely related to the cultivated watermelon (*C. lanatus* var. *lanatus*), which is consistent with the earlier hypothesis that *C. colocynthis* might be the progenitor of the cultivated watermelon (Whitaker, 1933; Shimotsuma, 1960). The analyses of the sequence variation at cpDNA regions also supported the relationship because some of the *C. lanatus* var.

lanatus accessions shared a unique substitution at the trnE-trnT region with all *C. colocynthis* accessions (Dane and Liu, 2007). However, our present result was inconsistent with that from a previous study by Levi and Thomas (2005) who found that *C. colocynthis* had higher similarity to *C. lanatus* var. *citroides* than the cultivated watermelon (*C. lanatus* var. *lanatus*) in chloroplast and mitochondrial genomes.

Although no consensus was reached on the ancestral species of cultivated watermelon, most previous phylogenetic studies showed that *C. lanatus* var. *lanatus* and *C. lanatus* vat. *citroides* were more closely related to each other than they were to other *Citrullus* species (Jeffrey, 2001). Different from previous studies, FISH mapping of rDNA genes by Guo et al. (2013) and our present study indicate that *Citrullus lanatus* var. *lanatus* had similar rDNA distribution pattern with *C. colocynthis* while *C. lanatus* var. *citroides* had similar rDNA distribution pattern with *C. ecirrhosus* and *C. rehmii*. Therefore, *Citrullus lanatus* var. *lanatus* and *C. lanatus* var. *citroides* appear to have been derived from different progenitors and they should belong to different taxonomic species.

Our result also supported the taxonomic treatment to place *A. naudinianus* in the genus *Acanthosicyos* instead of *Citrullus*. *A. naudinianus* appears distantly related to *Citrullus* species. Despite the similar number of rDNA signals in *A. naudinianus*, *Citrullus lanatus* var. *lanatus* and *C. colocynthis*, these species differ in chromosome number and 5S rDNA positions.

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Development of SNP Markers and Gene Mining in Watermelon and Melon

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Additional index words. watermelon, melon, CAPS, linkage map, QTL mapping

Abstract. Illumina HiSeq 2000 sequencing of watermelon (*Citrullus lanatus*) varieties Cream of Saskatchewan (COS), LSW-177, Huayuan Female Parent and melon (*Cucumis melo* L.) varieties MR-1, Topmark were performed. A large scale identification of single nucleotide polymorphisms (SNP) was performed and SNP data were employed to develop cleaved amplified polymorphic sequence (CAPS) markers. 2485 and 3156 potential CAPS markers were identified that could be used in watermelon and melon, respectively. The F_2 populations of LSW-177 (low sugar content, red flesh) × COS (high sugar content, white flesh), Huayuan Female Parent (high sugar content, red flesh) × LSW-177 and MR-1 (powdery mildew resistance) × Topmark (powdery mildew sensitive) were developed for genetic mapping and QTL/gene location using new CAPS markers and developed SSR markers. We detected 5 QTLs of lycopene content related, flesh color and one of them is a major QTL which located between CAPS *WII04E07-40* and *WII04E07-33* in watermelon chromosome 4, other QTLs located in chromosome 3, 4, 6, and 11, respectively. In watermelon, we located a center sugar (CS) related QTLs on chromosome 2 and 3 edge sugar (ES) related QTLs in chromosome 1 and 10. In the study of melon powdery mildew resistance gene location, we investigated individual powdery mildew disease index of 354 plants in the F_2 population. Major powdery mildew resistance gene was located using new CAPS markers.

Watermelon (*Citrullus lanatus*) and melon (*Cucumis melo* L.) are globally major vegetable fruits. Both of them have high production ranks among the fruit crops grow under protected conditions and open field in China, where 2010 production of watermelon and melon was 6,280,3725 tons and 1,278,500,0 tons, respectively. In recent years, high-throughput sequencing has been applied extensively to develop new molecular markers. SNP and CAPS markers have routinely been used in agricultural breeding programs; plants and animal variation studies, genome mapping and integrated mapping (Kole and Abbott 2008). CAPS markers have already proven useful in increasing marker resolution in Cucurbitaceae crops for genes related with plant disease. Chunqiu Zhang (2013) developed a new CAPS marker (*CAPS-DdeI*) which can be used as a universal marker for effective marker assisted selection in melon powdery mildew resistance breeding. Monica Morales et al. (2005) detected a locus between CAPS markers *M29* and *M132* which could produces severe yield losses in melon and cucumber crops. A CAPS marker (*PM4-CAPS*) associated with powdery mildew QTL *Pm-R* was developed by Fernando J et al. (2011) in the report. Takahiro Tezuka et al. (2009) found an AFLP marker linked to *Fom-1* and converted it into CAPS marker which tightly linked with the locus *Fom-1*. Kaishu Ling et al. (2009) demonstrated CAPS marker (*CAPS-2*) performed a high co-segregation with *ZYMV* resistance using F_2 and BC_{2R} populations. A CAPS marker (*Phe226*) that perfectly co-segregates with red and canary yellow flesh color phenotypes was developed by Haejeen Bang (2010). However, little research has been done on the linkage map construction and lycopene or sugar content QTLs/genes detection with CAPS markers. We have screened two crosses with three watermelon lines which have re-sequenced to exploit specific CAPS markers related with important agronomic characters and powdery mildew resistance in watermelon and melon.

Material and Methods

DNA was extracted from the young leaf tissue of the watermelon line COS, LSW-177, Huayuan Female Parent and melon line MR-1, Topmark. The five genomic DNA were re-sequenced by the IlluminaHiSeq 2000 high-throughput sequencing platform. Output data for each genome covered more than 20× of the watermelon and melon genomes. Referenced watermelon and melon genome were used to map the original data by BWA software. SNP mining of the assembled data was performed by SAMTOOLS software, extracting 1000bp sequence before and after the SNP locus by self-compiled script on perl. CAPS locus mining was detected by SNP2CAPS software using the sequence contained SNP locus. 2458 and 3156 pairs of CAPS locus appropriate to design PCR primers were found using 11 kinds of restriction endonuclease (*Eco*RI, *Bsa*HI, *Hind*III, *Mbo*II, *Pst*I, *Sca*I, *Bam*HI, *Mlu*I, *Asu*II, *Dra*I and *Pvu*I) from the crosses 'LSW-177×COS' and 'Huayan Female Parent×LSW-177', respectively. 15~25 CAPS locus evenly distributed on each chromosome were chosen to design PCR primers and PCR products were digested with restriction endonuclease. Touchdown PCR was performed by preheating for 7 minutes at 94 °C, and heating employing 30 cycles for 60 s at 94 °C, for 60 °C at 20 s, and then performed a 0.5 °C drop in temperature

Acknowledgements. This work was supported by the National Natural Science Foundation of China (31272186), China Agriculture Research Systen (CARS-26-02).

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each cycle, for 60 s at 72 °C, heating employing 10 cycles for 20 s at 94 °C, for 20 s at 45 °C,for 60 s at 72 °C, and post-heating for 7 minutes at 72 °C. The reaction mixture for enzyme digestion contained: 5 µl PCR production, 9 µl ddH_2O, 0.3 µl restriction enzyme (10U/µL). Incubated at 37 °C for 1~16 h. The enzyme-digested products were examined by 1% agarose gel electrophoresis.

The F_2 generations of the crosses 'LSW-177×COS' , 'Huayan Female Parent×LSW-177' and 'MR-1×Topmark' were screened for constructing the linkage map and detecting the QTL/gene(s) for lycopene content, sugar content and powdery mildew resistance, respectively. Phenotypic data was collected by lycopene content, the content of fructose, sucrose, glucose, and disease index of powdery mildew

Icimapping V3.3 software was used to construct linkage maps. Markers were grouped at a minimum LOD score of 3.0 and a maximum threshold rfs value of 0.35. Icimapping V3.3 software used Kosambi mapping functions to translate recombination frequency into map distance, and the order of the markers in each linkage group was determined by the method of maximum likelihood. The software package Map Chart 2.1 was used to graphically represent the linkage groups in the map. QTL analyses were performed using Icimapping V3.3 software. QTLs and their significance were calculated using interval mapping (IM), multiple QTL model (MQM) and permutation analysis. The QTL threshold base in the permutation analysis (1,000 permutation at $P = 0.05$) ranged between 2.0 and 2.3 for the various traits. A LOD score of 3.0 was used for detection of QTLs. QTL was named as "trait name+chromosome number+trait number."

Results and Discussion

The 2485 and 3156 potential CAPS markers that could be used in watermelon and melon were identified by the method of bio-information analysis, respectively. We selected 211 and 239 sequences with CAPS locus to design primers between the watermelon line LSW-177 to Huayuan Female Parent and COS by primer 5 software packages. The result indicated that 104 and 127 CAPS markers performed polymorphism after the reaction of restriction endonuclease. 142 pairs of polymorphic markers were exploited from 360 pairs of sequences derived from melon cross MR-1×Topmark

Two linkage maps with 82 and 144 markers were constructed, based on the segregation of the newly developed CAPS and SSR markers in 147 and 234 individuals of Huayuan Female Parent×LSW-177 (Pop 1) and LSW-177×COS (Pop 2) F_2 populations, respectively (Fig. 1 and Fig. 2). The map of Pop 1 included 14 linkage group while Pop2 map included 16 linkage groups. The lengths of Pop 1 and Pop 2 genetic maps were 1,298.5 cM and 2,039.5 cM with the average markers distances of 10.14 cM and 14.07 cM, repectively. According to the data of re-sequence, each CAPS locus has its physical location on the chromosomes and all the CAPS markers which come from the same chromosome were grouped into one linkage group. So the linkage groups could represent the 11 chromosomes of the watermelon.

Four QTLs related with flesh color (*FC3.1*, *FC4.1*, *FC6.1* and *FC11.1*) and one major QTL related with lycopene content (*LCYB4.1*) were detected with the linkage map. The four flesh color QTLs, located on chromosomes 3, 4, 6, and 11, could explain the phenotypic variation ranging from 34.68 % to 2.56 % and combined effects for flesh color of 43.68 % of the total variation. The lycopene content QTL was a major QTL on chromosome 4 explaining 83.5 % of the phenotypic variation. The lycopene QTL is tightly linked with CAPS markers *WII04E07-40* (0.35cM) and *WII04E07-33* (1.45cM). The flesh color QTL on chromosome 4 was located in the same region with lycopene content QTL indicating that flesh color in watermelon is controlled by lycopene content gene. T. Hashizume et al. (2003) mapped red flesh color

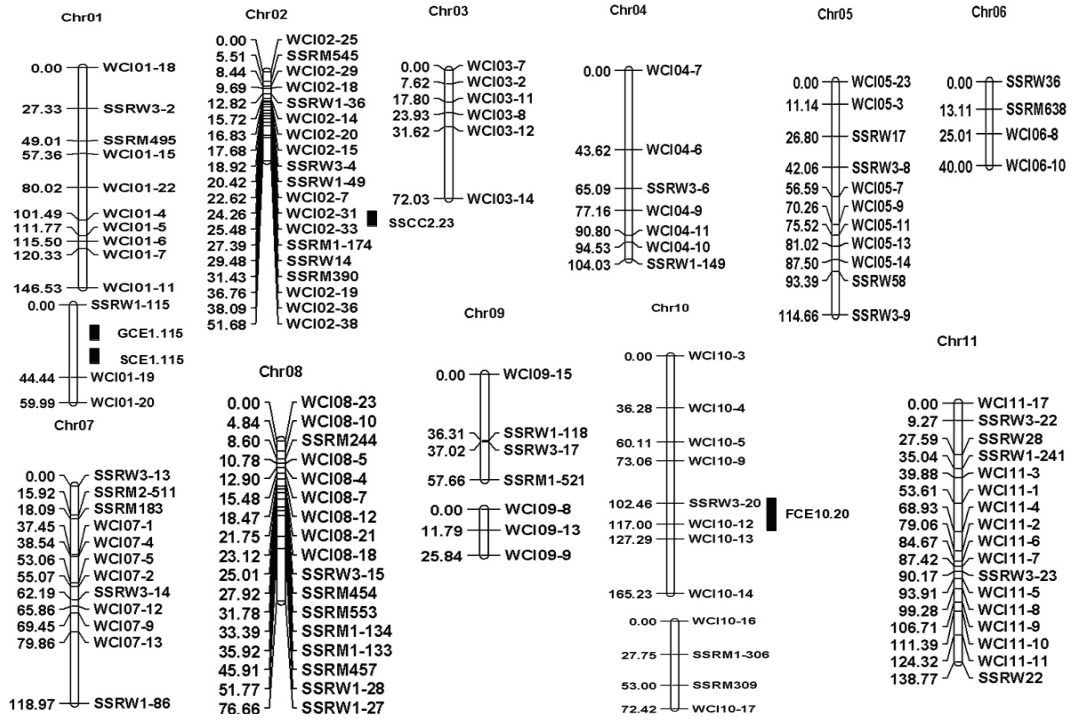

Fig 1. Construction of linkage groups of watermelon using CAPS and SSR markers and QTLs mapping for sugar content traits (black boxes).

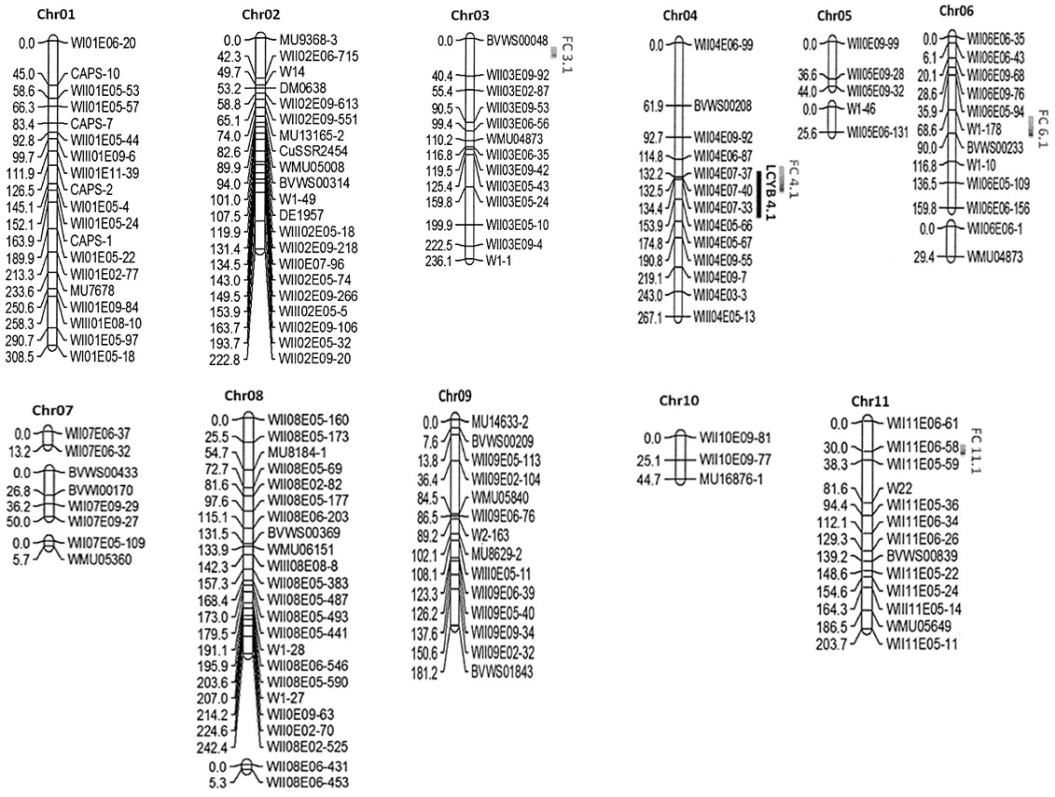

Fig. 2. Construction of linkage groups of watermelon using CAPS and SSR markers and QTLs mapping for lycopene content and flesh color traits (black boxes). Flesh color (*FC*), lycopene content (*LC*YB).

loci in watermelon using a comprehensive linkage map on Chr02 and Chr08 which were not detected in our research. The QTLs related with red flesh color on Chr02 and Chr08 were identified using a cross between red and yellow flesh genotypes. The Chr02 and Chr08 QTLs could be associated with the presence or absence of flesh carotenoids while the LSW-177×COS F_2 population was established from a cross of red and pale yellow flesh color. For the present research, many genes controlled the flesh color in watermelon and the flesh color performed a high correlation with the pigment (such as lycopene and β-carotene). In this research we detected just one major QTL for lycopene content and four QTLs related with the flesh color using the linkage map performed the same result as reported. In the present study, little research were aimed on the lycopene content QTL analysis in watermelon but the carotenoid biosynthetic pathway of watermelon was based upon carotenoid biosynthesis in plants (Isaacson et al. 2002, 2004). Stefania Grassi et al. (2013) reported that the procedure of many steps in watermelon were in common with tomato, yet suggested a complex different regulatory system for carotinoid biosynthesis between these fruits for many aspects.

One central sugar (CS) QTL related with the Brix content (*SSCC2.23*) in chromosome 2 was located; and three edge sugar (ES) QTLs associated with the content of fructose, sucrose, glucose in chromosome 1 and 10 (*FCE3.20*, *SCE1.115* and *GCE1.115*). The QTL related with ES (*FCE3.20*) on chromosome 10 could explain 11.79% of the phenotypic variation. The QTLs about sugar content have been reported by Ren Y. et al. (2014) that 9 QTLs were detected in chromosomes 2, 5, 6, and 8 using an integrated map and the QTLs which we detected were not in the same chromosomes as Ren Y. reported. The result indicated that there maybe more genes controlled the sugar content in the other chromosomes of watermelon.

In the study of melon powdery mildew resistance gene location, we investigated individual powdery mildew disease index of 354 plants in F_2. Major powdery mildew resistance gene was located using new CAPS markers. According to the data of disease index of powdery mildew, a major gene was performed to control the resistance of powdery mildew and the segregation rate performed 1:3 to resistance and sensitive with the 11.11of disease index.

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Determining the Relationships between Kirkagac and Other Melon Types by Using Morphological and Molecular Methods

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Additional index words. *Cucumis melo*, Kirkagac, morphological characterization, diversity, ISSR

Abstract. The relationship between Kirkagac and other types of melons was investigated by morphologically and molecularly characterizing 83 melon accessions, including those of 50 Kirkagac type melons (*Cucumis melo* var. *inodorus*). Kirkagac accessions were more morphologically similar to one another than they were to other accessions. No correlation was noted between the melon types and melon collection site. Wild melons, Kav 63, Kav 64, and Kav 66, were differentiated from other accessions for all observed characters. Inter-simple sequence repeat (ISSR) markers were used to assess genetic diversity. Among the 76 ISSR primers tested 13 were found to be scorable. A total of 110 bands were obtained of which 79 were polymorphic and 31 were monomorphic. The overall polymorphism rate was 71.8%. In Kirkagac accessions, the average similarity coefficient was 0.88 (range, 0.35–1.00).

A wide variety of melons (*Cucumis melo* L.) are cultivated in many countries (Pitrat, 2012). Turkey is the second largest melon producer in the world and produces 1,708,415 t melon from 102 000 ha area (Anonymous, 2012). Asian countries, including Turkey, Iran, India, Afghanistan, and China, comprise the secondary gene center of melon (Solmaz et al. 2010). In Turkey, winter melons, which are part of the inodorus group, are the most commonly produced. There are many accessions grown locally in different regions of Turkey. Among these, the most important are Kirkagac, Yuva, and Hasanbey accessions. The name "Kirkagac" is derived from the name of a town in the Aegean region of Turkey. However, these melons are produced in all parts of the country. Kirkagac melons vary in shape and size; they have a yellow surface with black points and stains. These melons taste sweet and are inodorous. They are not well known abroad, even though they are produced commonly in Turkey. Few studies (Sarı and Solmaz 2007; Sensoy et al., 2007; Solmaz et al., 2010, Aka Kacar et al., 2012; Sensoy and Şahin, 2012) have reported the morphological and molecular characterization of Turkish melons.

In this study, we characterized melon samples gathered from a variety of locations in Turkey. Morphological and molecular characterization was performed in order to identify the relationships among Kirkagac materials gathered from various places in Turkey, and between these melons and other species or subspecies of melon. This study provides information necessary for the protection genetic diversity in cultivated melons and establishes an infrastructure for breeding.

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Materials and Methods

This study was carried out at Manier Seed Company Tarsus Research Station and Cukurova University, Faculty of Agriculture, Department of Field Crops Molecular Biology Laboratory. We examined 83 melon accessions, of which 50 were Kirkagac type (*Cucumis melo* var. *inodorus*). Twenty-one of the Kirkagac accessions were collected from different provinces in Turkey. Two commercial hybrids and 2 open-pollinated varieties were also included. Of the remaining accessions, 56 were obtained from the genetic resource collection of Cukurova University Department of Horticulture and 6 were obtained from abroad.

Morphological characterization was based on 70 qualitative features according to UPOV criteria. DNA isolation was performed using the CTAB method developed by Doyle and Doyle (1987). We prescreened 4 accessions by using 76 inter-simple sequence repeat (ISSR) primers. We identified 13 scorable ISSR primers for use in ISSR analyses, which we performed according to Zietkiewicz et al. (1994).

ISSR fragments were scored based on their presence (1) or absence (0). The resulting data were used to generate a pair-wise similarity matrix by using Jaccard coefficients (Jaccard, 1908). The unweighted pair-group method with arithmetic mean (UPGMA) was employed to create clustering dendrograms using the NTSYS-PC program (version 2.00) (Rohlf, 1998). Principal component analysis (PCA) was performed using SAS (SAS Inst., 1990) software.

Results and Discussion

A high degree of morphological diversity was noted among

the examined accessions. However, the petals of all melon accessions were bright yellow.

The petals of Kirkagac accessions used in this study were bright yellow; ground color of the fruit skin before maturity was green, ground color of the fruit skin at maturity was yellow, secondary colors were present on fruit skin, and secondary colors were pointed and stained on the rind. Further, the fruit had a sweet taste, were inodorous; longitudinal sections of the seeds were elliptic, and the seeds were creamy yellow. As expected, Kirkagac melons shared more common morphological characters. These features can be used to identify the Kirkagac type.

The 83 melon accessions are clustered into 10 different groups based on morphological features (Fig. 1). These groups were A1, A2, B1, B2.1, C, D, E, F1, F2.1, and F2.2. All Kirkagac accessions, except Kav 67 (group F1), were clustered in sub-groups D, F2.1, and F2.2, whereas sub-groups D and F2.1 were composed of only Kirkagac accessions. Sub-group F2.2 contained 5 accessions of another inodorus group melon in addition to 37 Kirkagac accessions.

The first dimension (PC1) of PCA explained 12.3% of the morphological variation, the second dimension (PC2) explained

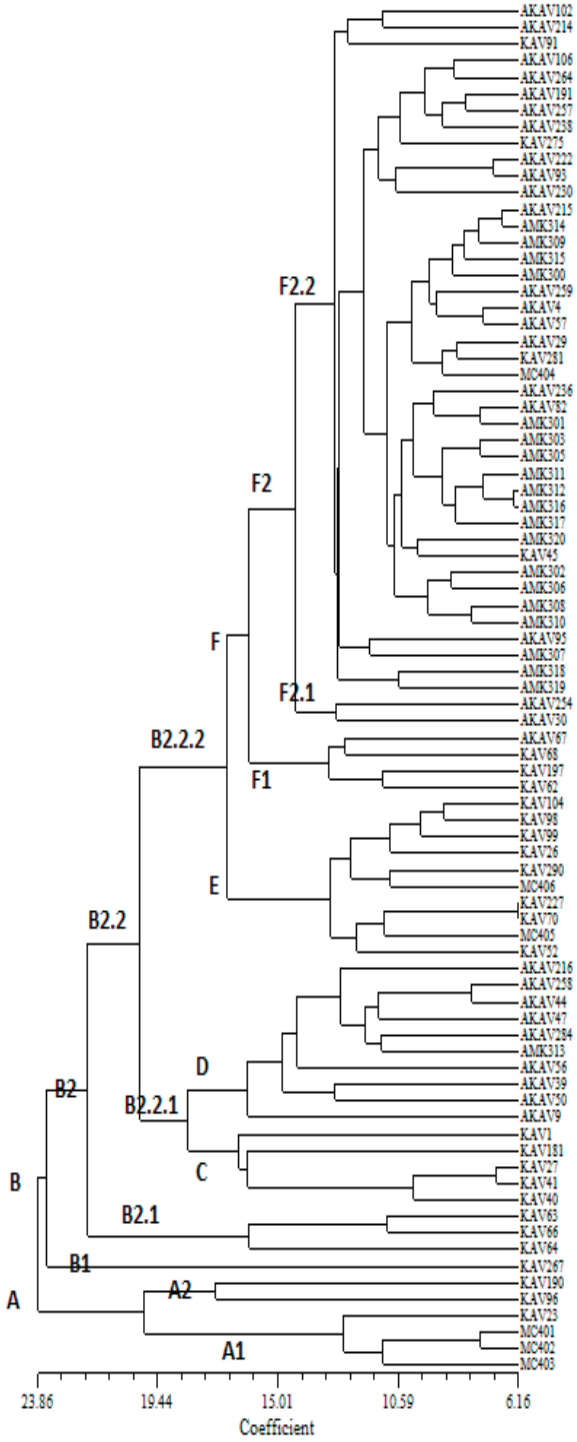

Fig. 1. Dendrogram of 83 melon genotypes characterized by 70 qualitative agro morphological traits using average method.

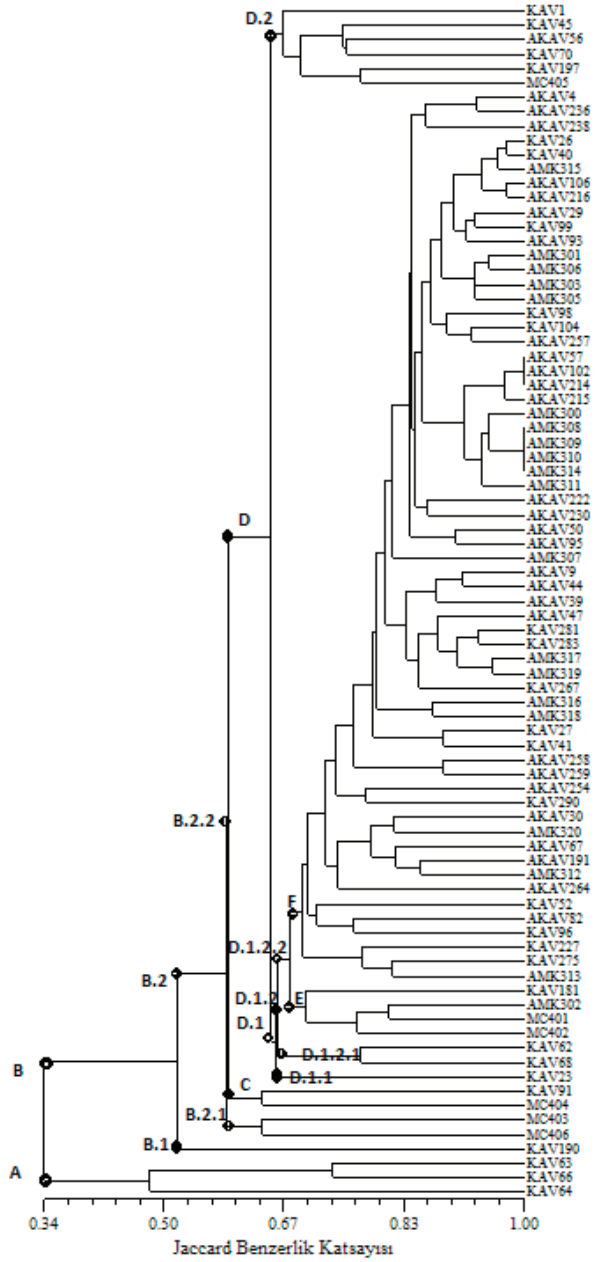

Fig. 2. Dendrogram of 83 melon accessions constructed using data obtained from 13 inter-simple sequence repeat (ISSR) markers by using the unweighted pair-group method with arithmetic mean (UPGMA).

Table 1. Number and percentage of polymorphic and monomorphic inter-simple sequence repeat (ISSR) bands for 83 melon accessions.

	All melon genotypes			Kırkagaç melon genotypes		
Primer no	Number of polymophic bands	Number of monomorphic bands	Percentage of polymorphic bands (%)	Number of polymophic bands	Number of monomorphic bands	Percentage of polymorphic bands (%)
UBC 808	3	3	50.0	1	5	16.7
UBC 809	5	3	62.5	3	5	37.5
UBC 811	5	2	71.4	4	3	57.1
UBC 813	6	1	85.7	4	3	57.1
UBC 814	2	1	66.7	0	3	0.0
UBC 826	6	5	54.6	3	8	27.2
UBC 827	8	1	88.9	5	4	55.6
UBC 841	4	4	50.0	1	7	12.5
UBC 866	5	3	62.5	3	5	37.5
UBC 880	11	0	100.0	7	4	63.6
UBC 886	3	4	42.9	3	4	42.9
UBC 888	13	1	92.9	9	5	64.3
UBC 890	8	3	72.7	4	7	36.4
Average	79	31	71.8	47	63	39.1

11% of the total morphological variation. Three groups were well separated from rest of the accessions (Figure not shown, Mancak, 2013). Group I included Kav 23, Mc 401, Mc 402, and Mc 403; group II included Kav 190, Kav 96, and Kav 267; and group III contained Kav 63, Kav 64, and Kav 66. All of the accessions in group III were wild.

Morphological characterization of the melon accessions showed high level of diversity in accordance with the previous studies (Sarı and Solmaz, 2007; Şensoy et al., 2007; Solmaz et al., 2010; Szamosi et al., 2010; Şensoy and Şahin, 2012). There was no correlation between accession grouping and collection site.

A total of 110 bands were generated using 13 ISSR primers (Table 1). Of these bands, 79 were polymorphic and 31 were monomorphic for all accessions. The rate of polymorphism was 71.8%. Primer no UBC 880 had the highest rate (100%) of polymorphism for all accessions, whereas primer no. UBC886 had the lowest rate (42.86%) of polymorphism. Although there have been few previous studies in which ISSR markers were used to characterize melons (Danin-Poleg et al., 1998; Fabriki Ourang et al., 2005; Sestili et al., 2008), our results confirmed that the ISSR method can be used to evaluate the genetic diversity of the Turkish melon germplasm.

Similarity coefficients among the 83 accessions were calculated using ISSR data and varied between 0.35 and 1.00, with an average coefficient of 0.88. The similarity coefficient between Kav 102 and Kav 57 was 1.00. Mk 308 and Mk 309; Mk 308 and Mk 310; Mk 309 and Mk 310; Mk 308 and Mk 314; and Mk 309 and Mk 314 accessions were also very similar to each other. Kav 70 and Kav 66 accessions were the least similar accessions and had a similarity coefficient of 0.35.

The melon accessions were clustered into 9 groups based on molecular characterization. These groups were A, B1, B2.1, C, D1.1, D1.2.1, E, F, and D2 (Fig. 2). Wild melon accessions (Kav 63, Kav 64, and Kav 66) were in group A and were separated from all other accessions. The Kav 190 genotype in subgroup B1 was separated from other accessions in group B. All Kirkagac accessions, except Kav 56 and Mc 302, were clustered in group F. None of the accessions of foreign origin were included in group F. There was no relationship between the places from which the accessions were collected and groups.

PCA performed for molecular data resulted in 5 well-defined groups (Figure not shown, Mancak, 2013). Three accessions (Kav 284, Mk 317, Mk 319) were separated from the others in group I. Group II consisted of 31 melon accessions, of which 25 were Kirkagac type. The Kirkagac accessions in this group were Mk 301, Mk 306, Mk 305, Mk 318, Kav 9, Mk 315, Kav 106, Kav 236, Kav 47, Kav 222**,** Mk 303, Kav 216, Kav 93, Kav 238, Kav 29, Kav 257, Kav 39, Kav 230, Kav 95, Mk 307, Kav 4, Mk 300, Mk 311, Kav 50, and Kav 191. Group III contained Kirkagac accessions Kav 215, Mk 310, Mk 309, and Mk 308. Group IV included only one Kirkagac genotype (Kav 30) and group V contained 39 accessions, of which 13 were Kirkagac.

In conclusion, with the exception of petal color, morphological characters varied between accessions. Kirkagac accessions shared more common characteristics (8 characters) with one another than they did with other accessions. As expected, wild melon accessions Kav 63, Kav 64, and Kav 66 were separated from other accessions in all analyses.

In future studies, it will be important to generate collections of Kirkagac melon accessions that have different agronomical features to be used in breeding programs.

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Non-allelic Factors Responsible for Chilling Tolerance at Seedling Stage in Cucumber PI 390953

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Additional index words. *Cucumis sativus*, cold, inheritance, vegetable breeding, sources of cold tolerance

Abstract. Cucumber (*Cucumis sativus* L.) is a chilling susceptible crop, and low temperature can be a limiting factor in early growth and development. In our previous studies, cucumber PI 390953 exhibited both high cold tolerance at seedling stage and high seed germination rate at low temperature. The objective of this study was to determine the type of gene action controlling chilling tolerance at seedling stage in PI 390953. Breeding line Gy 14 was used as the sensitive parent in crosses with PI 390953 to make F_1, F_2, and BC_1 generations. Cucumber seedlings at the first true leaf stage were placed in growth chambers at 4 °C for 7 h and moderate light intensity (photosynthetic photon flux of 500 µmol · m^{-2} · s^{-1}). Chilling damage (chlorosis and necrosis) on the first true leaf and growing point of plants was rated 14 days after the chilling treatment using a scale of 0 (no damage) to 9 (plant dead). We observed double recessive epistasis in the segregating F_2 generation of Gy 14 × PI 390953 with the segregation of 9 sensitive : 7 tolerant and moderately tolerant plants in F_2 population (χ^2=0.50 at P=0.48). We concluded that tolerance to low temperature at the seedling stage in PI 390953 is conditioned by non-allelic factors, where one or both in homozygous recessive condition would result in chilling tolerance.

Cucumber is one of the most susceptible crops to low temperature, a major limiting factor in cucumber growth and development. Under low temperatures, germination and seedling establishment are severely inhibited. Also, cold stress may cause seedling injury, and may reduce formation of flowers and fruit in later stages, causing yield reduction (Staub and Wehner, 1996). The physiological symptoms such as stunted growth, reduced photosynthetic capacity, necrosis, discoloration, and wilting are the main symptoms of chilling injury (Lyons, 1973). Chilling sensitivity may limit the geographical distribution of cucumber cultivation. Fluctuating temperatures often exist during the early part of the growing season in many regions of the world (e.g., northern and eastern Europe and USA).

In our previous studies we determined the genetic basis of cold tolerance in cucumber and proceeded later to transfer the trait into breeding lines or cultivars since there are no cultivars with cold tolerance. We have identified two cucumber accessions PI 390953 and PI 246903, both showing high cold tolerance at seedling stage, both under controlled conditions and in the field (Kozik et al., 2007, 2010). In contrast to PI 246903, accession PI 390953 also had a high seed germination rate under low temperatures in the growth chamber (11 and 13 °C) and in the field (Kozik et al., 2007). Genetic studies indicated that chilling tolerance at seedling stage in PI 246903 was controlled by a single dominant gene *Ch* (Kozik and Wehner, 2008), while low temperature seed germination ability in PI 390953 was controlled by non-allelic gene interactions (Kozik et al., 2012). The objective of this study was to determine the type of gene action controlling chilling tolerance at seedling stage in PI 390953.

Materials and Methods

The parental genotypes used in this study were sensitive Gy 14 (P_1) and tolerant PI 390953 (P_1), chosen on the basis of their reaction to low temperatures in our previous studies (Kozik et al., 2007). These lines were crossed to develop F_1, $F_{1reciprocal}$ (RF_1), F_2, and backcross populations BC_{1P1} and BC_{1P2}. All crosses were made by hand pollination in a greenhouse at the Research Institute of Horticulture.

Experiments were conducted under controlled environment conditions in the growth chambers of the Southeastern Plant Environment Laboratory at North Carolina State University (Thomas et al., 2005). Seeds were sown in peat pots (57 mm^2, 100 mL volume) filled with a substrate of gravel and peat in a 1 : 1 ratio and placed in flats. After seeding the populations, the flats were placed in growth chambers set at 26 °C day/22°C night temperatures under 12 h of combined fluorescent and incandescent light (from 0800 to 2000 HR). Light intensity (photosynthetic photon lux) was 650 and 44 µmol·m^{-2}·s^{-1}, respectively. Plants were watered with the standard phytotron nutrient solution (Thomas et al., 2005).

Cucumber seedlings at the first true leaf stage were placed in growth chambers at 4 °C for 7 h and a photosynthetic photon flux of 500 µmol · m^{-2} · s^{-1}. After the chilling treatment, plants were returned to the main growth chamber and placed under the same light and temperature regime as before. Chilling damage (chlorosis and necrosis) on the first true leaf and growing point of plants was rated 14 days after the chilling treatment using a scale of 0 to 9, where 0 = no damage, 1 to 2 = trace of damage, 3 to 4

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= slight damage, 5 to 6 = moderate damage, 7 to 8 = advanced damage, and 9 = plant dead (Smeets and Wehner, 1997).

Data were collected as means over all leaves on the plants within each generation. The experiment was a split-plot treatment arrangement in a randomized complete-block design with 10 replications. Each flat that constituted the plot contained three plants of each parent, six F_1 plants, six BC_1P_1 plants, six BC_1P_2 plants, and 25 F_2 plants.

The results of the test comparing the chilling injury of the cultigens and of hybrid populations were analyzed using the program STATISTICA 10.0 (StatSoft), and the Newman-Keul's test at $\alpha = 0.05$.

For the genetic analysis, plants of the hybrid populations segregating for chilling damage at seedling stage were grouped into three classes: tolerant (T; 0-3), moderate (M; 4-6), sensitive (S; 7-9). Data were tested for goodness-of-fit to theoretical ratios using the chi-square tests for F_2 population (Srb and Owen, 1955).

Results and Discussion

Seedlings of PI 390953 (P_2) showed low degree of mean chilling damage (2.5) while, plants of Gy 14 (P_1) exhibited high susceptibility (7.4) (Table 1). These results confirmed our earlier studies (Kozik et al., 2007, 2010).

The mean value of chilling damage for the F_1, F_2, BC_1P_1 and BC_1P_2 populations were not statistically different (Table1), and were higher than the midparent value (5.0).

On the basis of chilling damage we observed some variation within the hybrid populations and therefore the plants were classified as tolerant (T), moderately tolerant (M) and sensitive (S). In the F_1, and BC_1P_1 two groups of genotypes (M, S) were observed, with average percentage of M genotypes (48%, 42%, respectively) (Fig. 1). Plants of F_2 and BC_1P_2 populations segregated in three groups chilling damage (T, M, S) with a low percentage of T genotypes (12%, 6%, respectively), whereas they differed in the percentage of M and S genotypes. In F_2 generation 30% moderately cold-tolerant (M) and 58% sensitive (S) plants were observed. It was in the contrast with BC_1P_2 generation where the proportions were reversed (69, 25%, respectively).

The F_2 segregation ratios differed statistically from the ratios expected for theoretical models of a trait conferred by one, two, or three genes (data not shown). Then we tried to fit our data of F_2 population to six types of non-allelic gene interaction (Table 2). Chi-square estimates of these models showed double recessive epistasis for cold-tolerance at the seedling stage with theoretical segregation of 9 sensitive : 7 tolerant and moderately tolerant plants in F_2 population (χ^2=0.50 at P=0.48) for PI 390953. Our recent report provided information on the recessive character and non-allelic gene interactions (double dominant epistasis) conferring the cold germination ability in this cultigen (Kozik et al., 2012). Studies focused on the inheritance of chilling tolerance at the seedling stage are very limited. Therefore, the results reported herein provide the ultimate complementary evidence that chilling tolerance at the seedling stage and low temperature seed germination ability are inherited as separate traits, in line with our previous reports on this topic (Kozik et al., 2007, 2010, 2012). Although PI 390953 possess both chilling tolerance at seedling stage and good germinability at low temperature, it is also noteworthy that PI 246903 having single gene *Ch* expressing cold tolerance at young phase of plant development is also a good source of the trait in breeding programs for the development of new cultivars adapted to planting in the field in early spring.

Table 1. Mean of chilling injury in parental lines Gy 14 (P_1) and PI 390953 (P_2) and their F_1, F_2 and BC_1 populations.

Generation	P_1 Gy 14	P_2 PI 390953	F_1	F_2	BC_1P_1	BC_1P_2
DSI	7,4 c	2,5 a	5,5 b	5,7 b	5,8 b	5,5 b

Means followed in the line by the same letter are not significantly different at $\alpha = 0.05$.
Chilling injury ratings were based on 0–9 scale: 0 = no damage, 1–2 = trace of damage, 3–4 = slight damage, 5–6 = moderate damage, 7–8 = advanced damage, 9 = plant dead.

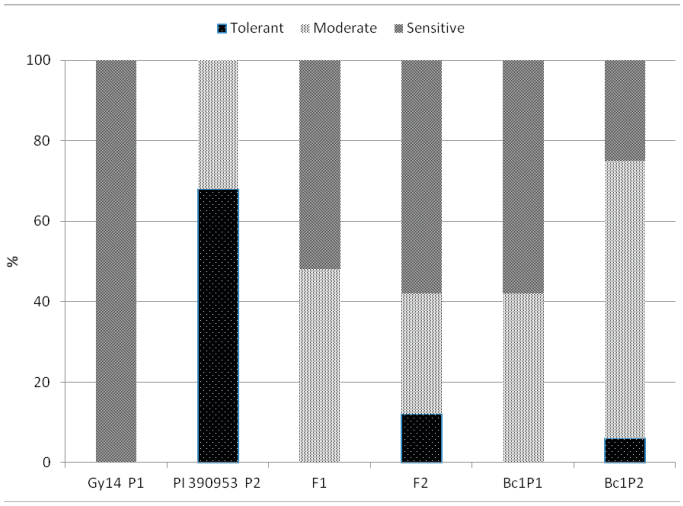

Fig. 1. Frequency distribution of cold-tolerant (T), moderately cold-tolerant (M), and cold-sensitive (S) genotypes in six generations of the cross of cold-sensitive (P_1; Gy 14) with cold-tolerant (P_2; PI 390953) cucumber.

Table 2. Genetic analysis of chilling tolerance at seedling stage in cucumber PI 390953.

Types of non-allelic gene interaction	F_2 segregation theoretical	F_2 segregation observed	χ^2	df	P
Additive	9 : 6 : 1				
	S : M : T	121 : 62 : 23	8.46	2	0.01
Recessive	9 : 3 : 4				
	S : M : T	121 : 62 : 23	44.5	2	0,00
Double recessive	9 : 7				
	S : M+T	121 : 85	0.5*	1	0.48*
Dominant	12 : 3 : 1				
	S : M : T	121 : 62 : 23	22.6	2	0,00
Dominant and recessive	13 : 3				
	S+M : T	183 : 23	12.0	1	0,00
Double dominant	15 : 1				
	S+M : T	183 : 23	5.03	1	0.02

*Significant at $\alpha = 0.05$.
S – genotype is sensitive (classes 7–9).
M – genotype is moderately tolerant (classes 4–6).
T – genotype is tolerant (classes 0–3).

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Microsatellite Marker-based Genetic Diversity of Seed-use Watermelon (*Citrullus lanatus* ssp. *vulgaris* var. *megalaspermus* Lin et Chao) Collections

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Additional index words. seed-use watermelon, genetic diversity, watermelon breeding, SSR marker

Abstract. Seed-use watermelon (*Citrullus lanarus* ssp. *vulgaris* var. *megaldspermus* Lin et Chao.) is an important market class in China. It is imperative to develop improved watermelon varieties for seed-use to meet the increasing demand from the seed processing industry. It is known that cultivated watermelon has a narrow genetic base, and the level of diversity in seed-use watermelons is not well studied. In this paper, genetic diversity of 60 seed-use watermelon germplasm lines from different geographic origins was evaluated with microsatellite (SSR) marker. From 350 watermelon SSRs, 12 were selected to fingerprint 60 seed-use watermelon genotypes. Of the 12 markers, each on average could detect 3.6 alleles. The genetic similarity coefficient among the 61 accessions was high ranging from 0.80 to 0.99 suggesting very close genetic relationships. A SSR-based dendrogram was developed, and clustering of the 60 lines was not associated with geographic origins or seed coat color. The result revealed the very narrow genetic base of the Chinese seed-use watermelon collections. Strategies to broaden seed-use watermelon breeding background are discussed.

The seed-use watermelon (SUW, *Citrullus lanatus ssp. vulgaris* var. *megalaspermus* Lin et Chao) is an important community in China. Based on the seed coat color, SUW in China could be classified broadly into two categories: black seeded watermelon which grows mainly in northwest China including Gansu, Xinjiang, Inner Mongolia and Ningxia provinces, and red seeded watermelon that is more popular in southern China like Guangxi, Anhui, Jiangxi, Hunan provinces. The SUW kernel is rich in protein, crude fat, multiple vitamins, mineral elements and other substances with health benefits. In recent years, with the rapid development of SUW processing industry, there is an increasing demand of seeds of seed-use watermelon in the market. It is imperative to breed SUW varieties for higher yield, better quality and stronger disease resistance.Understanding of the genetic relationship and genetic diversity could provide better guidance of use of SUW germplasm, which is, however lacking. Molecular markers have been widely used to evaluate genetic diversity in watermelon or cucurbit collections (e.g., Bredemeijer et al., 2002; Weising et al., 2005; Joobeur et al., 2006; Kwon et al., 2007; Weng, 2010). The objective of the present study was to use microsatellite or simple sequence repeat (SSR) markers to assess genetic diversity of 60 SUW accessions that belong to different eco-types.

Materials and Method

A total of 61 accessions were used including 60 SUW genotypes and one fresh market watermelon genotype. For each entry, seeds were sown in a potting soil mix in the greenhouse. At the 2–3 leaf stage, DNA was extracted from young leaf tissues (pooled from 10 plants of each accession) using the CTAB method (Murray and Thompson 1980).

SSR markers were selected from the literature (Levi et al., 2001; Joobeur et al., 2006) and the primers were synthesized commercially. PCR amplifications were conducted in a 20 µl volume reaction containing 2.0 µL 10× PCR buffer, 1.0 µL template DNA (30 ng/µL), 2 µL 25 mM $MgCl_2$, 1.6 µL 2.5 mM dNTPs, 0.5 µL each of 20 µM primers, 0.25 µL of 5U/ µL *Taq* polymerase, and 11.6µL ddH_2O. The temperature profile utilized for PCR amplification was as follows: 94 °C for 5 minutes, followed by 35−40 cycles of 94 °C for 30 s, 50−60 °C for 30 s, and 72 °C for 60 s, and a final extension of 5 minutes at 72 °C. The PCR products were resolved with polyacrylamide gene electrophoresis (PAGE, 6%) and displayed with silver staining.

PCR amplification results were assessed scored with the presence (1) or absence (0) of specific bands among the genotypes. The polymorphism information content (PIC) was calculated as $PIC = 1–\sum f_i^2$, in which f_i is the frequency of the i^{th} allele locus detected across all allele for the locus (Anderson et al., 1992). Cluster analysis was based on Jaccard's similarity coefficient (Jaccard, 1908) using unweighted pair group method (UPGMA) in NTSYS-PC version 2.1b (Rohlf, 2000) software package.

Acknowledgements. This study was supported by funds from the Inner Mongolia Science and Technology Project of China (20090707). We thank Jiming Wang and Jianping Sun for proving seeds of some entries used in this study, and Yiqun Weng for help revising the manuscript.

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Results

SSR polymorphism. Among 350 SSR markers, 12 showing the highest polymorphisms were selected (Table 2) to analyze genetic diversity of 60 seed-use watermelon genotypes. A total of 2,859 bands could be scored, but only 312 (10.9%) were used in diversity analysis. Among the 12 highly polymorphic SSR markers, one detected six alleles, one detected five alleles; for the remaining, 4, 4, and 2 markers detected 4, 3, and 2 alleles, respectively in the 60 SUW lines. On average, 3.6 alleles were detected per marker. The PIC value that reflected allele diversity and frequency among the test varieties, ranged from 0.183 0.786 with the average of 0.392 across 12 markers (Table 2).

Genetic diversity analysis of seed-watermelon genotypes. The SSR-based UPGMA clustering tree generated with the NTSYS software is shown in Fig. 2. The 61 genotypes could be divided into two large groups: Group 1 and Group 2. Group 1 could be further divided into two subgroups A and B. Group A was comprised of 11 SUW and one flesh-use (fresh market) watermelon (ZG18). The high similarity coefficient values (0.80-0.99) among these genotypes suggested that they had very close relations despite of their diverse geographic origins (6 from N. China, 5 from Northwest China and one from South China) (Table 1). ZG18 is a fresh-use watermelon variety which was clustered in the same group as ZG06 and ZG07 suggesting that the genetic distance between flesh-use and seed-use watermelons was not necessarily larger than that between seed-use watermelon genotypes. Group B included 33 watermelon genotypes, which in turn could be classified into two sub-groups (B1 and B2). Lines in B1 sub-group were heterogeneous including those from North (14), Northwest (9) and South China (4), Russia (2) and America (2).

Similarly, Group 2 could be divided into two subgroups A and B with Group A including nine 9 genotypes from North (4), Northwest (3), or South China (2), and Group B containing 7 genotypes from North (3) and Northwest China (4).

Discussion

In the present study, we examined 350 watermelon primer pairs, and selected 12 for investigating of genetic diversity of 60 SUW watermelon lines. The polymorphism level was very low, and for the 12 SSRs, each could detect only 3.6 alleles. The low genetic diversity among the 60 seed-use watermelon genotypes is consistent with previous findings that cultivated watermelon has a very narrow genetic base (Levi et al. 2001; Zhang et al. 2012).

Table 1. Information of 61 seed-use watermelon lines used for evaluation of gene.

Accession	Name	Ecotype[a]	Seed Color[a]	Variety[b]	Source (City, Province)[c]
ZG01	Hongxiaopian	N China	Red	A	Bameng, Neimenggu
ZG02	Xinzigua No.1	N China	Black	A	Bameng, Neimenggu
ZG03	Hezigua	N China	Black	A	Bameng, Neimenggu
ZG04	Heifeng	N China	Black	A	Bameng, Neimenggu
ZG05	Hezigua	N China	Black	A	Bameng, Neimenggu
ZG06	Chifengzigua	N China	Black	C	Bameng, Neimenggu
ZG07	Tongliaozigua	N China	Black	C	Bameng, Neimenggu
ZG08	Hongzhongpian	N China	Red	A	Bameng, Neimenggu
ZG09	Hezigua	N China	Black	A	Bameng, Neimenggu
ZG10	Hezigua	N China	Black	A	Bameng, Neimenggu
ZG11	Hongzhongpian	N China	Red	A	Bameng, Neimenggu
ZG12	Hongzhongpian	NW China	Red	A	Changji, Xinjiang
ZG13	Xinzigua	NW China	Black	A	Changji, Xinjiang
ZG14	Hedapian	N China	Black	A	Bameng, Neimenggu
ZG15	Hongxiaopian	N China	Red	A	Bameng, Neimenggu
ZG16	Hongzhongpian	N China	Red	A	Bameng, Neimenggu
ZG17	Heixiaopian	N China	Black	A	Bameng, Neimenggu
ZG18	Mengfeng No.6	N China	White, FM	A	Bameng, Neimenggu
ZG19	Hongxiu No.1	N China	Red	A	Bameng, Neimenggu
ZG20	Jingyuandaban No.1	NW China	Black	B	Lanzhou, Gansu
ZG20	Heidapian	N China	Black	A	Bameng, Neimenggu
ZG21	Mingguangixiaopian	N China	Black	A	Bameng, Neimenggu
ZG22	Heizhongpian	N China	Red	A	Bameng, Neimenggu
ZG23	Heixiaopian	NW China	Black	A	Changji, Xinjiang
ZG24	Hongxiaopian	S China	Red	B	Heizhou, Guangxi
ZG25-1	Daoxian Hongguazi	S China	Red	B	Daoxian, Huana
ZG25-2	Dabanhongguazi	S China	Red	B	Daoxian, Huana
ZG25-3	Hongguazi	S China	Red	B	Daoxian, Huana
ZG25-4	Hongguazi	S China	Red	B	Daoxian, Huana
ZG26	Daban No.1	NW China	black	B	Lanzhou, Gansu
ZG27	Heidapian	NW China	Black	B	Lanzhou, Gansu
ZG28	Hetaopizigua	NW China	Black	B	Lanzhou, Gansu
ZG29	Lanzhoudaban	NW China	Black	B	Lanzhou, Gansu

(continued)

Table 1 (continued). Information of 61 seed-use watermelon lines used for evaluation of gene.

Accession	Name	Ecotype[a]	Seed Color[a]	Variety[b]	Source (City, Province)[c]
ZG31-1	Dabanhongguazi	S China	Red	B	Daoxian, Huana
ZG31-2	Dabanhongguazi	NW China	Red	B	Lanzhou, Gansu
ZG32	Jianyangzigua	S China	Black	B	Jianyang, Fujian
ZG33	Langfangzigua	N China	Black	B	Wenan, Hebei
ZG34	Dabanguazi No.2	NW China	Black	B	Lanzhou, Gansu
ZG35	Guazigua	S China	Red	B	Xingfen, Jiangxi
ZG36	Hongguazi	NW China	Red	B	Yinchuan, Ningxia
ZG37	Hongzigua	Russia	Red	B	NGCWM, China
ZG38	Heizigua	N China	Black	B	Bameng, Neimenggu
ZG39	Jili (9126)F1	NW China	Black	B	Lanzhou, Gansu
ZG40	Huapizigua	NW China	Black	B	Xi'an, Shanxi
ZG41	Hongguazi	NW China	Red	B	Yinchuan, Ningxia
ZG42	Heipi(watermelon)	N China	Black	B	Bameng, Neimenggu
ZG43	Daban No.1	NW China	Black	B	Lanzhou, Gansu
ZG44	Guazigua	S China	Black	B	Nanxiong, Guangdong
ZG45	Zhengzhou Zigua	N China	Black	B	Zhenzhou, Henan
ZG46	Shanxi Hongzi	NW China	Red	B	Xi'an, Shanxi
ZG47	Dabanguazi	NW China	Black	B	Lanzhou, Gansu
ZG48	Ningxia Hongguazi	NW China	Red	B	Yinchuan, Ningxia
ZG49	Gaolan Zigua	NW China	Black	B	Lanzhou, Gansu
ZG50	GN-1	NW China	Black	B	Lanzhou, Gansu
ZG51	Linzi No.1	NW China	Black	B	Lanzhou, Gansu
ZG52	v0590157	Russia	Black	B	IVF-CAAS
ZG53	v0590159	Russia	Black	B	IVF-CAAS
ZG54	PI271983	America	Red	B	NGCWM, China
ZG55	PI385969—5	America	white	B	NGCWM, China
ZG56	PI482320	America	Black	B	NGCWM, China
ZG57	Hongzigua	N China	Red	B	Bameng, Neimenggu

[a]N = North, S = South, NS = Northwest, FW = flesh-use watermelon.
[b]A = F_1 hybrid, local; B = Cultivars or inbred lines; C = landraces.
[c]IVF-CAAS = Institute of Vegetables ad Flowers, Chinese Academy of Agricultural Sciences; NGWM = National Germplasm Center for Watermelons and Melon.

Table 2. Primer sequences of 12 microsatellite markers and their polymorphism information among 60 seed-use watermelon accessions.

No.	Forward	Reverse primer	# alleles	PIC value
1	GGAGTAGTGGTGGAGACATGG	TCCTTTCTCTTTCGCAAACTTC	2	0.183
2	AGACAGGGAAATCGCAGAGA	GGTTAAAGGACGTCGGGATT	4	0.433
3	TCTAGGATTAATTCCACATTCACAA	TTTACCGTGGGAGAGACTGG	3	0.406
4	TTGGGATGTAGATGTCCGGT	TCCCCAATCCAACTCCCTAT	6	0.786
5	TTGGGATGTAGATGTCCGGT	TCCCCAATCCAACTCCCTAT	3	0.546
6	CCCAACGCATAGTGTGTCTA	GAAGATAAGCAAGGCAACTGT	4	0.422
7	TTACTGGGTTTTGCCGATTT	AATTCCGTATTCAACTCTCC	3	0.231
8	CGCTGGATTTGTTTGTGAAAT	AATGTCGGGGAGTGTCACAT	3	0.234
9	TGATGATCCCACACGTCAAG	TGGTGAAAGGTGGTGTGAGA	4	0.345
10	TCTGAGTGTTTGACGGATGG	CAATCCCTTTGAGCCAGAGA	2	0.204
11	TTGTGTGCAGGGATTAACCA	CCGACCTGCAAAAGAATAGG	4	0.416
12	ACCTAATACGACGACGGTGG	TCTTTCAAATCGCCAGAACC	5	0.502

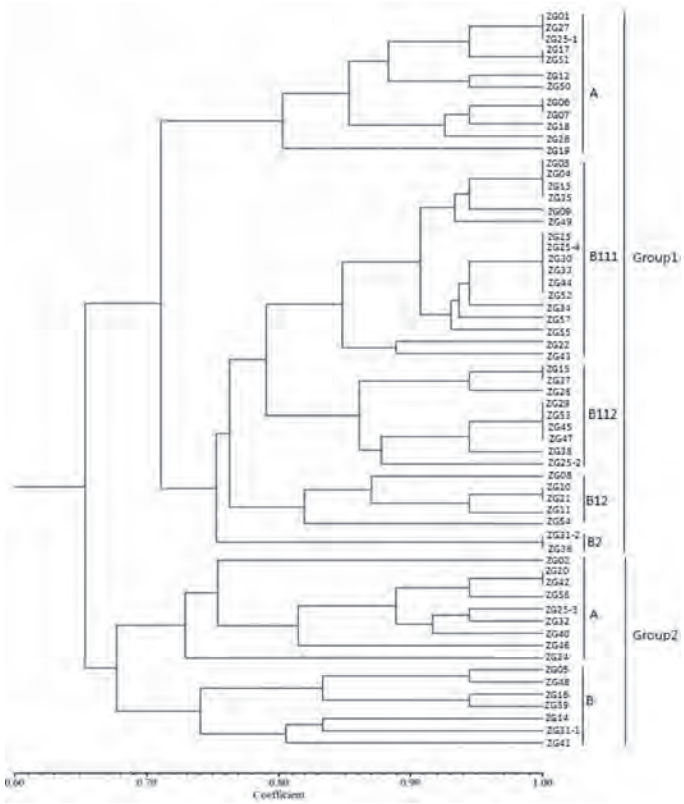

Fig. 1. Dendrogram depicting the classification of 61 watermelon accessions constructed using UPGMA based on SSR marker data. The major clusters and sub-clusters are marked to the right of the dendrogram. The scale at the bottom is Jaccard's coefficient of similarity.

Based on the 12 SSR data, a phylogenetic tree was constructed for 61 SUW genotypes (Fig. 1). Surprisingly, the grouping of these lines was consistent neither with their geographic origins (ecotypes), nor seed coat colors. These results were similar to the findings of Zhao et al. (1999), Zhang (2005), as well as Liu (2013) who investigated genetic diversity of seed-use watermelon germplasm with RAPD marker data. The most reasonable explanation of the result may be that the genetic diversity of the whole seed-use watermelon collection in China might be very low, and share the same or closely related parents. In addition, homonym and heteronym are very common in the collection.

The primary goal of the present study was to evaluate genetic diversity among seed-use watermelon lines, thus providing some guidance for exploring this gene pool for seed-use watermelon improvement. Previous studies and the present one all suggest that the Chinese cultivated seed-use watermelon collection has a narrow genetic base. Our data also suggest that genetic distance between flesh-use and seed-use watermelons is not necessarily longer than that between seed-use watermelon lines. It is possible the distinction between the two market classes is not significant except for some traits under selection during seed-use watermelon breeding. Therefore, the genetic resources from different market classes should be considered in breeding for seed-use watermelons.

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Watermelon Breeding Questions from 22 Years of Research

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Additional index words. *Citrullus lanatus, colocynthis, citroides*, genetics, gene linkage, allele

Abstract. Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] has small seeds that produce large plants, and small flowers that produce large fruit. Of the 62 genes for morphological and disease traits, why are none linked? There are many loci and alleles that affect the same trait in watermelon. Why is white flesh dominant to red? What is the difference between tiny seed in 'Sweet Princess', short seed in 'Baby Delight' and small seed in 'Small Seeded Dixielee'? Are the stripes on the watermelon fruit the dark area or the light area? Why are there only 4 of the 8 combinations for resistance to the 3 races of *Fusarium* wilt available as differentials? Why is it so difficult to combine disease resistance from PI accessions with quality from cultivars? Is colocynth more closely related to watermelon than citron? Why do small-fruited cultivars have low yield? Should we still breed tough but thick rind cultivars for protection during shipping (and for rind pickles)? Should we use taste to make selections since high fructose tastes sweeter than high sucrose? What constitutes good flavor when low-sugar watermelons are bred? Do cultivars from the eastern U.S. do poorly in the west, and vice versa? Should all cultivars have firm flesh?

Watermelon is an important crop worldwide, and much research has been done in the area of genetics and breeding to assist the process of cultivar development. During my 22 years of research on watermelon, questions have come up that are interesting to consider, especially in light of our knowledge of other crops. Even considering basic morphology, watermelon is an interesting species, with small seeds that produce large plants, and small flowers that produce large fruit (1 to 122.2 kg each).

Genes and linkage

There are 62 genes described for morphological traits and genetic resistance in watermelon (Wehner, 2012). That number is 43% of the number of genes (144) described for cucumber (*Cucumis sativus* L.). Cucumber gene linkage been studied for a longer time than watermelon. However, multiple linkage groups containing 2 to 14 gene loci each have been described for cucumber, but none for watermelon (not counting molecular markers). Why are there no reports of gene linkage in watermelon? One of the popular explanations is that watermelon has more chromosomes and shorter chromosomes than cucumber. Is that the reason?

Although there is little gene linkage in watermelon, there are numerous loci and, in some cases, multiple alleles at a locus that affect the same trait. For example, light green rind is caused by *g-1g-1 g-2g-2*. Flesh color is controlled by alleles at the *y* locus, and can be scarlet red (Y^{Scr}), coral red (Y^{Crl}), orange (y^{O}), or salmon yellow (*y*). However, canary yellow at the *C* locus is epistatic to the reds and yellows at the *y* locus. Thus, *CC* $Y^{Crl}Y^{Crl}$ is canary yellow, not coral red, and *CC yy* is canary yellow, not salmon yellow.

There are many cultivars with solid dark green rind color. 'Mountain Hoosier' and 'Early Arizona' have solid dark green rind, controlled by two loci with dominant alleles, *G-1G-1 G-2G-2*. It is possible to see faint stripes under the solid color of 'Sugar Baby', and 'Smokylee' has a gray smoke pattern over the solid color of its fruit. Is the dark green rind color of 'Sugar Baby' and 'Smokylee' controlled by the same two (*G-1 G-2*) loci as the other two cultivars? Another related question: are the stripes on the watermelon fruit the dark area or the light area? So, for example, is 'Allsweet' wide striped (dark area) or narrow striped (light area)?

In many plant species, white represents the absence of color, and is due to a defective color allele. However, in watermelon white flesh color in the fruit can be dominant or epistatic to other flesh colors, and the genes can be found in wild relatives such as citron. For example, *WfWf BB* or *WfWf bb* produces white flesh, *wfwf BB* is yellow flesh, *wfwf bb* is red flesh. Is this the same white color as that found in 'Cream of Saskatchewan', or is it controlled by a different gene than the citron white flesh color?

What is the difference between tiny seed (*TiTi*) in 'Sweet Princess', and short seed (*LL ss*) in 'Baby Delight'? Unfortunately, 'Baby Delight' is no longer available from germplasm collections for allelism tests. Short seeds is recessive to the medium size seeds (*LL SS*) of 'Klondike', so it is a different pattern from the inheritance of tiny seed. 'Sweet Princess' was released by Warren Henderson at North Carolina State University. The germplasm came from Warren Barham, the previous watermelon breeder at NCSU, who used a spontaneous mutant, with 'Charleston Gray' as the main donor for traits. There is also a trait for small seeds in 'Small Seeded Dixielee'. Is that inherited differently from tiny seeds and short seeds?

Disease resistance

Fusarium wilt resistance has been studied using differentials, usually common watermelon cultivars that are inbred lines. The differentials are available with increasing numbers of race resistances. For example, susceptibility (S) or resistance (R) to races 0, 1 and 2 in the differentials are as follows: S S S ('Black Diamond'), R S S ('Mickylee'), R R S ('Calhoun Gray'), and R R R (PI 296341). The increasing number of resistances seems to be different from the pattern where race resistance genes are

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inherited independently. The pattern is more like an increasing level of resistance to new populations of the *Fusarium* pathogen than an independent series of genes for resistance to several *Fusarium* races. Why are there no other combinations of resistance, such as R S R (races 0, 1, 2)?

Many PI accessions reported to have disease resistance have late maturity, low yield, and poor fruit quality. Breeders would like resistance combined with other elite traits. Why is it so difficult to combine resistance with earliness, yield, and quality? For example, PI 296341-FR (known since 1989) has resistance to *Fusarium* wilt race 2 caused by *Fusarium oxysporum*. Also, PI 189225 (known since 1965) has resistance to gummy stem blight caused by *Didymella bryoniae*. Much work has been done to combine resistance from those PI accessions with elite traits obtained by crossing with adapted cultivars. Yet, there still are no elite cultivars with resistance to *Fusarium* race 2 or gummy stem blight.

Is there limited recombination between the chromosomes of watermelon (*Citrullus lanatus* var. *lanatus*) and those of citron (*Citrullus lanatus* var. *citroides*)? Should breeders be working directly with citron to develop lines with higher quality and earlier yield instead of trying to produce watermelon with higher resistance? Colocynth (*Citrullus colocynthis*) seems to cross readily with watermelon. Colocynth is a different species, but citron is the same species as watermelon, just a different botanical variety. Is colocynth a closer relative to watermelon than citron? Perhaps we should be working with colocynth rather than citron to introduce new traits. Do we need to revisit the taxonomy of *Citrullus*?

Fruit yield and quality

Why do low yielding cultivars have small (‘Minilee’) or large (‘NC Giant’) fruit size, and high yielding cultivars have medium (‘Mt. Hoosier’) fruit size? Is it difficult for the watermelon plant to have many small fruit as a strategy for achieving high yield? It must also be difficult to fill a single large fruit as a strategy for producing high yield. Is the problem related to optimization of surface to volume ratio, or is it a problem of optimizing photosynthate transport from leaf to fruit?

The yield of a particular cultivar is dependent on the location it is being tested. Are there cultivars that have high yield in all locations? For example, some breeders have said that cultivars from eastern United States do poorly in the west, and cultivars from western United States do poorly in the east. If that is the case, we only need one cultivar. Is it sufficient to have just one elite cultivar, or should we be developing one specialized cultivar for each location?

Thick rind can be tender (‘Tendersweet Orange Flesh’), and thin rind can be tough (‘Petite Perfection’). Thick rind is useful for watermelon rind pickles, but that product seems to be disappearing from the market. Should all watermelon cultivars have tough but thin rind, or do we still need tough but thick rind cultivars, such as ‘Garrisonian’, ‘Smokylee’, and ‘Tom Watson’?

Should breeders select for sweet watermelons using °Brix for initial selection, and then use taste-testing to make final selections? After all, fruit with high content of fructose will taste sweeter but have the same °Brix as fruit with high content of sucrose. The specialty market for diabetics requires cultivars with low-sugar fruit. What constitutes good watermelon flavor, if low-sugar cultivars are developed? What is the optimum watermelon fruit texture to go with good flavor? Do we want soft flesh for watermelon cultivars sold for whole fruit (‘Allsweet’) and firm flesh for cultivars sold for cubed fruit (‘Crunchy Red’), or should all cultivars have firm flesh? Can watermelon flesh be too firm (citron)?

Future breeding objectives

Germplasm accessions related to watermelon have been collected from the dunes of the Kalahari desert. Thus, some accessions can survive hot, dry conditions and grow in deep sand. Also, there are watermelon ancestors such as green algae that live in salt water oceans, but watermelon cultivars do not tolerate salty soil or saline irrigation water very well. Would it be possible to breed watermelon for salt tolerance, or is that like breeding watermelons with C4 metabolism or nitrogen fixation capabilities?

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Breeding for Downy Mildew Resistance in Cucumber

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Additional index words. cucumber, downy mildew, breeding

Abstract. Cucurbit downy mildew remains the largest threat to cucumber production in Eastern and Great Lakes growing regions, resulting in up to 100% loss in affected areas. A resistance-breaking strain of the pathogen, *Pseudoperonospora cubensis*, emerged around ten years ago, and currently, there are no commercially available cultivars with effective resistance to the disease. Our breeding program has developed several lines that display high levels of resistance in east coast trials, and which show similarly low levels of symptoms without chemical control as standard cultivars under weekly fungicide applications. We are currently working to identify the resistance QTL underlying this resistance, and to introgress these resistance genes into multiple genetic backgrounds and market classes.

Cucurbit downy mildew (CDM), *Pseudoperonospora cubensis,* is a pathogen of watermelon, melon, cucumber, pumpkin and squash crops in the United States. There is a continuing need for cultivars with resistance to CDM in all cucurbit crops, but especially in the *Cucumis* species, cucumber and melon, where the disease is most severe. Downy mildew symptoms appear on the foliage as chlorotic, angular lesions that become necrotic, often killing the plant in a matter of weeks. New plantings of cucumber are infected by windblown spores that originate from sporulating crops.

Materials and Methods

Cucumber transplants were propagated according to standard practices. Disease ratings were taken on a whole plot basis following natural infection for true breeding plots and area under disease progress curve (AUDPC) was calculated from the percentage of diseased foliar area. For segregating populations, data was taken on only the most resistant plants. Yield was measured as number, weight and marketable number of fruit per plant. Our breeding approach utilized cuttings taken from resistant segregants identified in the field, rooted in the greenhouse and self-pollinated or inter-mated.

Results and Discussion

We screened a set of cucumber germplasm that included cultivars and accessions from the Cornell breeding program that were previously noted as having especially high levels of CDM resistance. Moderately resistant entries, 'Marketmore 97' and 'Ivory Queen', were used in breeding. Utilizing a pedigree approach, multiple downy mildew resistant lines were produced, of which DMR-NY264 has the most potential to be used immediately by growers (See Fig. 1). We are currently engaged in improving upon the quality and performance of this release.

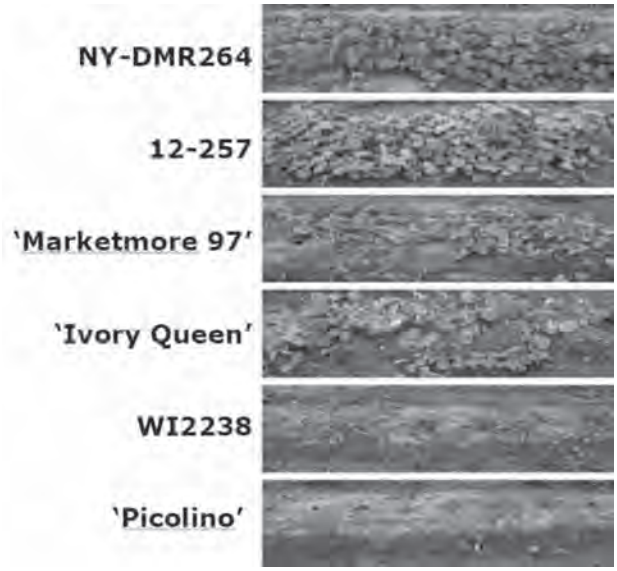

Fig. 1. Examples of cucurbit downy mildew resistance in cucumber. NY-DMR264 and 12-257 are recently released breeding lines from Cornell University. 'Marketmore 97' and 'Ivory Queen' are moderately resistant cultivars from Cornell University and Cook's Garden respectively. WI2238 is among the most resistant cultivars previously reported. 'Picolino' is a susceptible control.

A detailed description of our work has been published recently (Holdsworth et al., 2014).

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Using Genotyping-by-Sequencing to Identify SNPs Linked to the *Papaya Ringspot Virus* Resistance Trait in Bottle Gourd Useful for Marker Assisted Selection

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Additional index words. cleaved amplified polymorphism sequence, *Lagenaria siceraria*, next generation sequencing, potyvirus, watermelon, ZYMV.

Abstract. Bottle gourd [*Lagenaria siceraria* L.] is an important vegetable and a root stock for watermelon. Previously, we identified several sources of resistance in bottle gourd to potyviruses, including *Papaya ringspot virus* (PRSV) and *Zucchini yellow mosaic virus* (ZYMV). In an effort to elucidate the genetic basis of inheritance of resistance to these viruses, breeding populations were generated from two resistant lines (USVL1-8 and USVL5-5. Results demonstrated that inheritance of resistance to PRSV in bottle gourd was controlled by a single dominant gene. Resistance to ZYMV could be controlled by quantitative trait loci (QTLs). To facilitate molecular marker identification, we employed Genotyping-By-Sequencing (GBS) technology. In two experiments with 190 F_2 samples, GBS generated an average of 2.1 to 2.5 million reads per sample. Since the bottle gourd genome sequence was not available, alignments to the reference sequence were conducted using the closely related watermelon and cucumber genomes. After filtration, 171 high quality single nucleotide polymorphisms (SNPs) were identified. Two SNPs with close linkage to the *Prs* locus were located between 26.6 Mbp and 28.8 Mbp in the chromosome 1 of the watermelon genome. Based on these SNPs, cleaved amplified polymorphic sequence (CAPS) markers were designed, tested on F2 and BC1 bottle gourd populations, a close association to the *Prs* locus was revealed, suggesting the usefulness of these CAPS for marker-assisted selection in bottle gourd breeding. This work demonstrates the power of the GBS technology in translational genomic applications to a plant species whose genome sequence is not yet available.

Bottle gourd [*Lagenaria* siceraria (Molina) Standl] serves as a vegetable in many Asian diets. It has been used as an important rootstock for watermelon and other cucurbits (Davis et al., 2008; King et al., 2008). A strong genetic diversity was observed in the USDA germplasm collection of *Lagenaria* to various diseases and nematodes (Levi et al., 2009). In screening a core collection of 190 U.S. plant introductions (PIs) to *Zucchini yellow mosaic virus* (ZYMV), resistance was identified in 36 of these accessions (Ling and Levi, 2007). In addition to ZYMV, two advanced selected lines were shown to possess a broad spectrum resistance to multiple viruses in the family *Potyviridae*, including *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), and *Squash vein yellowing virus* (SqVYV) (Ling et al., 2013).

In recent years, some excellent achievements have been made on genome sequencing in a number of cucurbits, including cucumber (Huang et al., 2009), melon (Garcia-Mas et al., 2012), and watermelon (Guo et al., 2013). In comparison to the much attention and heavy investment on research to these three major cucurbits, very little genetic and genomic information is available for other minor cucurbits, including bottle gourd. Only approximately 10% of bottle gourd genome sequence has been obtained (Xu et al., 2011). Some levels of genetic diversity are observed among the Chinese bottle gourd collections (Xu et al., 2011). Levi and colleagues have also observed a strong genetic diversity against pests, diseases and nematodes in the USDA bottle gourd germplasm collections (Levi et al., 2009).

Due to its simple, robust, and cost-effective procedures, Genotyping-by-Sequencing (GBS) technology (Elshire et al., 2011; Poland et al., 2012) has quickly gained its popularity as a powerful next generation sequencing (NGS) tool for identification of single nucleotide polymorphism (SNP) markers in association with economically important traits (Liu et al., 2014). In the present study, we were interested in applying the GBS technology to identify SNPs with close association to the PRSV resistance locus in bottle gourd and in evaluating the usefulness of cleaved amplified polymorphic sequence (CAPS) for marker-assisted selection.

Materials and Methods

Host plant and genetic materials. Two families of F_1, F_2, F_3 and BC_1s were generated from two PRSV/ZYMV resistant lines (USVL5-5 and USVL1-8), derived from PI 381834 and PI 271360

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respectively, in crossing with a susceptible line (USVL10) derived from PI 181948. The families obtained from both resistant lines were named B1 and G2, respectively. Seeds were germinated on plastic trays filled with Metro-Mix 360 potting soil (SunGro Horticulture, Seba Beach, Canada) supplemented with slow-release fertilizer Osmocote (Scotts, USA) in an insect-proof greenhouse with temperature at 20 °C to 35 °C and 14–16 h of natural lighting period.

Virus isolates and inoculation. The ZYMV-FL culture (Provvidenti et al. 1984) and a local PRSV isolate collected in South Carolina (Ling et al., 2013) were used for inoculation. Virus cultures were propagated and maintained on Gray zucchini squash (*Cucurbita pepo* L.) or susceptible bottle gourd. A virus inoculum was prepared by macerating virus-infected leaves (1:5 w/v) in saline phosphate-buffer in a tissue extraction bag using a handheld roller or a Homex-6 tissue homogenizer (BioReba, Switzerland) and maintained on ice. Seedlings in 1–2 leaf stage lightly dusted with Carborundum were mechanically inoculated gently on cotyledons and leaves using a cotton swab soaked with the prepared tissue extract. To prevent any potential escape, second inoculation was carried out in a few days to a week. Symptom expression on the inoculated plants was evaluated 4 weeks post the first inoculation. Enzyme-linked immunosorbent assay (ELISA) was used for verification of a virus infection (Ling and Levi, 2007). Both symptom expression and ELISA readings were considered in determining the phenotype call to each test plant.

DNA extraction, library preparation, and sequencing. A small young leaf from each test plant was collected, processed accordingly or kept under frozen at –80 °C until use. DNA was extracted using a modified DNeasy protocol (Ling et al., 2009) and quantified using dsDNA BR kit for Qubit (Invitrogen). A subset of samples were digested with *Hin*dIII to test for cutting efficiency and the quality of DNA preparations were visualized on a 1% agarose gel. Two 96-well plates, each containing 95 samples (with one blank) of 25 µL/sample, were sent to the Institute for Genomic Diversity (IGD) at Cornell University (www.igd.cornell.edu) for GBS optimization, library preparation, DNA sequencing, preliminary data analysis and SNP calling as described (Elshire et al., 2011). Samples were digested with *ApeKI* endonuclease and barcoded to track individual libraries. Barcoded libraries from each plate were pooled and sequenced in one lane on an Illumina HiSeq 2000.

Data Analysis. Preliminary data analysis was performed by IGD using the TASSEL 3.0 GBS pipeline according to the standard GBS protocol (http://www.maizegenetics.net/Table/Genotyping-By-Sequencing/). Reads were aligned to the watermelon and cucumber reference genomes, provided by the Cucurbit Genomics Database (http://www.icugi.org) and the Department of Energy Joint Genomics Institute (http://www.jgi.doe.gov), respectively. SNP genotype files were provided by IGD in HapMap format. The SNP sets were filtered for missingness and minor allele frequency using the TASSEL 4.0 GUI (http://www.maizegenetics.net). SNP sets were again filtered for parental genotype using Microsoft excel.

Linkage mapping. After filtering, individual F_2 genotypes determined from phenotyping for PRSV resistance was added to the SNP sets and formatted for use in the mapping program JoinMap 3.0 (http://www.kyazma.nl/), which uses a regression mapping algorithm with the Haldane function and was run with the default parameters. Maps were generated using SNPs linked to *Prs* with a LOD of at least 4.0. SNPs were determined to be strongly associated to *Prs* if they were associated with a LOD of at least 10.0.

CAPS markers. Cleaved Amplified Polymorphic Sequence (CAPS) markers were designed by identifying the location of the relevant SNPs in the reference watermelon genome. The watermelon genome was downloaded from the Cucurbit Genomics Database (http://www.icugi.org) and visualized on the Integrative Genomics Viewer (https://www.broadinstitute.org/igv/home). Genomic sequences were extracted and inserted into the SeqBuilder program by Lasergene (www.DNAstar.com) for CAPS marker design. Primers were designed in exonic regions flanking the SNPs such that fragments were of an appropriate length for easy amplification and would yield characteristic banding patterns when digested with genotype-selective restriction endonucleases.

Results and Discussion

Inheritance of resistance to PRSV and ZYMV. All F1 individuals generated from crosses using the two resistant parents and the susceptible line were resistant to PRSV or ZYMV infection suggesting the virus resistance trait was likely inherited in a dominant Mendelian fashion. In F_2 populations, trait segregation for PRSV was observed in both breeding populations with ratios consistent with the segregation of a dominant monogenic allele (*Prs*) (F2-B1: 48R and13S, and F2-G2: 52R and 17S). However, for ZYMV, there were a slight skew toward resistance in the same F2 populations (F2-B1: 54R and 11S, F2-G2: 53R and 16S). There were nearly 1R:1S segregation in the BC1s populations (22S and 25R for PRSV, whereas 18R and 17S for ZYMV). Since both homozygous and heterozygous resistant F_2 plants would exhibit a resistance phenotype, F_3 offspring generated from randomly selected resistant F_2 lines were tested through inoculation with both PRSV and ZYMV. As expected, 2:1 ratio of heterozygous:homozygous genotypes in the F3 lines was observed to both viruses. F3 families derived from two thirds of resistant F_2 lines (27/43) were shown to be heterozygous with trait segregation and the other one third (16/43) were homozygous to PRSV resistance.

Genotyping-by-sequencing. Libraries for genotyping-by-sequencing (GBS) were created from 190 DNA samples. These included 102 F2-G2, 54 F2-B1, 4 F1-B1, 4 F1-G2, 4 of each parental line, and 14 individuals from both BC1s. DNA samples were digested with *Ape*K1, barcoded, and sequenced on Illumina HiSeq2000 in two separate sequencing experiments, yielding averages of 2,570,701 and 2,128,755 reads per sample. Data from the two runs were merged and analyzed. 1,374,490 unique tags were identified, giving a maximum coverage of 87.97 Mbp of the bottle gourd genome, or approximately 26 % of the estimated genome of 334 Mb (Xu et al., 2011). Of the unique tags, 593,249 (43.2 %) aligned to the watermelon reference genome and 314,864 (22.9 %) aligned to the cucumber reference genome.

Initial analysis resulted in sets of 11,589 and 5,679 SNPs for watermelon and cucumber alignments, respectively. A large portion of these SNP sets were attributed to differences between bottle gourd and the reference sequences, not variations within the bottle gourd population. To remove erroneous SNPs, these sets were filtered to have a minor allele frequency > 15% and missingness < 40% to yield sets of 946 and 472 SNPs.

Linkage analysis requires SNPs that are polymorphic between parental lines and within F_1 individuals, so SNP sets were filtered to accommodate these requirements. Some missingness within the parental set (≤ 25%) and some homozygosity within F_1 set

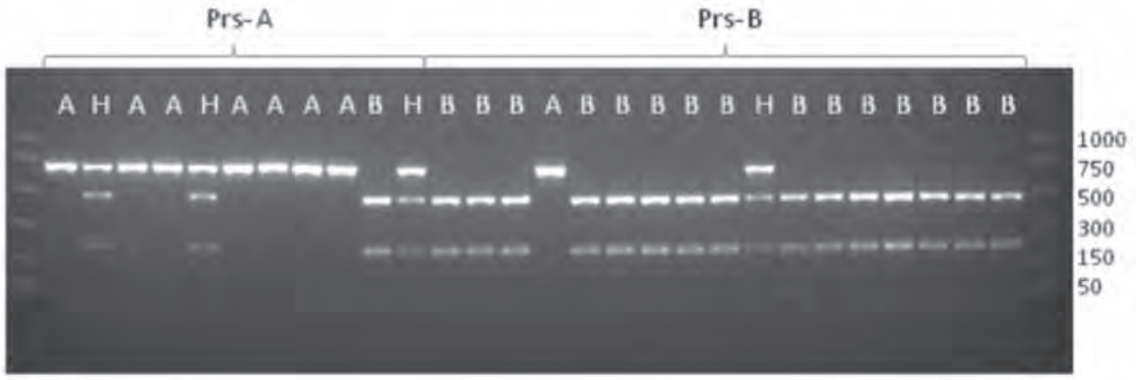

Fig. 1. Screening of F_2 samples with CAPS. In this example, the predicted genotype of *Prs* is either homozygous for the resistant allele (Prs-A) or homozygous for the susceptible allele (Prs-B). The banding pattern of the SNPs is seen and the genotype is presented in yellow, with A indicating homozygous for resistant parent genotype, B indicating homozygous for susceptible parent genotype, and H indicating heterozygosity. Recombination events between *Prs* and the marker are seen in discrepancies between *Prs* and CAPS genotypes.

(≤ 50%) was tolerated, to account for potential sequencing errors (confirmed later by CAPS marker analysis). This yielded a final set of 171 and 95 high quality SNPs for watermelon and cucumber alignments, respectively. Given the hypothetical genome size of 334 Mbp, the genomic coverage of 171 SNPs is approximately 1 SNP for every 2 Mbp. However, SNPs were not evenly distributed. Linkage analysis was performed on the two breeding populations for both watermelon and cucumber SNP sets, for a total of 4 separate analyses. Hypothetical genotypes of the *Prs* locus as determined by phenotyping were included in each analysis. All four analyses identified identical SNPs strongly linked with *Prs*. Data from the two breeding populations were merged and analyzed again yielded the same set of strongly linked SNPs. The two SNPs most closely linked to *Prs* were located at 26.6 Mbp and 28.8 Mbp on chromosome 1 of the watermelon genome and were related to *Prs* with recombination frequencies of 0.14 and 0.15, respectively. Linkage maps were created for all SNPs linked to *Prs* with an LOD of at least 4. This linkage map contained SNPs from 1st and 4th chromosomes of the watermelon genome. *Prs* was mapped between the two most closely linked SNPs, in the 26–29 Mbp region of the 1st chromosome of the watermelon genome. Similar linkage maps from identical SNPs arose from analysis of the cucumber SNP set, but this did not provide novel data.

CAPS Design and application. To confirm SNP association with *Prs*, two CAPS markers were designed from the two most closely linked SNPs. Flanking regions of the watermelon reference genome were used to design primers to amplify fragments surrounding the SNPs and restriction enzymes that selectively cut SNP genotypes were identified. CAPS marker fragments were amplified and products were of the length predicted by the watermelon genome reference and the restriction enzymes cut the parental lines as predicted. CAPS 1 and CAPS 2 were used to confirm the genotypes of all F_2 samples that were sequenced with GBS (Fig. 1). The genotypes derived from CAPS marker screening mostly agreed with the genotypes from GBS calling, with only minor discrepancies in 170 F_2 individuals analyzed. In a BC1s population with 76 individuals, CAPS genotypes were also closely linked to the phenotypes corresponding to the *Prs* locus.

Previously reported GBS experiments have yielded much greater number of SNPs, which would be necessary for a QTL genome-wide association study (Liu et al., 2014). The small size of our SNP sets can be partially attributed to limited alignment (only 43.2%) to the reference watermelon genome. The small size of SNPs could also indicate the lack of diversity between the resistant and susceptible parental bottle gourd lines used in this experiment. Despite this limited number of SNPs, given a low recombination frequency, SNP sets from both breeding lines were sufficient to identify closely linked SNPs and thus the genomic location of the *Prs* locus by aligning to either cucumber or watermelon reference genomes.

An NB-LRR sequence was recently identified as the gene responsible for PRSV resistance in melon (Brotman et al., 2013). Our future research is to determine if a similar sequence is also present in bottle gourd and whether it is also in control of PRSV resistance. In a simple inheritance of PRSV resistance in bottle gourd and with a close association of *Prs* to the identified SNPs, the CAPS markers developed will likely be useful for marker-assisted selection in bottle gourd breeding.

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Genetic Variation in Advanced Backcross Generations and Development of Predominately Gynoecious Line in Bitter Gourd (*Momordica charantia* var. *charantia*) from Its Feral Form (*Momordica charantia* var. *muricata*)

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Additional index words. variability, correlation, sex ratio, heritability, bitter gourd

Abstract. In the present study predominately gynoecious lines were extracted by crossing one commercial monoecious cultivar Pusa Do Mausami (*Momordica charantia* L. var charantia; medium long green fruits; 15–20 cm long) and one gynoecious line, DBGy-201 (*Momordica charantia* L. var muricata; short green fruits; < 5 cm long). High variance components for sex ratio and economic yield attributing traits were studied in three backcross generations BC_2F_4, BC_2F_5 and BC_2F_6 of bitter gourd. High values for genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) were recorded for sex ratio followed by number of fruits per plant, yield per plant (kg) and harvest index. Sex ratio was highly heritable in all three generations with maximum in BC_2F_6 (86.83 %) generation and highest genetic advance (6.59) suggesting the possibilities of high genetic progress through selection. Whereas, fruit number, yield and harvest index had maximum heritability in BC_2F_5 generation. The phenotypic and genotypic correlation coefficients indicated that sex ratio is negatively correlated with yield whereas number of fruits and harvest index had significant positive relationship with yield. Highest direct effect of the number of fruits per plant followed by sex ratio in BC_2F_4 generation stated that these traits in combination can be good criteria for selection in advanced generations in bitter gourd improvement breeding program. In BC_2F_6 population, one predominately gynoecious line (PDMGy) was extracted having very high percentage of pistillate flowers.

Bitter gourd (*Momordica charantia* L.) is one of the most important and nutritional vegetable crops belonging to the family cucurbitaceae. A wide range of variation in sex forms ranging from hermaphrodite to monoecious forms is observed in cucurbitaceous vegetable crops (Robinson and Decker-Walters, 1997). Gynoecious plants bear only pistillate flower at leaf axils however, monoecious plants produce staminate and pistillate flowers throughout the growing season. The monoecious bitter gourd accessions (*Momordica charantia* L. var charantia) produce staminate flowers from the start of reproductive phase till crop maturity and thus the staminate to pistillate flower sex ratio is relatively high (9:1 to 48:1), which also limits the production. The gynoecious habit (only pistillate flowers in a plant) was reported from feral form of bitter gourd i.e. *Momordica charantia* L. var muricata (Behera et al., 2006). However, the subsequent generations (including F_1) using gynoecious as one parent showed very high percentage of pistillate (female) flowers with high yield potential. Micro-propagation was exploited for maintenance and mass multiplication of gynoecious lines for hybrid seed production. But, maintenance of gynoecious lines is very difficult and these can be maintained either through sib-mating or in-vitro methods. These methods are very cumbersome and time consuming. Gynoecious lines can be temporarily induced to hermaphrodite flowers by use of silver ion containing compounds such as silver thiosulphate and silver nitrate. The subsequent generations using gynoecious as one parent has very high percentage of pistillate flowers and have high yield potential (Behera et al. 2006). Therefore, it is essential for the breeders to develop stable gynoecious lines or predominantly gynoecious (PG) lines for use as a variety or as parents in F_1 hybrid development. The development of lines with various levels of pistillate flowering ability may be accomplished by repeated selection and self-pollination of monoecious plants that are predominately pistillate. The available gynoecious lines may be intercrossed or crossed with monoecious or other sex types and reselected to develop new parental lines. In any of these approaches, obtaining stable gynoecy is difficult because gynoecious sex expression of bitter gourd is controlled by a single recessive gene *gy-1* (Behera et al., 2009).

Yield is a complex character, which depends upon many independent contributing characters. The study of genetic variation in respect of quantitative traits like yield and its components is essential for effective breeding strategy. So heritability is one of the popular indexes between the phenotypic and breeding value. Correlation and path coefficient determines the nature and magnitude of association among different quantitative traits and degree of relationship which help in measurement of direct and indirect effect of one variable over the other. The potential use of gynoecy in increasing cucumber yield was reported by previous studies. It was observed that the number of pistillate flowers was positively correlated with yield in some population season combinations. Moderate to highly significant positive correlations (r) between per cent pistillate nodes and yield were also observed in cucumber suggesting sex expression has potential for increasing yield through indirect selection (Cramer and Wehner, 2000). A positive correlation (r = 0.24–0.40) was observed with the number of females nodes on lateral branches and total fruit per plant (Fazio, 2001; Fan et al., 2006). Choice of gynoecious and monoecious parents is also an important consideration when

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developing a predominantly gynoecious lines (PG). Therefore, this study was designed to develop the stable PG line or converting a gynoecious bitter gourd cultivar to the predominantly gynoecious condition so it may be utilized to develop F_1 hybrids.

Materials and Methods

Present investigation was conducted during 2011–2013 at Experimental Farm of Division of Vegetable Science, IARI, New Delhi. All recommended cultural package practices were followed during the study. The experiment was laid out in randomized block design with three replications. The gynoecious parent (DBGy-201) utilised during experiment was pure inbred line with 100% pistillate flowers, short green fruits (<5cm long) and monoecious parent (Pusa Do Mausami) with usual staminate and pistillate flowers (11:1 to 30:1) with medium long green fruits (15-20cm long). The hybrid of DBGy-201 × Pusa Do Mausami was selfed using a staminate flower from the same plant to obtain F_2 seeds. The gynoeciuos plant in F_2 population was again crossed with the recurrent parent Pusa Do Mausami (PDM) to develop BC_1 and BC_2 population was developed by crossing the PDM with predominately gynoeciuos (PG) line. The successive backcross progenies were developed by selfing PG line followed by single plant selection. The plants were observed for the presence of pistillate flowers upto 20th node, higher male and female flower ratio (1:7 to 1:9) and better fruit traits were selected. For this study BC_2F_4 was selfed up to BC_2F_6 generation with 5% selection intensity in each backcross generation based on sex ratio of each plant. GCV (%), PCV (%), heritability (%), genetic advance and genetic gain was estimated for sex ratio, number of fruits, yield and harvest index. The analysis of variance was performed by SPSS (ver. 1.6) and the mean square was also evaluated on the basis of Panse and Sukhatme (1967).

Results and Discussion

The incremental rate of mean of sex ratio (6.54 to 27.88) from BC_2F_4 to BC_2F_6 was reported in this study. Consistent progress was observed with respect of donor parent (Pusa Do Mausami) up to BC_2F_6 generation which was the main target of improvement to extract predominantly gynoecious line. Number of fruits per plant in F_1 shows negligible difference over mid parent value (Table 1) but it reduced in BC_2F_4 generation and again increased in later two successive generations likewise the parents explain role of additive gene action and stagnant variability. Mean performance for yield and harvest index had same trend that is continuous progress in successive generations due to harvest index is dependent upon economic yield.

The results indicated that mean sum of square due to genotype was higher than mean sum of square due to replication for all the traits except for number of fruits in BC_2F_4, BC_2F_5 and BC_2F_6 generations (Table 2). The estimates of genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) indicated stabilized variability from BC_2F_4 to BC_2F_6 generations. Highest magnitude of GCV and PCV was obtained for sex ratio in BC_2F_5 generation whereas, number of fruits and harvest index had maximum variability in BC_2F_4 generation. Higher PCV value as compare to GCV for these traits stated that there is considerable influence of environment on expression of characters and selection based on phenotype may be useful for yield improvement. Similar results were reported by Islam et al. 2009. The magnitude of heritability is helpful for breeder to make decision for continuity of selection. Highest value of heritability was reported for sex ratio and it increased subsequently from BC_2F_4 to BC_2F_6 generation which may be due to selection in each generation changes the gene frequencies in the population but there was negligible increase from BC_2F_5 to BC_2F_6 generation which indicated that in advanced generations there is fixation of the traits. Heritability for fruit number, yield and harvest index was low but it increased from BC_2F_4 to BC_2F_5 with slight reduction in BC_2F_6 generations. Genetic advance and genetic gain was found to be highest for sex ratio followed by moderate to low values for number of fruits per plant, yield and harvest index. Hence, selection in early generations for high sex ratio can be effective. High heritability with high genetic gain for sex ratio in BC_2F_6 generation indicated significant contribution of additive genetic variance for this trait. Highest genetic gain was obtained in BC_2F_5 generations for sex ratio, number of fruits and yield per plant but harvest index was the maximum in BC_2F_4 generation although there was no significant difference among three generations for this trait. Moderate to low heritability accompanied with low genetic advance for number of fruits, yield per plant and harvest index suggested that characters are highly influenced by environment. These results are also in agreement with the results of Yadav et al. (2013) and Devmore et al. (2010).

The degree of association between two variables measured by correlation coefficient and analysis of its components, i.e. genotypic correlation coefficient estimates heritable and phenotypic correlations, are those which are inherent but also influenced by environment. These estimations provide information regarding effective selection for traits which are important for improvement in yield. The results of genotypic and phenotypic correlation coefficients between studied traits in three successive generations have been presented in Table 3. The results of correlation in BC_2F_4 generation for sex ratio indicated that genotypic correlation was significant and positive for number of fruits and yield per plant along with harvest index that is explained by strong direct effect (0.235) of sex ratio on yield. However, in BC_2F_5 generation it was negatively associated with yield and other traits that might be due to weak direct effect (0.051) of sex ratio on yield (Table 4). Again in BC_2F_6 generation, sex ratio showed strong and positive correlation with yield and harvest index except for number of fruits per plant. Phenotypic correlation was non-significant for number of fruits and harvest index but significant for yield to sex ratio in BC_2F_4 generation which confirmed considerable influence of environment on yield. However, in BC_2F_5 generation there was considerable negative association (phenotypic correlation) between sex ratio to other traits. Also in BC_2F_6 generation, phenotypically there was negative correlation between sex ratio to number of fruits, yield and harvest index. Dey et al., (2005) also reported that sex ratio, days to first male flower and node number to first female flower had negative effect on yield in bitter gourd. Therefore, improving yield through sex ratio cannot be obtained beyond a certain limit.

Strong and significant genotypic and phenotypic correlation was observed between number of fruits to yield per plant (Bhave et al., 2003) and harvest index in all three backcross (BC_2F_4, BC_2F_5 and BC_2F_6) generations that might be due to strong direct effect (1.46, 0.99 and 0.96, respectively) on yield. Maximum correlation was obtained in BC_2F_5 and BC_2F_6 generation that is also obvious from direct effect in these two generations. Correlation between number of fruits and harvest index showed almost same result as with yield that may be explained as yield and harvest index has strong genotypic and phenotypic correlation in all three backcross

Table 1. Mean performance of studied generations.

Traits	P_1 (DBGy- 201)	P_2(PDM)	F_1	BC_2F_4	BC_2F_5	BC_2F_6
Sex ratio	100% female	0.23 ± 0.01	5.03 ± 0.11	6.54 ± 0.06	17.71 ± 0.28	27.88 ± 0.33
No. of fruits	41.71 ± 0.20	18 ± 0.45	25.43 ± 0.50	24.28 ± 0.21	27.02 ± 0.19	33.55 ± 0.16
Yield per plant (kg)	1.86 ± 0.30	1.55 ± 0.01	1.72 ± 0.02	1.42 ± 0.01	1.56 ± 0.01	1.95 ± 0.01
Harvest index	55.82 ± 0.21	60.72 ± 0.31	63.17 ± 0.39	51.97 ± 0.20	58.93 ± 0.14	64.03 ± 0.86

Table 2. Mean square of genotype (MSG), replication (MSR) and error (MSE), range, coefficient of genotypic variation (GCV), coefficient of phenotypic variation (PCV), heritability (h^2) genetic advance (GA) and genetic advance over mean among the studied traits in bitter gourd.

Traits	Generation	MSG	MSR	MSE	Range	GCV (%)	PCV (%)	Heritability (%)	Genetic advance	Genetic gain
Sex ratio	BC_2F_4	1.38	0.68	0.16	5–8.4	9.73	11.55	70.98	1.10	16.88
	BC_2F_5	26.79	2.30	1.70	12.2–26.6	16.32	17.91	83.07	5.43	30.65
	BC_2F_6	37.19	10.62	1.78	20.10–36.0	12.32	13.22	86.83	6.59	23.65
Fruit number	BC_2F_4	8.33	11.60	4.13	20–30	4.87	9.68	25.33	1.22	5.05
	BC_2F_5	8.36	0.47	2.76	30–40	3.90	6.14	40.29	1.78	5.10
	BC_2F_6	5.73	1.42	2.14	40–48	2.51	4.19	35.85	1.35	3.10
Yield	BC_2F_4	0.014	0.012	0.010	0.90–1.35	3.42	9.55	12.87	0.02	2.53
	BC_2F_5	0.017	0.014	0.001	1.38–1.75	4.20	5.76	53.07	0.09	6.30
	BC_2F_6	0.10	0.003	0.004	1.80–2.15	2.24	3.92	32.82	0.05	2.65
Harvest index	BC_2F_4	8.66	0.21	3.67	47.12–57.45	2.48	4.44	31.16	1.48	2.85
	BC_2F_5	4.18	1.79	1.48	55.12–62.45	1.61	2.62	37.70	1.20	2.03
	BC_2F_6	1.53	0.29	0.59	62.05–66.26	0.87	1.48	34.81	0.68	1.06

Table 3. Phenotypic and genotypic correlation coefficients of bitter gourd traits in BC_2F_4, BC_2F_5 and BC_2F_6 generations.

Traits	Generations	F/G	No. of fruits	Sex ratio	Harvest index
Sex ratio	BC_2F_4	F	0.120^{ns}		
		G	0.218^{*}		
	BC_2F_5	F	-0.322^{**}		
		G	-0.599^{**}		
	BC_2F_6	F	-0.161^{ns}		
		G	-0.221^{*}		
Harvest index	BC_2F_4	F	0.950^{**}	0.086^{ns}	
		G	1.009^{**}	0.118^{ns}	
	BC_2F_5	F	0.957^{**}	-0.293^{**}	
		G	0.971^{**}	-0.538^{**}	
	BC_2F_6	F	0.989^{**}	-0.159^{ns}	
		G	0.993^{**}	-0.235^{**}	
Yield	BC_2F_4	F	0.913^{**}	0.184^{*}	0.862^{**}
		G	1.059^{**}	0.500^{**}	1.052^{**}
	BC_2F_5	F	0.939^{**}	-0.364^{**}	0.910^{**}
		G	1.026^{**}	-0.579^{**}	1.003^{**}
	BC_2F_6	F	0.939^{**}	-0.122^{ns}	0.932^{**}
		G	1.026^{**}	-0.185^{*}	1.019^{**}

*, **,ns are significant at 5%, 1% and non- significant respectively, (F= phenotypic, G= genotypic)

generations. Yield per plant also found to be positively correlated with fruit weight, number of fruits per plant and fruits L: D ratio in the study of Dalamu et al., (2013); Lawande et al., (1989); Sundaram (2010), and Sharma and Bhutani (2001) in bitter gourd.

Path analysis helps in partitioning the correlation coefficients between direct and indirect effects of component characters on yield. It provides a measure of relative importance of each independent variable to prediction of changes in the dependent one. Genotypic path coefficient analysis indicated highest magnitude of direct effect (Table 4) in BC_2F_4 generation for number of fruits followed by sex ratio. Correlation of number of fruits (r = 0.941) with yield was also highly significant indicating that the perfect association between these two characters (Bhave et al., 2003; Dey et al., 2005) and one can rely upon number of fruits per plant to select high yielding types in advanced generations for bitter gourd. Whereas, in BC_2F_5 and BC_2F_6 generations, all independent variable (sex ratio, number of fruits and harvest index) had positive direct effect on yield which was highest for number of fruits per plant (Srivastava et al., 1976) followed by harvest index and sex ratio.

Table 4. Path analysis of studied traits in BC_2F_4, BC_2F_5 and BC_2F_6 generations.

Traits	Generations	Direct effect	Sex ratio	No. of fruits	Harvest index	Correlation with yield (r)
Sex ratio	BC_2F_4	0.235	–	0.318	–0.053	0.279
	BC_2F_5	0.051	–	–0.593	–0.036	–0.484**
	BC_2F_6	0.045	–	–0.213	–0.016	–0.150
No. of fruits	BC_2F_4	1.462	0.051	–	–0.453	0.941**
	BC_2F_5	0.991	–0.03	–	0.065	0.979**
	BC_2F_6	0.966	–0.009	–	0.070	0.974**
Harvest index	BC_2F_4	–0.450	0.027	1.474	–	0.898**
	BC_2F_5	0.068	–0.027	0.962	–	0.950**
	BC_2F_6	0.071	–0.010	0.958	–	0.966**

Residual effects BC_2F_4= -0.192, BC_2F_5= -0.054, BC_2F_6= -0.054

In summary, the present study suggested that GCV, heritability and GA for sex ratio was high and it can be used for bitter gourd improvement program. Meanwhile, simultaneous evaluation of correlation and path coefficient as well as the higher relationship between number of fruits per plant and yield indicated that number of fruits per plant can be suitable for direct selection index in yield increase. In BC_2F_6 population, one predominately gynoecious line (PDMGy) was extracted having very high percentage of pistillate flowers and high yield potential of 1.95 kg/plant as against 1.55 kg/plant in Pusa Do Mausami (commercial check). The fruits of this novel genotype also reach early edible stage in about 45 days from sowing with medium long (12–16cm), medium thick (4.5–5.2 cm dia) and fruit weight 85g. This novel genotype has tremendous potentiality in terms of yield per unit area.

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QTL Analysis of Cucumber Fruit Shape Based on Elliptic Fourier Descriptors

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Additional index words. *Cucumis sativus,* genetic linkage map, elliptic Fourier coefficient, principal component analysis, SSR marker

Abstract. Fruit morphological traits, such as shape and color, are of great importance in cucumber, because they relate directly to the commercial value of the products, and thus targets for breeding. We report here on analyses to quantitatively evaluate genetic variability of fruit shape based on elliptic Fourier descriptors, and to detect quantitative trait loci (QTL) associated with fruit shape by using recombinant inbred lines (RILs). We used cucumber cultivars of five major cucumber types (Beit-alpha, European greenhouse, northern- and southern Chinese, pickling, and slicer), and a population of 111 F8 RILs for QTL analysis. Fruit shape was quantitatively evaluated by principal components analysis of elliptic Fourier descriptors. This method describes an overall shape mathematically by transforming contour coordinates into Fourier coefficients, and summarizes these coefficients by principal component analysis. There was a significant large genotypic (cultivar) effect on the scores of fruit shape components, and significant relationships between these scores and traditional evaluations. The QTL mapping analysis for each shape component (principal component scores) based on composite interval mapping indicated that many genes are clearly involved in the fruit shape of cucumber. These results indicate that these methods provide effective, objective indicators of fruit quality traits, with considerable promise for application in scientific research and breeding programs. The results of QTL analyses will contribute to development of more informative DNA markers for fruit shape and to clarifying the genetic mechanism of fruit shape.

Cucumber is an extremely important crop with regard to production value, especially in East Asia, and breeders have vigorously pursued improvements in yield, disease resistance, fruit quality, and other economically important traits. Of the various fruit quality traits, fruit shape is considered to be one of the most important (along with color, texture, and flavor). Fruit shape attributes such as ratio of length to width (L/D ratio) and overall curvature are important objects of improvement in cucumber because they directly influence the commercial value of the product. While breeding efforts for many fruit vegetables have been dedicated to improving fruit shape, very little progress has been made on identifying the genetic mechanisms that govern fruit shape attributes except for L/D ratio, so experimentally derived knowledge of the genetics of these traits is very limited. The difficulty in evaluating fruit shape characteristics other than L/D ratio is one of the main barriers to substantial progress.

Recent improvements in computer performance, combined with reductions in the cost of digital imaging hardware and software, have contributed to the increasingly widespread use of digital image processing in biological and agricultural research. Several approaches have been used to quantify the variation in shapes of biological organs such as seeds, leaves, fruits, and roots. One approach uses simple measurements such as length, width, area, and perimeter. These measurements have the advantage of simplicity, but do not capture shape features in sufficient detail for more complex morphologies. Another approach captures the underlying factors that define a shape using a set of parameters such as coordinate values or descriptors obtained by means of Fourier analysis. One of the most effective methods in this category involves a combination of elliptic Fourier descriptors (EFDs) with principal-components analysis (PCA) (Kuhl and Giardina, 1982; Rohlf and Archie, 1984). This method describes the overall shape mathematically by transforming coordinate information for the image contours into EFDs, then summarizing the EFDs by means of PCA. The combination of EFDs and PCA has been successfully used in genetic and evolutionary studies of plant organ shapes (Iwata et al., 2002; Yamanaka et al., 2001; Yoshioka et al., 2004; Yoshioka et al., 2005; Iwata et al., 2010).

We report here on analyses to quantitatively evaluate genetic variablity of fruit shape of five major cucumber types (Beit-alpha, European greenhouse, northern- and southern chinese, pickling, and slicer), and to detect quantitative trait loci (QTL) associated with fruit shape by using recombinant inbred lines (RILs).

Materials and Methods

Plant materials. We used 10 cucumber cultivars (*Cucumis sativus* L.) that represent a high degree of genetic variability

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across five cucumber types, including three Japanese old cultivars (northern- and southern Chinese type cultivar, 'Aofushinari', 'Kaga-aonaga-fushinari', 'Natsufushinari'), three of the latest commercial F1 hybrid cultivars ('High-green 21', 'Fresco 100', 'Magical 2'), one European greenhouse type cultivar ('Marianna'), slicer type cultivar ('Poinsette 76'), Beit-alpha type cultivar ('Atar') and pickling type cultivar ('Patton'). For autumn cultivation, seeds were sown on 9 September, 2011 and the seedlings were transplanted on 27 September. At the Naro Institute of Vegetable and Tea Science (Mie, Japan), three plants of each cultivar, arranged in a randomized block design, were grown in a plastic greenhouse. All plants set fruit approximately a month after transplanting. From most plants, we randomly sampled six to eight fruits weighing 90–120 g, from each plant. In addition, we used a population of 111 F8 RILs was derived by single-seed descent from a cross between CS-PMR1 and 'Santou' for QTL analysis of fruit shape. CS-PMR1 is a progeny of PI 197088 and has round type fruit. Santou is a Japanese native cucumber cultivar that has long type fruit. Four plants of each line, arranged in a randomized block design, were grown in a plastic greenhouse during the autumn of 2007. We randomly sampled approximately 10 fruits weighing around 120 g per line.

Shape analysis. We obtained fruit images by using a digital image camera and saved each image in RGB color format with 256 grey levels, i.e. 8-bit resolution, per channel (red, green, blue). After we converted each fruit image into binary image by a threshold method, we obtained the closed contour of the fruit and chain-coded them (Freeman, 1974). The coefficients of the elliptic Fourier descriptors (EFDs), which were normalized to avoid variations related to the size, rotation and starting point of the contour trace, were then calculated from the chain-code data by the procedure proposed by Kuhl and Giardina (1982). By this procedure, we approximated the shape of each fruit by the first 20 harmonics, and thus calculated 80 (4 × 20) standardized EFDs. The 80 coefficients could be classified into two groups related to symmetrical variations and asymmetrical variations (Iwata et al., 1998; Yoshioka et al., 2004). We hereafter referred to the former group as symmetrical group, the latter as asymmetrical group. To summarize the information contained in the coefficients, we performed a PCA based on a variance-covariance matrix. In PCA analyses, we used all coefficients in the evaluation of 10 cucumber cultivars of five major cucumber types, and used only the coefficients of the symmetrical group in the evaluation of RILs. To determine the effect of each PC on fruit shape, we recalculated the coefficients of the EFDs, letting the score on a particular PC be equal to the mean ± 2 SD (standard deviations), while keeping the scores of the remaining components as means. EF-PCA was carried out by a program package "SHAPE" developed by Iwata and Ukai (2002). We used the scores of the PCs as the characteristics of fruit shape in the following analyses.

QTL analysis. The genetic linkage map used in this study was constructed based on 111 RILs derived by single-seed descent from a cross between CS-PMR1 and 'Santou' and is described in a previous report (Fukino et al., 2008, 2013). The map was constructed with 295 markers (289 simple sequence repeats [SSRs], 5 sequence characterized amplified regions [SCARs], and 1 inter simple sequence repeat [ISSR]) and contains seven linkage groups spanning 693.0 cM, with an average marker distance of 2.4 cM. The fruit shape of RILs used for map construction (Fukino et al., 2013) were evaluated as described above, and the genotype information obtained during map construction was used here for QTL analysis. QTL mapping analysis for each principal component of fruit shape was performed by composite interval mapping (CIM) using Windows QTL Cartographer v2.5 (Wang et al. 2007). The parameter settings for CIM were as follows: model 6, forward and backward stepwise regression model, five maximum background marker loci, window size 10, and 1-cM walking speed along chromosomes. The LOD thresholds for QTL detection in interval mapping and CIM were determined by 1000 permutations.

Results and Discussion

Evaluation of fruit shape of 10 cucumber cultivars of five major cucumber types. A good summary of the elliptic Fourier coefficients was provided by the first three principal components (PC). Figure 1 show the effect of each PC. These reconstructed shapes indicated that the first PC is good measures of the ratio of length to width (L/D ratio) of the fruit, accounting for 84.5% of the overall fruit shape variation. The second PC expressed the horizontal skewness from the mean fruit shape (Fig. 1): the differences between left and right part in blossom-end and flower-end part of fruit. The third PCs is associated with the curvature of the blossom-end and flower-end part of fruit. The significant differences among the 10 cultivars in the first and third PCs were observed (data not shown). Scatter-plots clearly indicated the wide variation of the fruit shape characteristics (Fig. 2).

Evaluation of fruit shape of RILs. A good summary of the elliptic Fourier coefficients of the symmetrical grous was provided by the first four principal components (PC). The reconstructed shapes indicated that the first PC is good measures of the ratio of length to width (L/D ratio) of the fruit, accounting for 94.2% of the overall fruit shape variation. The second to fourth PCs expressed are associated with the curvature of the blossom-end and flower-end part of fruit.

QTL analysis for fruit shape. A total of 21 QTLs were detected by CIM: six QTLs for the first PC and five QTLs for each of the second to fourth PCs. Figure 3 show the positions and LOD scores of QTLs associated to fruit shape characteristics. The LOD thresholds for QTL detections of the PCs ranged between 2.74 to 2.88. The LOD scores for QTLs of the first PC ranged from 3.60 to 14.90, and the phenotypic variation explained (R2) ranged from 3.04 % to 16.65 %. At all QTLs, the 'Santou' allele lowered the first PC score, indicating that the 'Santou' allele increases L/D ratio of fruit (long shape). The LOD scores for QTLs of the second PC ranged from 3.0 to 7.83, and the phenotypic variation explained (R^2) ranged from 6.37 % to 19.66 %. At most of these QTLs, the

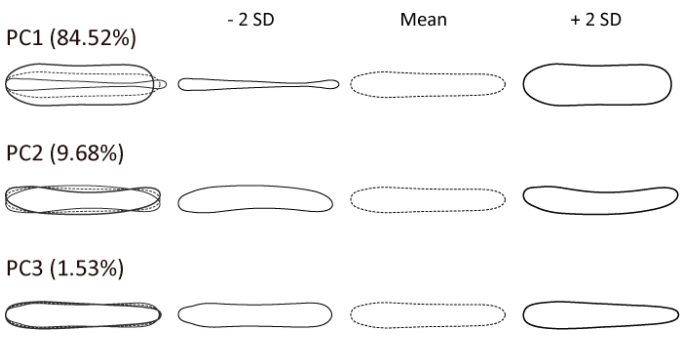

Fig. 1. Effect of each principal component (PC) on fruit shape of cucumber. Dashed line, thick solid line, and thin solid line stand for mean, mean +2 SD, and mean –2SD.

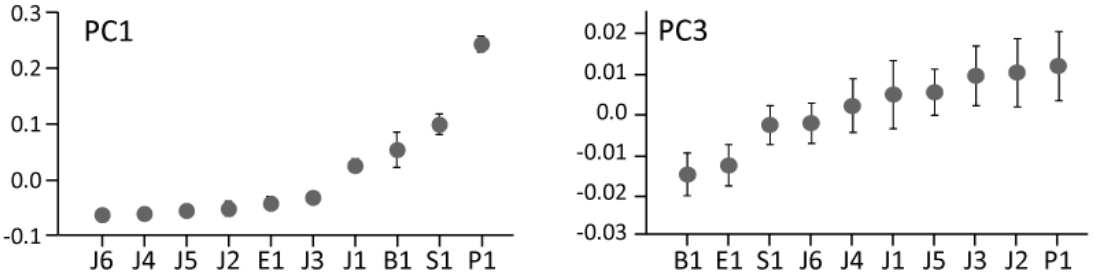

Fig. 2. Variations of the first and third principal component (PC) among the 10 cultivars of five major cucumber types (B1: beit alpha, E1: european greenhouse, P1: Pickling, S1: Slicer, J1-J3: Japanese old cultivars, and J4-J6: Japanese current F1 hybrid cultivars). Vertical bars indicate standard deviations.

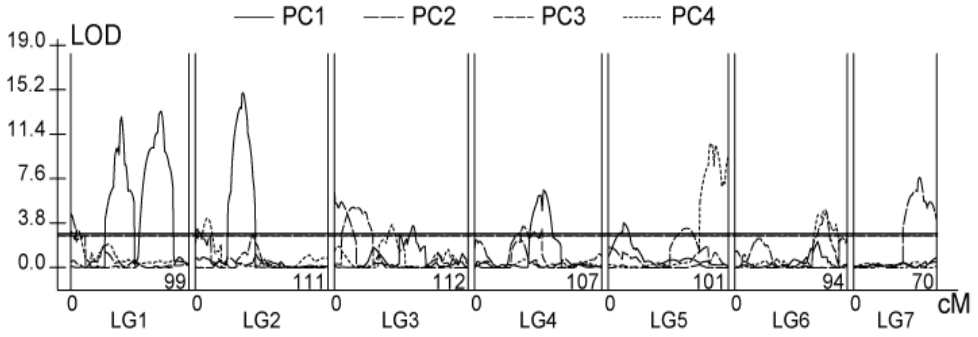

Fig. 3. Positions and LOD scores of QTLs associated to fruit shapes. Horizontal lines represent LOD thresholds at the 0.05 level of significance.

'Santou' allele lowered the second PC score, indicating 'Santou' allele reduces swelling of lower (blossom-end) half of fruit. The highest LOD scores for the third and fourth PCs were 5.18 and 10.66, and the phenotypic variation explained (R2) were 14.0 % and 26.0%, respectively. These QTLs may have a significant influence on the swelling of stem-end and blossom-end of fruits.

In conclusion, fruit shape analysis based on elliptic Fourier descriptors provide effective, objective indicators of fruit quality traits, with considerable promise for application in scientific research and breeding programs. Many genes are clearly involved in the fruit shape of cucumber. The results reported here will contribute to development of more informative DNA markers for fruit shape and to clarifying the genetic mechanism of fruit shape.

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Quantitative Trait Loci (QTL) Mapping of Resistance Genes for *Melon Yellow Spot Virus* in Cucumber (*Cucumis sativus* L.)

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Additional index words. genetic linkage map, MYSV, spotted wilt, SSR marker, *Tospovirus*, viral disease

Abstract. ***Melon yellow spot virus*** **(MYSV) is a serious thrips-transmitted virus of cucurbits in Japan, and has also been reported in Taiwan, Thailand and China. Two isolates of MYSV, a melon isolate MYSV-S and a cucumber isolate MYSV-FuCu05P, have been reported in Japan. In this study, we conducted QTL mapping of resistance genes in a cucumber line 27028930 for each isolate with simple sequence repeat (SSR) markers. The line 27028930 was crossed with a susceptible cultivar 'Tokiwa' to produce an F_2 population, which was used for resistance tests and construction of a genetic linkage map. The data suggest a single dominant gene for resistance to MYSV-S, and it was mapped in Chromosome 3. Two QTLs for resistance to MYSV-FuCu05P were detected in Chromosome 1 and 3. Using SSR markers linked to the loci, we examined the associations between genotypes of the markers and resistance in F_2 population derived from a cross of different parental lines, which showed the effectiveness of these markers for selection of MYSV resistant plants.**

Melon yellow spot virus (MYSV) is a devastating virus of cucurbits in Japan, which is a member of the genus *Tospovirus* and transmitted in a persistent manner by melon thrips (*Thrips palmi* Karny) (Kato et al., 1999; Kato et al., 2000; Takeuchi et al., 2001). MYSV has also been reported in Taiwan, Thailand and China. MYSV induces chlorotic spots, mosaic mottling, yellowing on leaves, and sometimes causes mosaic patches or mottling on fruits. These symptoms cause yield loss and reduced fruit quality. The disease is called spotted wilt. Two isolates of MYSV have been reported in Japan, MYSV-FuCu05P and MYSV-S. They were originally isolated from cucumber (*Cucumis sativus*) and melon (*C. melo*), respectively, and the host range in cucurbits differs between the isolates: MYSV-S also infects *Citrullus lanatus*, *Cucurbita maxima* and *Lagenaria siceraria,* while MYSV-FuCu05P only infects *C. sativus* and hardly infects *C. melo* (Kato et al., 1999; Takeuchi et al., 2001).

One of the most effective methods for disease control is the use of resistant cultivars. However, no cucumber or melon cultivar has been reported to be resistant to MYSV. Sugiyama et al. (2009a) evaluated 398 cucumber accessions for resistance to MYSV-FuCu05P and MYSV-S, and found an accession 27028930 originating from Thailand had resistance to both isolates. All tested plants of 27028930 were systemically infected by MYSV-FuCu05P, but symptoms of infected plants of 27028930 were mild. No systemic infection by MYSV-S developed in 27028930 plants at 20 °C. Higher incubation temperature (25 °C or 30 °C) facilitated systemic infection by MYSV-S, but the percentage of infected plants was lower compared with a susceptible cultivar (Sugiyama et al., 2009b). These results show that the accession 27028930 can be used as resistant material for breeding.

In this study we identified QTL conferring resistance to 2 isolates of MYSV, and developed DNA markers linked to the resistance.

Materials and Methods

Plant materials. Cucumber accessions 27028930, 'Tokiwa', 'Kyuri Chukanbohon Nou 4', 'Kyuri Ano 4', and 'High Green 21' were used as parental lines. The accession 27028930 is an MYSV resistant accession originating from Thailand. 'Tokiwa' and 'Kyuri Chukanbohon Nou 4' are susceptible inbred cultivars. 'Kyuri Ano 4' is an MYSV resistant inbred line which is the progeny of a cross of 27028930, 'Tokiwa' and 'Encore 10'. 'Encore 10' and 'High Green 21' are susceptible F_1 hybrid cultivars. These accessions were used to develop F_1 and F_2 populations.

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Ninety-two F_2 ('Tokiwa' × 27028930) plants, 10 plants of each parental line, and 10 F_1 plants were used for genetic linkage map construction and QTL analysis for resistance to MYSV-FuCu05P. Ninety-three F_2 ('Kyuri Chukanbohon Nou 4' × 'Kyuri Ano 4') plants, 12 plants of each parental line, and 12 F_1 plants were used for association test between marker genotypes and MYSV-FuCu05P resistance. Ninety-one F_2 ('Tokiwa' × 27028930) plants, 10 plants of each parental line, and 10 F_1 plants were used for genetic linkage map construction and linkage analysis for resistance to MYSV-S. Ninety F_2 ('High Green 21' × 27028930) plants, 8 plants of each parental line, and 8 F_1 plants were used for association test between marker genotypes and MYSV-S resistance.

Virus materials. Two MYSV isolates, MYSV-FuCu05P and MYSV-S, were originally isolated from cucumber (Sugiyama et al., 2009a) and melon (Kato et al., 2000), respectively. The isolates were rub-inoculated onto cotyledons of cucumber cultivar 'Shimoshirazu' when the cotyledons were fully expanded. Systemically infected leaves were stored at –80 °C and used as inocula.

MYSV resistance tests. Plants for MYSV-FuCu05P resistance test were mechanically inoculated with MYSV-FuCu05P 7 days after sowing. The inoculated plants were grown in a growth chamber at 20°C under 14-h light/10-h dark condition, and then planted in a greenhouse. The disease severity index (DI) on true leaves was recorded as following: 0 = no symptoms, 1 = only mild vein yellowing, 2 = only slight mosaic mottling with no yellowing, 3 = mild yellowing, 4 = yellowing, 5 = severe yellowing, and 6 = dead. The average DI from 5th to 14th true leaves of each plant was calculated, and the calculated DI score for each plant was used in QTL analysis.

Plants for the MYSV-S resistance test were mechanically inoculated with MYSV-S 7 days after sowing, and grown in a growth chamber in the conditions described above. The third true leaves were sampled at 28 days post inoculation (dpi), and virus was detected using DAS-ELISA analysis as described previously (Sugiyama et al., 2009b).

SSR marker analysis. The SSR markers from previous studies (Ren et al., 2009; Fukino et al., 2008; Yuan et al., 2008; Watcharawongpaiboon and Chunwongse, 2008) were screened for polymorphisms between parents. Genomic DNA was isolated from young leaves using DNeasy 96 Plant Kit (Qiagen). PCR products were labeled by post-PCR labelling (Kukita and Hayashi, 2002) or using labeled primers with fluorescent dyes (6-FAM, VIC, NED, PET). The sizes of PCR products were analyzed using 3730xl DNA analyzer and GeneMapper software (Applied Biosystems).

Genetic linkage map construction and QTL analysis. The F_2 populations and SSR markers were used to construct genetic linkage maps. Linkage maps were constructed with MAPMAKER/EXP 3.0 (Lander et al., 1987). The previously reported assignment of linkage groups to corresponding chromosomes (Ren et al., 2009) was used. QTL analysis was performed using composite interval mapping with Windows QTL Cartographer v2.5 (Wang et al., 2012). The putative QTLs were estimated from the calculated LOD threshold score after 1,000 permutation tests.[the LOD threshold score which was calculated in the permutation test could be specified]

Results and Discussion

Evaluation of resistance to MYSV-FuCu05P. After inoculation of MYSV- FuCu05P, 'Tokiwa' plants showed severe yellowing on leaves, while 27028930 plants showed mild yellowing. The mean DI scores of 27028930 and 'Tokiwa' plants at 76 dpi were 3.3 ± 0.18 (SD) and 4.8 ± 0.20, respectively. The mean DI score of F_1 ('Tokiwa' × 27028930) plants was 3.9 ± 0.15, and the DI scores of F_2 ('Tokiwa' × 27028930) plants ranged from 2.2 to 5.7.

Evaluation of resistance to MYSV-S. MYSV-S was not detected in each 27028930 plant, while it was detected in all 'Tokiwa' plants. MYSV-S was detected in 1 out of 10 F_1 ('Tokiwa' × 27028930) plants, 1 out of 10 F_1 (27028930 × 'Tokiwa') plants, and 27 out of 91 F_2 ('Tokiwa' × 27028930) plants. These results suggest that the resistance is controlled by a single dominant gene (*Sws*; resistance to spotted wilt by MYSV-S) in agreement with the expected segregation ratio ($\chi^2 = 0.91$; $P = 0.19$).

Genetic linkage map construction and QTL analysis for resistance to MYSV-FuCu05P. An F_2 ('Tokiwa' × 27028930) population was used for MYSV- FuCu05P resistance test, and a genetic linkage map was constructed using the F_2 population. The map is comprised of 80 SSR markers, which span 691 cM, with an average marker interval of 8.6 cM and contains 7 linkage groups. Two significant QTLs contributed by 27028930 were detected for MYSV-FuCu05P resistance by composite interval mapping (Fig. 1). One QTL was identified in chromosome (Chr.) 1, and explained 24.1 % of the phenotypic variance with a maximum LOD of 11.3. The other QTL was identified in Chr.3, and explained 23.6 % of the phenotypic variance with a maximum LOD of 10.0.

Genetic linkage map construction and linkage analysis for resistance to MYSV-S. Another F_2 ('Tokiwa' × 27028930) population was used for MYSV-S resistance test, and a genetic linkage map was constructed using the F_2 population. The constructed map is comprised of 66 SSR markers, spans 709 cM, with an average marker interval of 11.8 cM and contains 7 linkage groups. We performed a linkage analysis for MYSV-S resistance, and an MYSV-S resistant gene (*Sws*) was mapped in a position close to the QTL for MYSV-FuCu05P resistance in Chr. 3.

Association between marker genotypes and resistance. In order to assess whether SSR markers linked to the QTL could be used to select MYSV resistance, we tested their association with resistance in F_2 populations derived from a cross of different parental lines. SSR markers linked to the QTL in Chr.1 and Chr.3, and F_2 ('Kyuri Chukanbohon Nou 4' × 'Kyuri Ano 4') population

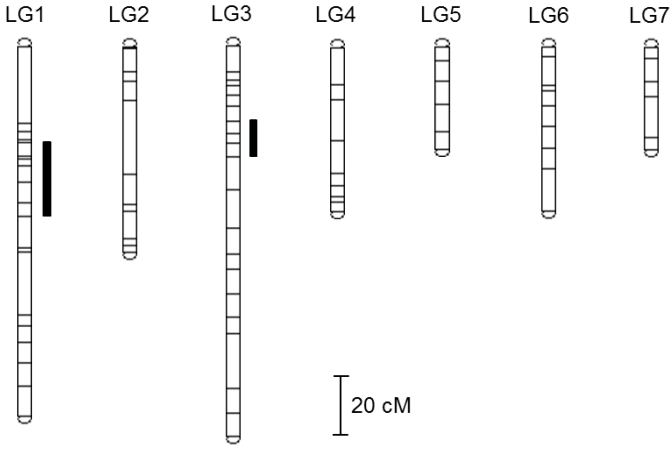

Fig. 1. Linkage map based on the F_2 ('Tokiwa' × 27028930) population. Linkage group (LG) numbers are shown on top. Horizontal lines indicate the positions of SSR markers. Black bars indicate significant QTLs with LOD >3.2 for MYSV-FuCu05P resistance.

Table 1. Marker genotypes and disease index (DI) scores in MYSV-FuCu05P inoculated F_2 population derived from a cross of 'Kyuri Chukanbohon Nou 4' (S) and 'Kyuri Ano 4' (R).

Population type	No. of plants	Marker genotype[a] (Chr.1/Chr.3)	Mean DI score[b]
S	12	B/B	5.8 ± 0.2
R	12	A/A	1.6 ± 0.1
F_2 (S × R)	5	A/A	2.5 ± 0.7 a
	7	A/B	3.8 ± 0.5 b
	10	B/A	3.1 ± 0.5 ab
	5	B/B	4.7 ± 0.4 c

[a] Genotypes of SSR markers linked to the QTLs in Chr.1 and Chr.3. Marker genotype designation: A, homozygous for 'Kyuri Ano 4' allele; B, homozygous for 'Kyuri Chukanbohon Nou 4' allele.
[b]Values followed by the same letter are not significantly different at the 5% level, as determined by the Tukey-Kramer HSD test.

were used to examine the relationship between the markers and MYSV-FuCu05P resistance in F_2 plants (Table 1). The mean DI score of F_2 plants homozygous for both alleles derived from a resistant line 'Kyuri Ano 4' was 2.5 ± 0.7 (genotype A/A) at 64 dpi. The mean DI score of F_2 plants homozygous for both alleles derived from a susceptible cultivar 'Kyuri Chukanbohon Nou 4' was 4.7 ± 0.4 (genotype B/B). The mean DI scores of genotype A/B and B/A were 3.8 ± 0.5 and 3.1 ± 0.5, respectively. The DI score of genotype A/A was significantly different from the DI scores of genotype A/B and genotype B/B at the 5% level. These results show that the SSR markers linked to the QTLs can be used to select for resistance to MYSV-FuCu05P.

An SSR marker linked to *Sws* in Chr.3 and F_2 ('High Green 21' × 27028930) population was used to examine the relationship between the marker genotype and MYSV-S resistance in F_2 plants. MYSV-S was detected in all 21 plants homozygous for the allele derived from a susceptible cultivar 'High Green 21'. MYSV-S was detected in only 5 out of 28 plants homozygous for the allele derived from a resistant line 27028930 and in only 9 out of 41 plants heterozygous for the allele. These results show that the SSR marker linked to *Sws* in Chr.3 can be used to select for resistance to MYSV-S.

In conclusion, we have demonstrated that SSR markers linked to the QTLs detected in this study are effective for selection of MYSV resistant plants.

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Development of Recombinant Sub-NILs Containing Smaller Introgressions of QTL *ssc5.1* Determined to be Involved in the Accumulation of Sugar via MAS and High Throughput SNP Genotyping

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Additional index words. *Cucumis melo*, NILs, QTL, SNP, °Brix, sucrose

Abstract. We used a set of 48 near-isogenic lines derived from the intraspecific cross between an *inodorus* and *conomon* type melon to perform QTL analyses of Brix (°Brix) and soluble sugar content in three locations in summer 2011 and 2012. A QTL was detected in NIL SC5-1 *(ssc5.1*) in the three locations which decreased °Brix by 25 % to 35 %, and sucrose content by 71 % in one location compared to the PS control. To develop recombinant sub-NILs containing smaller introgressions of SC, NIL SC5-1 was crossed with PS to generate an F_2 population. A set of 48 SNPs was developed covering the SC introgression of approximately 1.92 Mb contained within scaffolld00022 in order to refine the positions of the smaller introgressions of recombinants. Seventeen segregating lines containing SC introgressions of various sizes (between 20 Kb and 1.8 Mb) were identified. The F_3 seedlings derived from the segregating F_2 lines were genotyped to identify those fixed for SC or PS alleles. The °Brix assessment was repeated on mature fruits in one location. Means varied between 10.6 and 14.9 °Brix and the *ssc5.1* QTL was detected in 5 lines that decreased °Brix from 12 % to 29 % compared to the PS control. The F_4 seed was obtained and a larger experiment with more replications will be performed in 3 locations in 2014 to confirm the effect of the QTL in the sub NILs.

Important genetic and genomic tools have been developed in the past several years in melon (*Cucumis melo* L.), such as saturated genetic maps (Diaz et al., 2011) and EST databases (Clepet et al., 2011). Paramount among these has been the genome sequence of DHL92, and resequencing data of its parental lines "Piel de Sapo" line T111 (PS), an *inodorus* type melon, and the accession PI 161375 'Shongwan Charmi' (SC), a *conomon* type (Garcia-Mas et al., 2012). A comparison of resequenced individuals to a reference genome offers a means to identify and characterize genetic polymorphisms, of which single nucleotide polymorphisms (SNPs) are the most important and abundant. The abundance of SNPs in the genome, coupled with the diversity of technologies for performing multiplex assays that can range from genotyping single SNPs at a time to over one million in parallel (Perkel, 2008) make them a powerful tool for genetic mapping and marker assisted breeding.

Sugar content in melon is the major determinant of both fruit quality and consumer acceptance, and is a primary target for crop improvement. The major sugar that accumulates during melon fruit ripening is sucrose (Burger et al., 2003) and this accumulation is developmentally controlled by metabolism of carbohydrates occurring in the fruit sink (Hubbard et al., 1989). Variation in sucrose content has been shown to account for nearly all the variation in total sugar content in both low and high sugar accumulating accessions (Burger et al., 2003). Sugar accumulation in melon is a multigenic trait with low heritability and a large environmental interaction (Eduardo et al., 2007).

A QTL analysis of °Brix and soluble sugar content was performed in 48 near-isogenic lines (NILs) in 3 locations in 2011 and 2012. Following the identification of a QTL on LGV that was stable across environments, a combination of high-throughput SNP genotyping and MAS was used to develop sub-NILs containing smaller introgressions of the QTL.

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Materials and Methods

A set of 48 melon NILs derived from the diverse intraspecific cross of PS x SC was evaluated in three locations [Valencia (VAL), Caldes de Montbui (CDM) y Cabrera de Mar (CAM), Barcelona] in the summer of 2011 and 2012. Development of NILs is described in Eduardo et al. (2005). Each plant was allowed to set one fruit. Fruits were harvested 55 days after pollination in all locations. °Brix was measured from juice of mature fruits with a hand refractometer. Soluble sugar measurements from frozen fruit samples were performed at INRA-Bordeaux using Nuclear Magnetic Resonance (NMR) Spectroscopy.

Statistical analysis of °Brix and soluble sugar content was performed on data of five fruits/NIL using JMP (version 8.0.1 for Windows, SAS institute, Cary, NC) The effects of SC introgressions were studied by comparing the mean °Brix value of each NIL against PS as control and using the Dunnett contrast with Type-I error $\alpha \leq 0.05$ (Dunnett, 1955).

To develop sub-NILs containing smaller introgressions of *ssc5.1*, NIL SC5-1 was first crossed with PS to generate the F_1 hybrid. F_1 plants were grown in the summer of 2012, self pollinated, and F_2 seeds collected from mature fruits. An F_2 population was then grown in the spring of 2013. Leaf samples were taken at the two-leaf stage and DNA was extracted using the modified CTAB method (Doyle and Doyle, 1990). After genotyping, F_2 recombinant plants were transferred to the greenhouse in CDM.

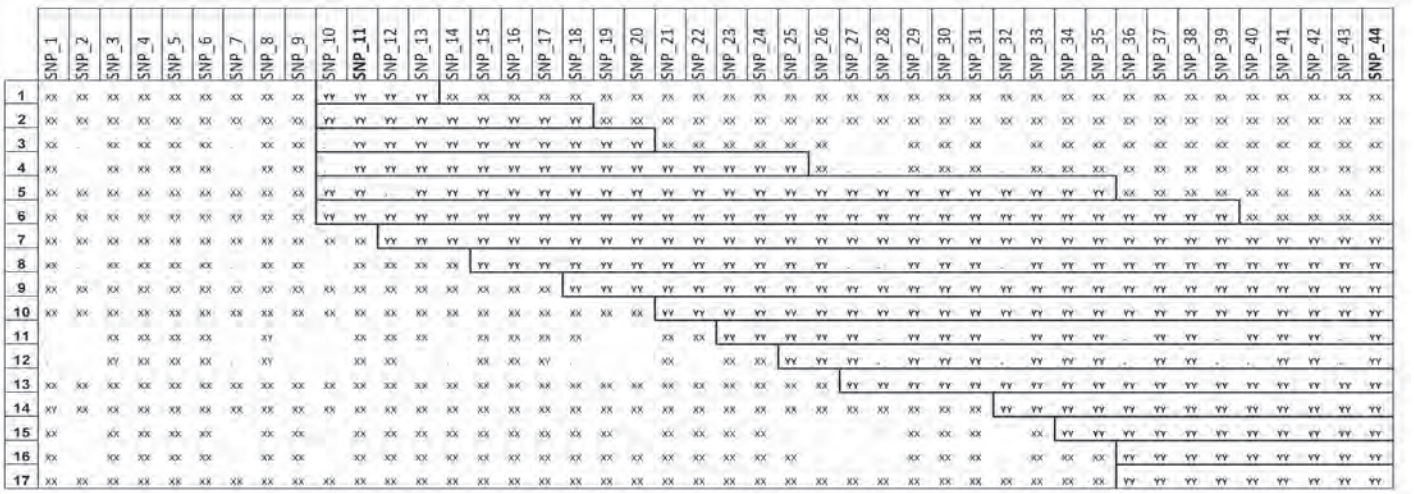
To refine the positions of the smaller SC introgressions contained in selected recombinant lines, SNPs were developed using the SUPER pipeline (Sanseverino et al., 2013) based on resequencing data of SC, PS and the reference sequence of DHL92 generated from Illumina paired-end sequencing (Garcia-Mas et al., 2012). SNP calling was done in iterations as described in Argyris et al. (in preparation). After filtering, a set of 48 SNPs was chosen.

The F_2 lines were genotyped with the SNPs, and subsequent lines were selected containing genetic introgressions of various sizes. The segregating lines were self-pollinated and seeds collected from mature fruits. F_3 seedlings derived from selected F_2 lines were genotyped to identify lines fixed for SC or PS alleles. Four plants of each line were selected and were transferred to the greenhouse in CDM in summer 2013, self-pollinated, and °Brix assessment of mature fruits was performed.

Table 1. Means (± SD) of °Brix and soluble sugar content (mg/g fresh weight) of NILs and parental lines in three locations in 2011 and 2012. Numbers in bold are significantly different with arrows denoting increase or decrease in trait value relative to the PS mean ($P \leq 0.05$).

Trait	Location	T111 (PS)	PI 161375 (SC)	NIL mean	NIL min	NIL max	SC5-1
	VAL	10.6 ± 0.4	11 ± 0.9	10.5 ± 1.7	5.4	14.2	**6.9 ± 1.2**↓
Brix (°Bx)	CDM	10.5 ± 1.3	6.9 ± 1.4	10.2 ± 1.5	5.1	13.3	**7.8 ± 1.5**↓
	CAM	11.9 ± 1.2	8.2 ± 0.5	9.7 ± 1.4	6.8	14	**8.7 ± 0.7**↓
	VAL	43.1 ± 14.3	–	41.2 ± 13.2	1.6	89.9	**14 ± 6.4**↓
Sucrose	CDM	43.9 ± 11	15.1 ± 11.2	44.1 ± 18.6	2.8	106.9	32.1 ± 19.8
	CAM	48 ± 8.6	26.0 ± 7.2	37.6 ± 12.3	8.7	73	33.2 ± 10
	VAL	11.7 ± 1.9	–	13.1 ± 2.6	6.5	21.9	**16.4 ± 1.3**↑
Glucose	CDM	18.2 ± 6.9	16.3 ± 3.3	18.3 ± 5.4	8.5	45.2	17.2 ± 3.4
	CAM	17.9 ± 2.6	15.1 ± 2.4	18.9 ± 4.4	5.8	40.1	20.9 ± 2.5
	VAL	8.4 ± 1.7	–	10 ± 1.9	5.6	19.4	**12.4 ± 1.2**↑
Fructose	CDM	16.1 ± 2.8	14.6 ± 4.0	16.7 ± 4.0	7.8	28.5	14.6 ± 4.5
	CAM	14.2 ± 2.5	12.0 ± 1.8	13.8 ± 2.7	8.6	25.8	15 ± 1.7

Table 2. Genotypes of 17 F3 subNILs fixed for PS (XX), SC (YY), or heterozygous (XY) alleles outside and within the *ssc5.1* QTL interval. Flanking SNP markers used in the recombinant screening are in bold. Missing data denoted by (.).



Results and Discussion

Mean °Brix for the NIL population grown in 2011–2012 was significantly different across locations, ranging from 9.7 in CAM to 10.5 °Brix in VAL (Table 1). Means of individual NILs ranged from 5.1–14.2 °Brix. One NIL (SC5-1) was significantly lower in all three locations (6.9, 7.8, and 8.7, °Brix respectively) corresponding to 35, 25, and 27% less than the PS control in VAL, CDM, and CAM, respectively.

Of the soluble sugars, sucrose concentration showed the widest variation in NILs, ranging from 1.6–106.9 mg·g^{-1} across locations, with a mean from 37.6–44.1 mg·g^{-1} (Table 1). Those for glucose and fructose were lower with less variation, ranging from 13.1–18.9 and 10–16.7 mg·g^{-1}, respectively. In VAL, SC5-1 showed significantly decreased sucrose content (14 mg·g^{-1}), corresponding to a 71% reduction compared to the PS control (43.1 mg·g^{-1}). In contrast, glucose and fructose content were significantly augmented (16.4 and 12.4 mg·g^{-1}, respectively) corresponding to a 33% to 37% increase in the trait, respectively, compared to the control. SC5-1 also showed decreased sucrose content in CDM and CAM, although the differences compared to T111 were not statistically significant. The effect on glucose and fructose in these locations was variable.

SC5-1 was detected as having significantly reduced °Brix in multiple environments. In VAL, this was also accompanied with significantly reduced sucrose, and increased fructose and glucose concentrations. This indicated the presence of putative QTLs in the region of the SC introgression contained on LGV controlling sugar accumulation. The position of this QTL cluster is within the confidence interval of QTL for °Brix detected in the same NILs; with F_2 and DHL populations derived from the PS × SC cross (Diaz et al., 2011; Monforte et al., 2004); as well as with QTL for °Brix and sucrose detected in a RIL population derived from distinct parental lines (Harel-Beja et al., 2010). Given that the QTL was detected in a NIL in multiple locations and corresponded to a cluster of QTL for multiple traits in distinct populations reinforces the idea that mechanisms of sugar accumulation can be conserved across a wide range of germplasm and that *ssc5.1* is a stable QTL suitable for further investigation through the development of sub-NILs.

Recombinant sub-NILs containing smaller introgressions of *ssc5.1* were developed via MAS and high throughput SNP genotyping based on the results of the QTL analysis. Three-hundred F_2 recombinants within the *ssc5.1* QTL interval were genotyped first with two flanking SNP markers (SNP_11 and SNP_44) and another located inside the QTL interval (SNP_29) and 83 lines were selected. The size of the original SC introgression containing *ssc5.1* in NIL SC5-1 corresponded to a genetic distance of approximately 1.92 megabases (Mb) containing 317 genes on genome scaffold00022. To refine the positions of the smaller introgressions of selected recombinant lines, 48 SNPs were selected to cover a genomic interval including the QTL of 2.9 Mb for an average of 1 SNP per 61 kilobases (kb). Following the genotyping with flanking and internal SNPs, 17 F2 recombinants segregating for SC alleles within the *ssc5.1* interval were selected. Measurements of mean °Brix of the F_3 progeny sub-NILs fixed for SC alleles in the QTL interval and with *ssc5.1* introgressions ranging in size from 100 kb to 1.8 Mb (Table 2) varied between 10.6 and 14.9 °Brix (Fig. 1).The *ssc5.1* QTL was detected in 5 lines that decreased °Brix content by 17 to 23% compared to PS. This preliminary data indicated that the 5 lines have alleles of SC in common in a region of approximately 80 kb on scaffold00022.

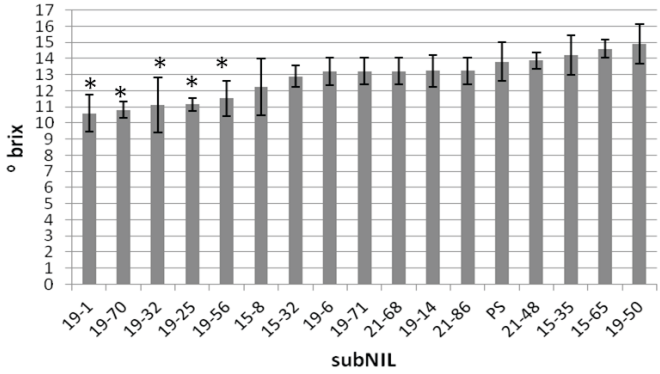


Fig. 1. Means (n = 4) of °Brix of subNILs and parental line (PS) (n = 5) serving as control grown in 2013 in CDM. Asterisks denote subNILs with significantly lower mean °Brix values compared to PS ($P \leq 0.05$). Error bars indicate ± SD.

A larger experiment with F_4 seed and more replications will be performed in 3 locations in 2014 to confirm the effect of the QTL in the sub NILs and its precise position. This work demonstrates the rapidity with which new germplasm can be developed for an important fruit quality trait, and the possibility to narrow large genomic intervals containing hundreds of genes to small intervals with very few genes in a short period of time using available genomics tools in melon.

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Fine Mapping of the Flesh Color Controlling Genes in Watermelon (*Citrullus lanatus*)

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Abstract. Flesh color of watermelon (*Citrullus lanatus*) is an important trait predominantly determined by a network of the carotenoid biosynthetic pathway. Here, we report the map-based cloning and characterization of the genes that control white-flesh in U.S. Plant Introduction (PI) 296341-FR (*C. lanatus* var. *citroides*) and red-flesh elite Chinese cultivar 97103 (*C. lanatus* var. *vulgaris*). Genetic analysis of the F_1, F_2, recombinant inbred lines (RILs) and backcross lines derived from the recombinant inbred lines and 97103 showed the genotype of PI296341-FR *WfWfBB,* and97103 *wfwfbb.* Using the RILs population, we localized these two genes in a large region in linkage group 2 and 4. RILs lines which only contain one of these two loci were selected to fine mapping the related gene. With distinct BILS-F2 population, we have cloned the *LCYB* as *B* gene, which control the red flesh color formation. And the epistatic *Wf* gene was localized in a small region in chromosome 2.

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] flesh color is an important trait. Watermelon exhibits a wide range of flesh color. Henderson et al. (1998) described five flesh colors: white, salmon yellow, canary yellow, orange, and red. Flesh color of watermelon is predominantly determined by the different carotenoid accumulated, which also contributes to the nutritional value of the fruit through the health-promoting function of carotenoids (Bang, 2010). To maximize the health-promoting benefits of carotenoids through increased consumption, characterization of carotenoid synthesis and accumulation in watermelon is essential. Understanding the molecular and genetic components controlling the carotenoid biosynthetic pathway is fundamental for targeted breeding aimed at improving carotenoid-producing watermelon cultivars (Tadmor, 2004; Lewinsohn, 2005).

The varied flesh colors of watermelon are attributed to different carotenoid composition and concentrations (Holden, 1999; Tadmor et al., 2004). Through HPLC analysis, major carotenoids of representative samples of watermelon fruits were clear (Tadmor et al., 2004). The canary-yellow and the pale yellow fruit accumulate the violaxanthin and neochrome (Bang, 2010), and the major carotenoid in red-fleshed watermelon is lycopene (Bang, 2005), and high level β-carotene together with colorless precursors, phytoene and phytofluene also accumulated (Kang et al., 2010). A red-orange variety has been shown to have increased levels of β-carotene at the expense of lycopene (Tadmor et al., 2004). There are no carotenoid accumulation in white-flesh watermelon, but accumulation of colorless phytofluene and light yellow ζ-carotene in white flesh watermelon was also reported (Kang et al., 2010).

Genetic studies on watermelon flesh started in early 1940s. Poole (1944) first described the color inherence between the canary yellow, red flesh and salmon yellow in Golden Honey. The flesh-color determination in watermelon is complex and controlled by several genes to produce red, orange, salmon yellow, canary yellow or white flesh (Henderson 1989, 1991; Robinson et al., 1976). Shimotsuma (1963) suggested *Wf* produced white flesh and the recessive allele *wf* allowed color to develop, and *wfwfyy* control flesh to develop red color (*Y* was renamed to *B* by Henderson in 1989). Brilliant yellow flesh color called canary yellow (*C*) is dominant to red flesh (*c*) (Poole, 1944). The coral red flesh (*Y*) is dominant to orange flesh (y^o) and salmon yellow (*y*), and orange flesh (y^o) is dominant to salmon yellow (*y*). These above loci have commercial utility in watermelon breeding. It was not known whether *B* for yellow flesh and *C* for canary yellow flesh are the same locus (Henderson, 1998). With the development of molecular biology, the carotenogenesis pathway and the related genes have been comprehensively investigated during fruit development, especially in tomato, carrot, cauliflower and other plants (Sandmann, 2001; Bramley, 2002; Clotault et al., 2008; Lu et al., 2006). Unlike the cases for tomato mutants, the identities of predominant carotenoids in several watermelon color mutants were not fully established (Kang et al., 2010). In Bang (2007) research, the watermelon *LCYB* orthologous gene was regarded to as C locus which is responsible for the color determination between canary yellow and red.

Recombinant inbred lines (RILs) as a segregation population were widely used to dissect complex quantitative traits into component loci and study their relative effects on specific trait by quantitative trait loci (QTL) analysis (Mauricio, 2001).QTL mapping provides a means of identifying specific chromosomal regions associated with ecologically important traits. Such results can be coupled with further genetic dissection (Mackay, 2001). Heterozygotes by testcrosses (TC) or backcrosses (BC) from a RI population were widely used to detected non-additive gene/QTLs (Li et al., 2001; Luo et al., 2001; Mei et al., 2003). A particularly useful type is derived from residual heterozygous lines (RHLs). The BCF1 and the BCF2 population from the parental RIL carried the same allele as the recurrent parent but was heterozygous for special other loci were used for QTL mapping (Mei et al., 2005).

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With the development of the draft genome of watermelon (Guo et al, 2013) and the BCF population, we can use map-based cloning to analysis the important traits.

In our research, we first constructed a F_2, RILs populations derived from the cross between 97103(red) and PI29634-FR (white). With the genetic and linkage map analysis, two main QTL for flesh color decision loci were found in the RILs population. The genetic background of each RIL was clear and shown in our previous paper (Ren, 2012). In order to positive cloning these two flesh color decision (FCD) loci, the BCF2 populations derived from certain BCF1 lines which only contain one of these two loci were used to map-based cloning. We cloned the LCYB gene (Cla005011) as B locus and narrow down the *Wf* locus in a small region in chromosome 2.

Results

Flesh color inheritance. Four crossed population between 97103 and PI296341-FR were evaluated to determine the flesh color inheritance (Fig. 1). The F_1 generation had all fruits with white flesh. Upon visual observation, the flesh color produced in F2 population was classified into three categories, white, pale yellow and red. The F_2 plants segregated 116:7(white and yellow: red). In the 103 RILs (F9, n =1 03) populations, we found 54 white: 21 yellow: 28 red. We also developed 96 backcross lines (BCF1) derived from cross between the RILs and 97103. The flesh color phenotype of 49 BCF_1 lines showed red and yellow as the parents RILs lines. While BCF_1 lines of the 54 white-flesh RILs lines turn to white with different degree red or yellow color. The result of chi-square goodness-of-fit test of the segregation ratios of the F_2 and RILs and BCF_1 populations are shown in Table 1.

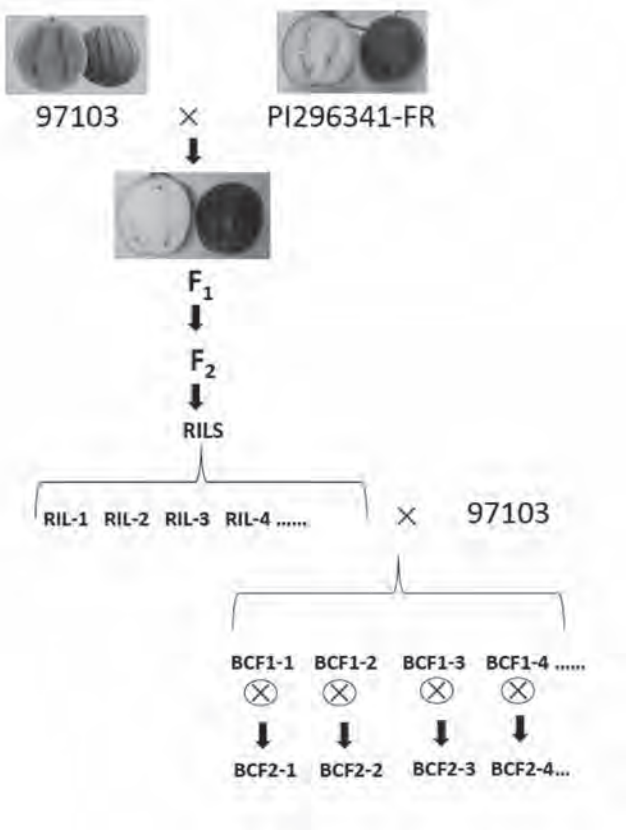

Fig. 1. Construction of watermelon populations with the flesh color trait.

It seems that there are two genes control the flesh color between 97103 (*wfwfbb*) and PI 296341-FR (*WfWfBB*), As excepted the *Wf* is epistatic to *B*, which control white flesh, the B locus control yellow and red flesh formation.

Primary location of FCD loci using high quality genetic maps. We have constructed the high quality genetic map for watermelon using the some RILs lines. Using the flesh color degree as a marker, we found two mayor loci associated with flesh color determination (Fig. 2). These two FCD loci were localized on LGs 2 and 4 (LOD > 5). The QTL explained 24.0887% (LOD = 10.3) and 9.74% (LOD = 6.2) of the phenotypic variation for flesh color determination. The most effective FCDa was mapped between the molecular markers BVWS00009 (Chr2:24521599) and BVWS00338 (Chr2: 34243536) in chromosome 2,whereas FCDb mapped to in chromosome 4 between the molecular markers BVWI00330 (Chr4:8826777) and BVWS02367 (Chr4:13845145). As a population for mapping QTL, RILs can provide additional insights into the mechanisms underlying loci interaction. Further analysis showed that the RILs lines which contain PI296341-FR FCDa sequence were all original white flesh color. The RILs with the 97103 FCDa and FCDb showed red-flesh color. The results showed that FCDa region contain the regarded epistatic *Wf* gene, and the *B* gene was localized in FCDb region.

Segregation of Wf and B genes. According to the genetic analysis, the genotype of the parents, 97103 and PI296341, was *wfwfbb* and *WfWfBB*, whereas the RILs have four genotype: *WfWfBB*, *WfWfbb*, *wfwfBB* and *wfwfbb*. To develop the molecular markers linked to the *Wf* gene, four white flesh RIL (*WfWfbb*) lines were isolated to construct the RILs × 97103 F_2 populations. The flesh colors of these F_2 populations were analyzed for mapping the *Wf* gene. In one of these four BCF_2 populations, the flesh color all turned to red, and the other three populations, the number of white flesh plants were less than expected. The observed data of the F_2 progeny was not a good fit to 1:2:1(Chi-squared test). In one429 BCF_2 population, 262 plants had red flesh and only 60 were white flesh (Table 2).

For B gene cloning, the three white or yellow RIL (*wfwfBB*) were chosen randomly to construct the BCF2 populations. In one F_2 population, flesh phenotype segregated 638 yellow: 199 red), suggesting that in this RIL the yellow flesh is dominant to red flesh at a single locus (Table 3).

Map-based cloning of Wf and B. To isolate the *Wf* gene, we used 60 plants with pure white flesh in 429 F_2 individuals derived from three heterozygote RILs (*Wfwfbb*) which contain FCDa region. Using more markers, the candidate region of *Wf* was narrowed down to the 1200 kb between the markers BVWS00114 (Chr2:28314166) and BVWS00201(Chr2:30509668) (Fig. 3). In this region the Watermelon Genome Automated Annotation System in Cucurbit Genomics Database (http://www.icugi.org/cgi-bin/ICuGI/index.cgi) predicted 229 ORFs, the regarded *Wf* gene should be one of them. In transcriptome analysis of 97103 (Guo, 2011), there are 13 candidate genes did not express in flesh. To ultimately determine the *Wf* gene awaits the setup of either a larger population screening platform or genetic transformation protocols.

In order to clone *B* gene, we used 199 red flesh F_2 individuals derived from heterozygote FCDb plants of special white and yellow flesh BIL (*wfwfBb*) lines for fine mapping. Using the genome sequence of 97103 and the SNP between 97103 and PI296341-FR, we designed more markers in this region to positive clone the *B* gene. With specific markers, we localized the B gene within 8,885,138 to 8,990,056 in chromosome 4. There are 7 predicted

Table 1.Segregation of white (W), yellow (Y) and red (R) flesh colors in the F_1, F_2, RILs and backcross progeny derived from a cross between 97103 and PI296341-FR watermelon.

Generation	Genotype	Observed White:yellow:red	Expected White:yellow:red	χ^2	*P*
97103(PA)	wfwfbb				
PI296341FR(PB)	WfWfBB				
F_1	WfwfBb	10:0:0	1:0:0	0	1
F_2	segregating	116(W+Y):7	12:3:1	0.00815	0.92805
RILs	segregating	54:21:28	2:1:1	0.005094	0.97485
BCF1(RILs*PA)	segregating	44:25:27	2:1:1	0.02949	0.98536

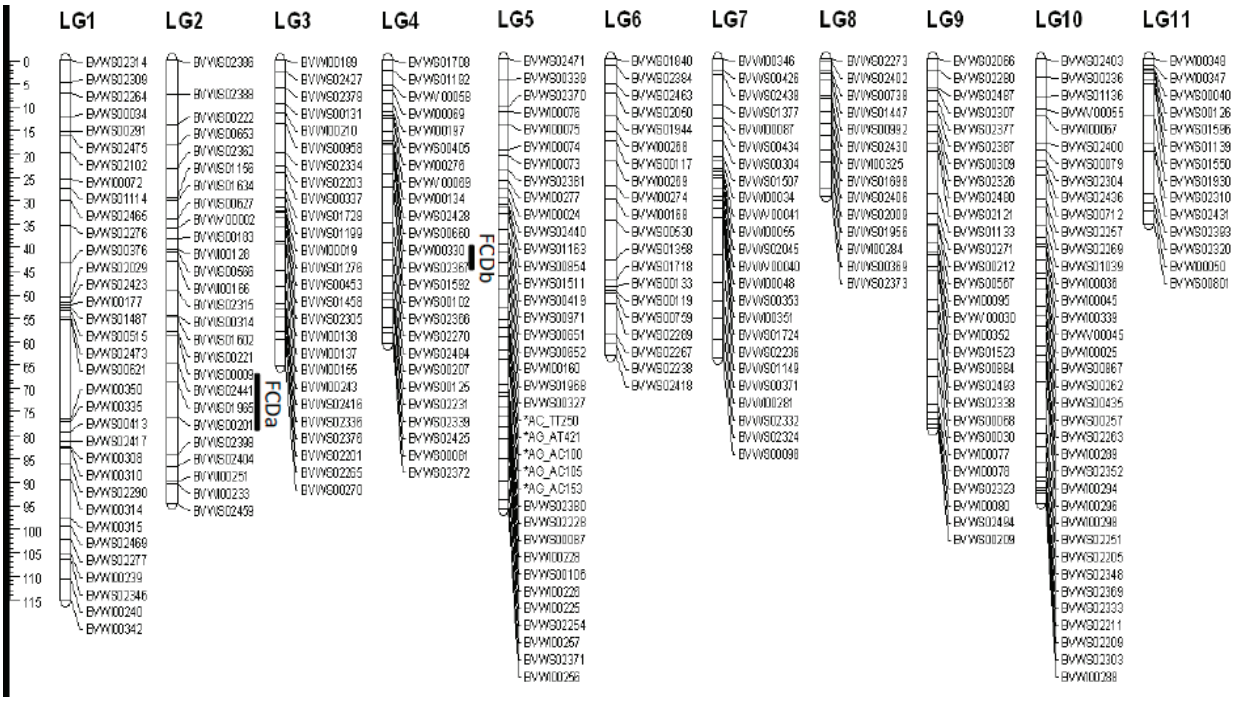

Fig. 2. Quantitative trait loci (QTL) identified for the flesh color determination genes in *Citrulluslanatus* var. *vulgaris*(97103) ×*C. lanatus* var. *lanatus* (PI 296341-FR) RILs population (F9, n = 103). The length of the bars is equal to the 1-LOD support interval and the number in parentheses is the percentage of phenotypic variation explained by the QTL (R^2). The shaded area on linkage group (LG) 2 and 4 indicates the location of a QTL associated with flesh color decision region in the previously reported population (Ren, 2012). The figure was produced using MapChart Version 2.1 (Voorrips, 2002).

open reading frames in this region, one of them Cla005011 is regarded as lycopene β-cyclase (LCYB) (Figure 4). We also analyzed the Cla005011 nucleotide sequences in all F2, RILs, BCF_1, BCF_2 populations. The coincidence of the *LCYB-97103* allele without the *Wf* locus and the red flesh suggested that this Cla005011 gene function as regarded *B* locus.

Discussion

The Wf may encode an upstream regulator which control flesh color and brix in watermelon. We have reported the comparison and construction of an integrated linkage map of watermelon obtained from four independent component maps, including the position of quantitative trait loci (QTL) controlling economically important traits. Two QTL were detected for brix accumulation are located on chromosome 2 and both have $R^2 > 20\%$ (Ren, 2014). And *Wf* gene was contained in the main QTL loci. The flesh color and sugar accumulation phenotype always linked, and our data provided strong evidence that the main QTL on chromosome 2 in watermelon is a combinatorial locus. This provides further compelling evidence that watermelon chromosome 2 harbours a combinatorial QTL for fruit ripening. Several single gene mutants in tomato have been identified that have a global effect on ripening and texture. We hypothesize the same high level regulator was located in watermelon chromosome 2. Together with downstream effectors, this locus can affect watermelon fruit flesh color and sugar accumulation.

The epistatic interaction between Wf and B is imbalanced in BILs population. Shimotsuma (1963) suggested the flesh color of F_2 generation of a cross between white and red flesh inbred lines obtained a ratio of 12 white (*Wf*_ _ _):3 yellow (*wfwfB*_):1 red (*wfwfbb*), and *Wf* was regarded as epistatic to *B*. In our research, the *Wf* gene in wild type watermelon which blocks the carotenogenesis pathway only maintained in F_2 and RILs population. In BILs (RILs*97103), the flesh turned more red, and *WfwfBb* white, *Wfwfbb* white with red, *wfwfBb* yellow with red, *wfwfbb*

Table 2. Flesh color phenotypes in the F_1, F_2 population derived from a cross between 97103 and RILs (WfWfbb) watermelon.

Generation	Genotype or source	Observed flesh color	Observed	Expected	χ^2	*P*
97103(PA)	wfwfbb					
RILS(PB)	WfWfbb					
F_1	Wfwfbb	White	21			
F_2	segregating	segregating	429			
	F_2-wfwfbb	White	60	107		
	F_2-Wfwfbb	red and white	107	215		
	F_2-WfWfbb	red	262	107	299.6822	< 0.0001

Table 3. Flesh color in the F_1, F_2 population derived from a cross between 97103 and RILs (wfwfBB) watermelon.

Generation	Genotype e	Flesh color	Observed	Expected	χ^2	*P*
97103(PA)	wfwfbb	red				
RILS(PB)	wfwfBB	yellow and white				
F_1	wfwfBb	yellow and white	10			
F_2	segregating	segregating	837			
	F_2-wfwfBB/Bb	yellow with white or red	638	628		
	F_2-wfwfbb	red	199	209	0.00329	0.95423

red. The BILs (*Wfwfbb*) lines were not totally white flesh, and some white flesh lines had colored blotches. *Wf* can't block the *bb* gene to generate the red color. It seems the Wf is an upstream regulator in watermelon flesh development, which needs the other downstream factors to regulate the B expression.

Function of LCYB alleles in different flesh color cultivar. In our research we found the *LCYB* in the FCDb region, and this gene sequence variation is coincident with the red flesh formation in different population. So we regarded this LCYB gene as the B locus. Bang et al. (2007) found the candidate gene for color determination between red and canary yellow. They suggested that the same *LCYB* gene might be the former *C* gene which control the canary yellow formation. The red-fleshed watermelon accumulates not only lycopene but alsoβ-carotene. The canary-yellow and the pale yellow fruit accumulate the downstream production of carotenogenesis pathway, such as violaxanthin and neochrome. All these means the LCYB as the important enzyme in carotenogenesis pathway can catalyze the lycopene to β-carotene in red and canary yellow cultivars. Maybe the differentiations of protein stability, or catalysis activity, or the difference in promoter region that regulate the gene expression, influence the variation of carotenoid accumulation in different flesh color cultivars.

There are other loci controlling flesh color in watermelon genome. The genetic models on flesh color inheritance proposed so far suggested that several genes are involved in watermelon flesh color determination (Gusmini and Wehner, 2006; Henderson et al., 1998; Shimotsuma, 1963; Poole, 1944; Porter, 1937). In our QTL analysis, the two main peaks in the LOD trace contained five distinct sub-peaks, which exceeded the significance threshold ($P < 0.05$) for fruit flesh color formation. The absence of significant QTL indicates that many loci of small effect must underlie the observed genetic variation in fruit flesh.

We also found the *Wf* lost the epistatic function in BILs lines with more 97103 loci. These data suggested that there are other loci affecting watermelon flesh color formation. The significant flesh color variation in watermelon suggests that the mechanisms regulating carotenoids accumulation varies among watermelon genotypes. Understanding the mechanisms may provide new insights into the regulation of carotenogenesis in fruits.

Materials and Methods

Plant materials. The cross between *C. lanatus* var. *vulgaris* 97103 and a wild *C. lanatus* var. *lanatus* accession PI296341-FR (the African citron watermelon was evaluated in Beijing (39.48°N, 116.28°E).The F1, F2, RILs and backcross (BC)-F2 populations were generated by controlled pollinations in the greenhouse. Briefly, 10 F1 plant was self-pollinated to obtain F2 seed. One hundred twenty-three F2 individuals and 103 RILs were genotyped. Ninety-six BILs lines were generated based on the cross between 97103 and 96 distinct RILs. Seed of each watermelon variety were sown at the same time and showed a uniform fruit development. Freshly opened female flowers were hand-pollinated and tagged to identify fruit development stage. All fruit for evaluation were harvested at full maturity and cut longitudinally, photographed, and categorized into different flesh color group.

Leaf or flower tissues from greenhouse grown plants were used for DNA extraction according to the method of Skroch and Nienhuis (1995). Total RNA was extracted from watermelon fruit flesh using the TRIzol Reagent (Invitrogen, USA).mRNA was purified from the total RNA using the Oligotex mRNA Midi Kit (QIAGEN, Germany)followed by DNase I treatment according to manufacturer's instructions.

Flesh control QTL detection and integration of previous QTL. In order to ensure the fruits were ripe, we divided the RIL population into early-maturing, mid-maturing and late-maturing subgroups according to previous maturity data of each RIL line. The early-maturing and mid-maturing fruits were harvested 30 and 35 days after manual pollination, respectively. The late-maturing citron-type fruits were harvested 40-45 days after pollination to ensure the ripeness. Each fruit was cut lengthwise and immediately classified for flesh color. The flesh color of RILs were calculated as 1-4 grade, white 1, white and yellow 2, yellow 3, red 4. For linkage analysis the flesh color loci, the Join Map program version 4.0 was used. FCD QTLs were integrated into the map within the marker intervals according to the location presented in the original publications (Ren et al., 2012).

Population construct for fine mapping of Wf and B loci. To identify the *Wf* gene, three white flesh RILs with *WfWfbb* loci

with were crossed with line 97103. F_2 progeny resulted of these BILs (*Wfwfbb*) lines with the white flesh phenotype were used to fine map the *Wf* locus.

To identify the *B* gene, the yellow flesh RILs with *wfwfBB* loci with was crossed with line 97103. F2 progeny resulted of this BILs (*wfwfBb*) lines with the red flesh phenotype were used to identify the B locus.

Marker designer. Sequence-tagged site markers were designed based on the SNP and Indel between sequences of 97103 and PI296341-FR re-sequencing data. The BVWS markers were listed in the referenced paper (Ren et al., 2012). The new designer markers were named according to their physical positions.

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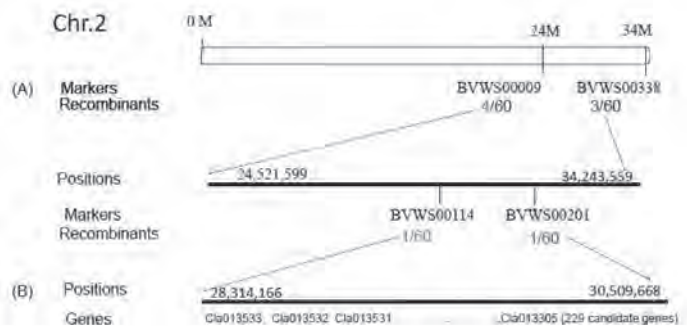

Fig. 3. High-resolution mapping of the *Wf* gene. (A) The FCDa locus on chromosome 2 mapped in crosses between 97103 and PI 296341-FR. The locus lies close between the Marker BVWS00114 and BVWS00201 in chromosome 2, and the position was localized between Chr.2: 28,314,166…30,509,668. (B) Location of 229 putative ORFs in the 2200-kb region spanning two crossovers on either side of the *Wf* gene. The numbers indicate the number of recombinants. The presumed ORFs were predicted using Cucurbit Genomics Database.

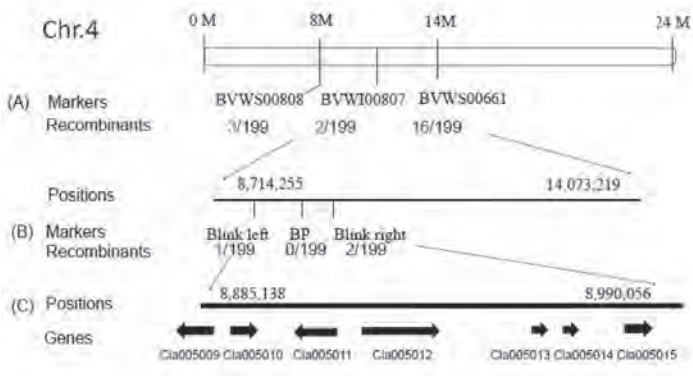

Fig. 4. Map-based cloning of the *B* gene in 97103. **(A)** The B locus was mapped in the chromosome 4 between markers BVMS00807 and BVWS00661.The numerals indicate the number of recombinants identified from 166 F2 red flesh plants. **(B)** Fine-mapping of D27 with markers developed based on the Indel between 97013 and PI296341-FR. The B gene was narrowed to an 105-kb genomic DNA region between markers Blink left and Blink right a and cosegregated with marker BP. **(C)** Detailed view of ORFs within the 105-kb mapping interval showing the candidate 7 ORFs, The LYCB-like Cla005011 (ORF3) is the candidate for B.

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Progress in Cucumber Molecular Breeding

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Additional index words. marker, resistance diseases, fruit quality-related genes, QTL, MAS

Abstract. Cucumber (*Cucumis sativus* L.) is the leading vegetable of protected cultivation in China. Recently we have made significant progress in gene mapping and Marker-Assisted Selection (MAS). Here we present the development and use of molecular markers for cucumber breeding in our lab.

In cucumber breeding, a large range of methods can be used to improve disease resistance, yield, fruit appearance, other fruit quality characteristics, and sex expression. The genome sequence of the cucumber provides an invaluable new resource for genetic improvement, basic biological research and breeding of cucurbits. We will present the development and use of molecular markers for cucumber breeding in our lab.

Resistance diseases gene mapping

The most effective way of plant protection is the production of cucumber hybrids with resistance to multiple diseases. Therefore, breeding of cucumber hybrids with a combination of multiple diseases is a major objective (Table 1).

Scab. Scab can cause serious losses for cucumber production, especially in protected culture such as high tunnel production. Resistance to cucumber scab is dominant and is controlled by a single gene, *Ccu*. The population included 148 individuals derived from the cucumber inbred line 9110 Gt crossed with line 9930. The *Ccu* gene was mapped Chr.2 of cucumber. The flanking markers SSR03084 and SSR17631 were linked to the *Ccu* gene with distances of 0.7 and 1.6 cM, respectively. The veracity of SSR03084 and SSR17631 was tested using 59 diverse inbred lines and hybrids, and the accuracy rate for the two markers was 98.3% (Zhang et al., 2010). Using 1,944 F_2 plants population, the two closest markers, Indel01 and Indel02, were 0.14 and 0.15 cM away from the target gene *Ccu*, respectively, and the physical distance between the two markers was approximately 140 kb. In this region that contained four nucleotide binding site (NBS)-type resistance gene analogs (RGAs)(Kang et al., 2010).

Powdery Mildew. The cucumber inbred lines K8 (resistant) and K18 (susceptible) were used to study the inheritance of resistance to powdery mildew. Four QTLs named *pm5.1*, *pm5.2*, *pm5.3* and *pm6.1* for the resistance gene to powdery mildew were detected in this study. The QTL of *pm5.2* located on Chr.5 was the major QTL. Four NBS resistance genes were found in the region of *pm5.2* (Zhang et al., 2011).

Downy Mildew. Five QTL for resistance to downy mildew were detected: *dm1.1, dm5.1, dm5.2, dm5.3* and *dm6.1*. Linked SSR markers for the five QTL were identified: SSR31116, SSR20705, SSR00772, SSR11012, SSR16882 and SSR16110. Six and four NBS-type RGAs were predicted in the region of *dm5.2* and *dm5.3*, respectively. (Zhang et al., 2013a).

Fusarium Wilt. A set of 148 F9 recombinant inbred lines (RILs) derived from the cross 9110Gt×9930 were used to study the inheritance of fusarium wilt resistance and detect quantitative trait locus (QTL) conferring the resistance in cucumber. The major QTL was placed in the region of SSR03084~SSR17631 within the genetic distance of 2.4cM on Chr.2. The validation of SSR17631 linked to *Foc* was tested using 46 diverse germplasms. The accuracy rate for SSR17631 selecting resistant materials was 87.88% and it could to be used to screen cucumber resources with fusarium wilt resistance in molecular marker-assisted selection (MAS) breeding (Zhang et al., 2014).

Target Leaf Spot. The cucumber inbred lines 931(susceptible) and hardwickii (resistant) were used to study the inheritance of resistance to cucumber target leaf spot. The resistance to cucumber target leaf spot is recessive and controlled by a single gene *cca-2*, and *cca-2* locus was mapped on Chr.6 The flanking markers SSR10954 and SSR16890 were linked to the *cca-2* gene with distances of 0.6 and 1.0 cM, respectively (Yang, 2012).

Gummy Stem Blight. Gummy stem blight resistances are controlled by different genes. We use different tissue to screen disease resistance: seedling, cut stem, leaves in vitro, adult plant. Four QTL for resistance to gummy stem blight were detected: *gsb3.1, gsb5.1, gsb5.2* and *gsb6.1* (Unpublished).

Watermelon Mosaic Virus. Inbred line 65G and 228 were used as the experiment materials for genetic analysis and gene mapping of watermelon mosaic virus resistance gene. Genetic analysis showed that the resistance to watermelon mosaic virus is recessive and controlled by a single gene *wmv*. *wmv* gene were mapped on Chr.6, the loci SSR10449 and SSR16005 were found to flank *wmv* locus (Zhou, 2012).

Cucumber fruit quality-related genes mapping

Cucumber fruit quality is a broad term and includes: flavor (bitterness), fruit visual appearance (e.g. size, shape, fruit skin color, spine color, flesh color), and so on. The development and use of molecular markers for cucumber fruit traits in our lab is depicted in Table 1.

Bitterness Gene Mapping. Bitterness in cucumber is also due to the presence of cucurbitacins and the trait has a complex inheritance mechanism. We were interested in studying the inheritance of cucumber fruit and foliage bitterness and to locate them on a current linkage map using a RILs population derived by crossing 9110Gt × 9930. It was concluded from the inheritance analysis that there were 2 loci controlling fruit bitterness in the population. One locus was in the same position as the location previously identified for *bi-1*, and another locus was for *bi-3*. The

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Table 1. Gene mapping results.

Traits	Gene	Chr.	Population	Reference
Scab	*Ccu*	2	9110Gt×9930	Zhang et al., 2010
Powdery mildew	*pm*	5,6	K8×K18	Zhang et al., 2011
Downy mildew	*dm*	1,5,6	K8×K18	Zhang et al., 2013a
Fusarium wilt	*Foc*	2	9110Gt×9930	Unpublished
Target leaf spot	*cca-2*	6	931× hardwickii	Yang, 2012
Gummy stem blight	*gsb*	3,5,6	931× hardwickii	Unpublished
Watermelon mosaic virus	*wmv*	6	65G×228	Zhou, 2012.
Bitterness	*bi-1, bi-3*	5,6	9110Gt×9930	Zhang et al., 2013b
	Bt-2	5	931× hardwickii	Unpublished
Glossy fruit skin	*G*	5	1101×1106	Dong et al., 2013
White fruit skin	*w*	3	1507×1508	Dong et al., 2012
Glabrous	*gl-2*	2	9110Gt×NCG-042	Yang et al., 2012
Black spine	*B*	4	GY14×NC76	Liu et al., 2013
Red mature fruit	*R*	4	NCG127×9930	Liu et al., 2014
Heavy netting mature fruit	*H*	5	PI205996×PI263079	Wang et al., 2014
Virescent leaf	*v-1*	6	9110Gt×9930	Miao Han., 2011
Plant height	*Ph*	1,2,5,6	9110Gt×9930	Miao et al., 2012a
Cotyledon length	*Cl*	1,3,5,6	9110Gt×9930	Miao et al., 2012b
Cotyledon width	*Cw*	1,3,5	9110Gt×9930	
Hypocotyl length	*Hl*	5,6	9110Gt×9930	
The first pistillate flower node	*Fpfn*	3,6	9110Gt×9930	
Days to anthesis	*Da*	1	9110Gt×9930	

locus of *bi-1* was located on Chr.6. The locus of *bi-3* was on Chr.5 (Zhang et al., 2013b). Based on PI183967×931 population the *Bt-2* gene was fine-mapped between 2 SNP markers on Chr.5, at a physical distance of 304.9 kb, containing 41 candidate genes. (unpublished).

Visual Appearance. The appearance of vegetables is a primary criterion in making purchasing decisions. Therefore, breeding of cucumber with good appearance traits is a major objective in our lab.

Glossy fruit skin is one of the highly valuable appearance quality traits related to the market values of cucumber. In previous studies the glossy fruit skin gene control by single recessive gene *d*, but in this study, we found it is controlled by single dominant gene. And *G* gene was mapped to Chr.5 of cucumber. The flanking markers with CS28 and SSR15818, genetic distances of 2.0 and 6.4cM, respectively (Dong et al., 2013).

Inbred line 1507(dark green fruit skin) and 1508(white fruit skin)were used as the experiment materials for genetic analysis and gene mapping of white fruit skin in cucumber. Genetic analysis showed that a single recessive nuclear gene *w*, and *w* was mapped to Chr.3 of cucumber. The flanking markers SSR23517 and SSR23141 were linked to the *w* gene (Dong *et al.,* 2012).

The genetic analysis showed having trichomes or not is determined by a single nuclear gene. *gl-2* gene was mapped to a linkage group with 11 SSR makers corresponding to Chr.2. The flanking markers SSR10522 and SSR132751 were linked to the *gl-2* gene with genetic distances of 0.6 and 3.8 cM, respectively. This two markers were tested using BC1P2 population, and the accuracy rate was 94.4 % and 91.6 % (Yang et al., 2011).

Cucumber inbred lines GY14 with white fruit spines and NC76 with black fruit spines were used as the experiment materials for genetic analysis and gene mapping for black fruit spine. The *B* gene were mapped on Chr.4 of cucumber. The two closest flanking markers SSRB-181 and SSRB-130 were 2.0 and 1.6 cM, respectively. Two flanking markers to predict the spine color for marker-assisted selection (MAS) breeding was 96.8 %and 96.2 % (Liu et al., 2013).

Genetic analysis and gene mapping were carried out on red mature fruit in cucumber using NCG127 (red mature fruit) and 9930 (yellow mature fruit) as experimental materials. The results showed that a single dominant nuclear gene *R*. The *R* gene was mapped to a linkage group corresponding to Chr.4 of cucumber. The flanking markers UW019319 and UW019203 were linked to the *R* gene with genetic distances of 0.8 and 0.7 cM, respectively (Liu et al., 2014).

Cucumber inbred lines PI205996 without heavy netting mature fruit and PI263079 with heavy netting mature fruit were used as the experiment materials for genetic analysis and gene mapping. The heavy netting of mature fruit trait of PI263079 was controlled by one dominant nuclear gene (*H*). It was located on the Chr.5 of cucumber delimited in a physical distance of 297.7 Kb. Flanking markers SSR13006 and SSR-90 were 3.6 and 1.7 cM away from the *H* gene, respectively (Wang et al., 2014).

Other traits

Virescent Leaf Gene Mapping. Based on the cucumber genome sequence, new markers were developed to fine mapping *v-1* gene. Then *v-1* gene was fine mapped to Chr.6 between SSR18405 and CAPS15170-4 markers, with genetic distances of 0.27cM and 0.15cM, respectively. In this region identified 5 candidate genes (Miao, 2010).

Plant Height QTL Mapping. Phenotypic data of 148 RILs, which originated from 9110Gt ×9930, were investigated four times in different seasons. 11 QTLs were detected for three plant height-associated traits. These QTLs were mapped on Chr.1, 2, 5 and 6, respectively. Five QTLs explained phenotypic variation more than 10 %. Three QTLs (27.3 %) were found to be expressed consistently under four cropping seasons in greenhouse cultivation. QTL cluster was detected on Chr.1.Compared with previous map, *de* gene controlling determinate habit in cucumber was found

in the region of QTLs mapped to the long-arm of Chr.6. Based on the results in this study, we speculated there are at least four genes controlling plant height (Miao et al., 2012a).

Seedling Traits QTL Mapping. 19 QTLs were detected for five traits: cotyledon length (*Cl*), cotyledon width(*Cw*), hypocotyl length(*Hl*), the first pistillate flower bearing node (*Fp fn*), days to anthesis(*Da*). These QTLs were mapped on Chr.1, 3, 5, and 6, respectively. 17 QTLs (89.5 %) were major QTLs ,which explained more than 10% of the phenotypic variation. Three QTLs were repeatedly detected in different seasons in greenhouse cultivation. Their LOD values varied between 3.28 ~ 15.25, which explained 6.0 % ~ 37.8 % of the phenotypic variation (Miao et al., 2012b).

According this result, we can know the location of genes on chromosomes, and using this information for fine mapping and clone gene. Mapping information from these studies opens the way for marker-assisted selection of these horticultural important genes in cucumber, and this result will promote the process of cultivated cucumber breeding.

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Selection of Multiple Disease Resistant Genotypes of *Cucumis melo* L.

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Additional index words. soil-borne diseases, *Fusarium oxysporum* f. sp. *melonis, Monosporascus cannonballus,* powdery mildew

Abstract. This study was carried out to select melon genotypes with resistance against Fusarium wilt, Monosporascus root rot and powdery mildew. Plants of 150 melon accessions were collected and evaluated in soil artificially infested with *Fom* race 1 which was isolated from diseased melon plants grown in Suwon, Korea. The fourteen genotypes exhibited significant resistance against *Fom* race 1. Screening of melon genotypes with resistance to *M. cannonballus* and powdery mildew was conducted under infested greenhouse. Thirteen melon germplasm and sixteen F_1 hybrids between melon genotypes were used in the study to select melon with resistance to *Monosporascus cannonballus* and powdery mildew. The four genotypes and three F_1 hybrids exhibited resistance against *M. cannonballus* and only one accession, PI 414723 showed high resistance to powdery mildew. The four accessions namely, 'Tuyona', 'Saxovot', 'Wondae', and 'PI 414723' were selected to serve as rootstocks with resistance to *Fom* race 1 and against *M. cannonballus*. PI 414723 was reported to have resistance to powdery mildew, ZYMV, CABYV, *Aphis gossypii* Glover, Fusarium wilt, and Papaya Ringspot Potyvirus (PRSV). In this study, PI 414723 also exhibited highest level of resistance to *M. cannonballus.* The selected melon genotypes with resistance to *Fusarium* wilt and *Monosporascus* root rot are considered to be potential and valuable source in breeding of melon rootstocks.

Soil-borne diseases aggravated by continuous cropping cause severe yield loss in melon cultivation of the major production area in Korea.

The soil-borne pathogen *Fusarium oxysporum* f. sp. *melonis* (*Fom*) is believed to be the most destructive pathogen of melon (Lee 1994, Oda 1995). *Fom* attacks melon at any growth stage, even before sprouting, but mainly when the fruit is ripe (Mas et al. 1981). This causes either slow wilting accompanied by progressive yellowing, or sudden wilting without prior yellowing. Other related symptoms may also appear. Based on the host resistance genes associated with variants of this pathogen, Fom isolates were classified into four physiological races designated 0, 1, 2, and 1,2 (Risser et al., 1976).

M. cannonballus was reported to induce sudden wilting in melon which also has become a major production problem worldwide (Martyn & Miller 1996). Root infection and damage occurs at all developmental stages, but increase in water demand during fruit development and maturation can lead to vine collapse due to loss of water-uptake capacity (Martyn and Miller, 1996).

Powdery mildew which is caused by the fungi *Erysiphe cichoracearum* and *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*) occur as a white powdery growth on the upper surfaces of leaves and on the stems of infected plants. Fruits are usually not directly affected, but their size and growth may be stunted.

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Materials and Methods

The experiment was conducted at the National Institute of Horticultural & Herbal Science in Suwon, Korea (37°18'23"N, 126 58'40"E).

Plants of 150 melon accessions were evaluated in soil artificially infested with *Fom* race 1 which was isolated from diseased melon plants grown in Suwon, Korea. Isolates of *Fom* 1 were grown on potato dextrose agar (Difco) in plastic petri dishes of 5.5 cm diameter at 25 °C for 8 days. Sterile distilled water (5 ml) was added to each petri dish, and colonies were dislodged using a sterilized glass rod. The suspension was filtered through two layers of sterile cheesecloth and the conidial suspension was adjusted to 1×10^6 conidia mL^{-1} using a hemacytometer. Roots of 20-day-old plants were dipped into 1×10^6 conidia/mL for one minute and then transplanted into pots. Disease ratings were recorded three weeks after inoculation.

Screening of melon germplasm with resistance to monosporascus root rot and powdery mildew was conducted under infested greenhouse conditions. Thirteen melon lines and sixteen F_1 hybrids between melon genotypes were used in the study. Melon plants were transplanted (18 July 2013) in a randomized complete-block design using three replications. Each bed was 190 cm long and intra-row spacing was set at 45cm.

Results and Discussions

Out of a total of 150 melon genotypes screened, 14 exhibited significant resistance to *Fom* race 1 (Table 1). Among the 24 melon lines and 16 F_1 hybrids between melon genotypes, PI 414723 showed high resistance, four genotypes and three F_1 hybrids

exhibited moderate resistance against *M. cannonballus* and only one accession, PI 414723 showed high resistance to powdery mildew**.** The four accessions 'Tuyona', 'Saxovot', 'Wondae' and 'PI 414723' were selected to serve as genotypes with resistance to *Fom* race 1 and *M. cannonballus* (Table 2). PI 414723 was reported to have resistance to powdery mildew, ZYMV, CABYV, *Aphis gossypii* Glover, Fusarium wilt and Papaya Ringspot Potyvirus (PRSV). In this study, PI 414723 also exhibited the highest level of resistance to *M. cannonballus*.

The selected melon genotypes with resistance to *Fusarium* wilt, *Monosporascus* root rot and powdery mildew are considered to be a potentially valuable source for breeding of melon rootstocks. Furthering breeding programs by combining the desirable characteristics such as disease resistance, cold and salt tolerance would address the needs of new hybrid cultivar melon production under greenhouse conditions.

Table 1. Disease severity of major melon genotypes against *Fusarium oxysporum* f. sp. *melonis* race 1.

Accession	Disease severity[z]	Accession	Disease severity
K145188	HR	IT190391	MR
K145216	HR	IT190845	MR
Wondae	HR	IT190934	MR
PI 414723	HR	IT191010	MR
Tuyona	HR	IT224900	MR
Saxovot	HR	IT235425	MR
Akbal	HR	IT207200	SR
Hasanbey	HR	IT119799	S
Charantais Fom-2	HR	Daejeong	S
IT138001	HR	Earlselite	S
IT138010	HR	Homrunstar	S
IT190255	HR	Oltin Tepa	S
IT190256	HR	Charantais T	S
IT190325	HR	Charantais Fom-1	S
IT190182	MR	Powerking	S
IT190344	MR	Vedrantais	S

[z]Disease severity: HR = highly resistant, MR = moderately resistant, SR = slightly resistant, S = susceptible.

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Table 2. Disease severity of melon genotypes against *M. cannonballus* and powdery mildew.

Accession	Disease severity[z]		Accession	Disease severity	
	M. cannonballus	Powdery mildew		*M. cannonballus*	Powdery mildew
PI 414723	HR	HR	PI 414723 × Saxovot	SR	S
Daejeong	MR	S	K145188 × Daejeong	SR	S
Oltin Tepa	MR	S	K145216 × Daejeong	SR	S
Saxovot	MR	S	K145188 × Tuyona	SR	S
Tuyona	MR	S	K145188 × Tuyona	SR	S
Wondae	SR	S	Tuyona × K145216	SR	S
K145188	SR	S	Wondae × Tuyona	SR	S
K145216	S	S	K145188 × Oltin Tepa	SR	S
Hasanbey	S	S	K145216 × Oltin Tepa	SR	S
Gaegurichamoe	S	S	Wondae × Oltin Tepa	SR	S
Earlselite	S	S	Wondae × Saxovot	SR	S
Homrunstar	S	S	Saxovot × Oltin Tepa	S	S
PI 414723 × Daejeong	MR	S	Saxovot × K145188	S	S
PI 414723 × Tuyona	MR	SR	K145216 × Saxovot	S	S
PI 414723 × Oltin Tepa	MR	S			

[z]Disease severity: HR = highly resistant, MR = moderately resistant, SR = slightly resistant, S = susceptible.

Advances in Biotechnical Approaches for Breeding of Pumpkins (*Cucurbita pepo* L.)

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Additional index words. Styrian oil pumpkin, zucchini, haploid induction, X-ray irradiation, adventitious regeneration, endopolyploidy, genome doubling

Abstract. To enhance breeding of cultivated pumpkins, two biotechnological approaches were tested on two types of *Cucurbita pepo* L.: the styrian oil pumpkin and the zucchini type. The first approach was focused on the development of a parthenogenetic haploid induction protocol based on pollination with X-ray irradiated pollen. Several factors affecting haploid production were tested such as genotype of mother plants and pollen donor, irradiation dose and season. All factors had a profound effect on fruit set, embryo formation and haploid embryo regeneration. A limited number of haploids were obtained along with diploid, triploid and tetraploid plants. Due to a relatively low frequency of haploid embryos and the heterozygous status of diploid regenerants (revealed by SSR marker analysis) experiments were further focused on the application of higher irradiation doses. In order to maintain pollen germinability after prolonged irradiation, exposure of pollen to high air humidity during irradiation was tested. Positive effects were observed therefore the method was used in subsequent experiments. Additionally, the application of plant growth regulators (4-CPPU, 2,4-D) in weekly intervals after pollination was tested. In the second approach the improvement of tolerance to fusarium rot was attempted using fusaric acid (FA) as a selective agent during in vitro adventitious regeneration from cotyledonary explants. Surprisingly, FA at low concentrations (5 mg/L) stimulated regeneration and at medium concentration (10 and 20 mg/L) induced genome doubling. Moreover, lower endopolyploidy status of explant tissues was shown to correlate with higher regeneration ability.

Among the cultivated species of the genus *Cucurbita*, *Cucurbita pepo* L. is the economically most important. It is native to North America but nowadays grown worldwide and characterized by high polymorphism of plant and fruit characteristics. Fruits are used as vegetables for human consumption or as livestock fodder whiles the seeds are processed to extract oil. Based on fruit shapes, it is divided in eight edible-fruited groups such as Acorn, Scallop, Crookneck, Straightneck, Pumpkin, Vegetable Marrow, Cocozelle, and Zucchini.

The aim of our work was to develop new biotechnological approaches to enhance breeding of styrian oil pumpkin and zucchini. The first approach was focused on haploid induction for the production of homozygous lines which would be further used for breeding of hybrid cultivars. The second approach was based on induced somaclonal variation through tissue culture procedures with fusaric acid as a selective agent.

Materials and Methods

Haploid induction. Fifteen *C. pepo* (11 hull-less accessions, 4 zucchini cultivars) and one *C. moschata* accession were used for haploid induction experiments conducted in spring and summer seasons from 2011 to 2013. Plants were grown in the greenhouse and open-field using standard agronomic practices. Male and female flowers were isolated 1 day prior to anthesis to avoid undesirable crosses. The next morning the anthers were collected, placed in petri dishes, and irradiated using an X-ray unit (RX-650; Faxitron Bioptics). Female flowers were pollinated immediately after irradiation and re-isolated (Košmrlj et al., 2013).

Fruits were harvested 4 weeks after pollination. Seeds were extracted aseptically in a laminar flow and rescued embryos were cultured on solid E20A medium (Sauton and Dumax de Vaulx, 1987) at 23 °C with a 16-hour photoperiod. The ploidy level of regenerated plantlets was determined by flow cytometry using DAPI staining according to Bohanec (2003). To test possible homozygosity of diploid regenerants caused by spontaneous chromosome doubling, published SSR markers (Gong et al., 2008) and a touchdown PCR protocol (Formisano et al., 2012) were used.

Due to relatively low haploid induction rates, further steps were taken to improve the efficiency of the induction protocol described above. Styrian oil pumpkin hybrid 'GL Opal' pollen was used to evaluate the effect of higher relative humidity during irradiation on germinability. The germination tests were conducted with pollen from anthers collected at anthesis. To achieve high humidity (HH) conditions, the petri dishes containing pollen were placed in larger petri dishes filled with water. The germinability was assessed with hanging drop culture in liquid Brewbaker and Kwack (1963) medium optimized for styrian oil pumpkin pollen; hereafter referred to as BK. The pollen was allowed to germinate at 23 °C in the dark for 2–3 hours. In addition, the diameter of pollen grains was measured using Lucia Cytogenetics 2 software (version 2.3; Laboratory Imaging, Czech Republic). A detailed description of the protocol is given in Košmrlj et al. (in press). This improved protocol was used in the following haploid induction experiments with styrian oil pumpkin and zucchini cultivars.

Acknowledgements. The authors wish to thank Viktorija Dolenc, Damijana Kastelec, Aleš Kladnik, and Shoko Kojima for their assistance in the study. The study was financed by the Slovenian Research Agency, grant numbers P4-0077 and 1000-10-310191, and Ministry of Agriculture and the Environment project "Breeding of oil-seed pumpkins" [V4-1116 (C)].

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Endopolyploidy analysis, adventitious regeneration, and genome doubling. Samples of different plant organs (leaf, hypocotyl, epicotyl, cotyledon, and root) of 1 month old 'GL Opal' greenhouse-grown plants were collected and analyzed by flow cytometry using DAPI staining (Bohanec, 2003) in order to reveal differences in the endopolyploidy pattern.

For analysis of endopolyploidy and adventitious shoot regeneration, seeds of styrian oil pumpkin 'GL Opal' and four zucchini cultivars were sterilized and allowed to germinate on MS medium (Murashige and Skoog, 1962) including vitamins (Duchefa Biochemie B. V.) supplemented with 30 g/L sucrose and 8 g/L agar, with pH adjusted to 5.8 before autoclaving. The endopolyploidy in different sections of 7-day old 'GL Opal' cotyledons was analyzed by DNA image cytometry according to Vilhar et al. (2001). Samples were prepared and their DNA content was measured by the protocol of Dolenc Koce et al. (2003).

Basal cotyledonary explants of the same age were inoculated on MS medium containing different concentrations of growth regulators (6-benzylaminopurine (BA), zeatin, meta-topolin (mT), and N6-[2-Isopentyl]adenine (2iP)), para-aminobenzoic acid (PABA) and FA, 30 g/L sucrose and solidified with 8 g/L agar (pH 5.8). Cotyledonary cultures were kept at 23 ± 1 °C and 16-h photoperiod for 3 to 4 weeks. Regenerated shoots were subcultured on MS medium containing 0.1 mg/L BA and kept under the aforementioned conditions before they were subjected to further analyses. Ploidy level of regenerants was analyzed with flow cytometry and DAPI staining according to Bohanec (2003).

Results and Discussion

Haploid induction in styrian oil pumpkin. The major aim of our study was the development of a parthenogenetic haploid induction protocol based on pollination with X-ray irradiated pollen (0, 50, 100, 150, 200, 300, and 350 Gy). We tested several factors known to affect haploid production such as genotype of female parent and pollen donor, irradiation dose, and season. Fruit set largely decreased at 200 Gy, whereas a decline in embryo formation was observed at 100 Gy. Although increased X-ray doses affected the formation of embryos, some were formed even at the highest tested irradiation treatment (350 Gy). These findings are in contrast with published results in *C. pepo* (Kurtar et al., 2002), in which fruit set was achieved at gamma ray doses up to 400 Gy, but no embryos were formed at doses > 50 Gy. Among nine tested female parents large differences were found. The best parthenogenetic response was found in 'Turkey #2' (10.0%), 'Gleisdorfer Ölkürbis' (4.4%), and 'Naked Seed' (3.9%), whereas 'Slovenska Golica' remained unresponsive. However, the sample number in 'SlovenskaGolica' was low as a consequence of contamination in tissue culture. In 2011, most accessions tested as female parents responded positively to haploid induction by induced parthenogenesis, whereas in 2012, haploid induction rates were generally lower. This variation between years can probably be attributed to weather conditions. 'GL Opal' and 'White Acorn' were efficient when used as pollen donor among six accessions tested. Generally, the highest frequency of haploids was observed when pollen irradiated at 200 and 300 Gy was used for pollination. In total, 3830 putatively partenogenic embryos were subjected to ploidy analysis and four ploidy levels (n, 2n, 3n, 4n) were detected with the majority being diploid. The unexpected determination of tetraploids clearly correlated with increased dose delivered to pollen grains and so far we were unable to find similar reports in studies of parthenogenesis. In agreement with many gynogenic haploid induction studies (reviewed by Bohanec 2009) no spontaneous chromosome doubling could be confirmed among analyzed regenerants. However, our results show that the use of SSR markers is efficient since the majority (96.8%) of heterozygotes was discriminated by the first two loci tested. Moreover, three loci were sufficient to confirm the heterozygocity and consequently zygotic status of 99.6% regenerants.

Optimization of haploid induction. As the observed haploid frequencies were relatively low and no spontaneously doubled haploids were obtained we focused on the application of higher irradiation doses (> 350 Gy). Based on the Brewbaker and Kwack (1963) protocol we optimized the germination method for styrian oil pumpkin pollen, which is known to have short longevity even under natural conditions (Nepi et al., 2010). Therefore it was crucial to ensure optimal conditions during irradiation and subsequent germination. For germination tests different pH values and the addition of sucrose, mannitol, and polyethylene glycol to the BK germination medium were tested. The optimal medium condition was pH 9 and 12.5% (w/v) sucrose, while other tested components were not effective. In order to maintain pollen germinability after prolonged irradiation, exposure of pollen to HH during irradiation was tested and compared to irradiation at room humidity (RH). It was found that HH significantly improved germination at doses ≥ 350 Gy. Pollen diameter measurements revealed a major variability in pollen size (diameters ranged from 79.2 to 196.5 µm) and 2 subgroups in the pollen population. Following irradiation, HH conditions allowed germination of larger pollen grains compared to the nonirradiated control and RH, which contradics the finding of Brewbaker and Emery (1962) that larger pollen exhibits greater radiosensitivity.

After a comparison with the results obtained with irradiation under RH conditions (Košmrlj et al., 2013) we expected embryos to be formed after pollination with pollen irradiated with up to 600 Gy using HH, whereas only doses up to 500 Gy seem feasible with RH. The assumption was tested and confirmed in 2013 on three cultivars of styrian oil pumpkin and four zucchini cultivars. Fruits, diploid embryos and haploid embryos were obtained from flowers pollinated with pollen irradiated with doses up to 600 Gy. The persistence and further growth of obtained fruits was stimulated by the application of plant growth regulators (4-CPPU or 2,4-D) in weekly intervals after pollination. Some of the results are presented in Table 1.

After irradiation with higher doses higher yields of haploids were expected based on literature data where relatively more haploids (Chalak and Legave, 1997; Grouh et al., 2011), or even exclusively haploids (Sauton and Dumas de Vaulx, 1987), were obtained by pollination with pollen irradiated at higher doses. In our experiments the higher irradiation doses caused a substantial increase

Table 1. Results obtained in haploid induction experiments performed on four zucchini type cultivars (Bianca di Trieste, Elite, Greyzini, Nano verde di Milano). 97 fruits were obtained from 153 flowers pollinated with pollen irradiated with doses from 300 Gy to 600 Gy and two haploid embryos were regenerated.

Irradiation dose (Gy)	No. of pollinated flowers	No. (%) of fruits obtained	No. of embryos obtained from 100 seeds
300	23	13 (56.5)	1.28
400	19	13 (68.4)	0.33
500	81	51 (63.0)	0.05
600	30	24 (80.0)	0.34

in the number of empty seeds per fruit compared to lower doses. Although the number of haploids per pollinated fruit remained low, the percentage of haploids per rescued embryo increased.

Endopolyploidy pattern and adventitious regeneration in styrian oil pumpkin. A high level of endopolyploidy has been reported in the family Cucurbitaceae (Barow and Meister, 2003). Furthermore, it has been shown to be organ specific. Our results confirm both as levels up to 64C in studied organs of styrian oil pumpkin plants have been observed. The lowest endopolyploidy extent has been found in the leaves, whereas the hypocotyl, epicotyl, and cotyledon were identified as the most endoreduplicated organs. In cucumber, endopolyploidy has been correlated with the adventitious shoot regeneration ability (Colijn-Hooymans et al., 1994). Furthermore, in *Cucurbita* spp., the basal part has been reported to be the most responsive for adventitious shoot regeneration (Ananthakrishnan et al., 2003; Lee et al., 2003; Kathiravan et al., 2006; Zhang et al., 2008; Kim et al., 2010) and our results show that this section contains a majority of putatively mitotically active cells (2C and 4C). We can therefore conclude that a lower endopolyploidy extent in cotyledons correlates with higher regeneration ability. Further studies of the endoreduplication pattern through developmental stages of plants are needed as explant age is also reported to be one of the main factors for adventitious shoot regeneration in *Cucurbita* spp.

After subjecting basal cotyledonary explants to various BA-based media, 2iP, PABA, and FA were found most effective in stimulating adventitious regeneration, while mT was the least effective. All regenerants from cytokinin-based media were diploid. Surprisingly, FA, added to media as a possible selective agent inducing increased tolerance to *Fusarium*, not only promoted regeneration at low concentrations (5 mg/L) but also induced genome doubling when medium concentrations (10 and 20 mg/L) were used. Doubling rates were comparable to those of traditional antimitotic treatments in related species (Košmrlj et al., in preparation).

*Adventitious shoot regeneration in zucchini. G*enome doubling was later confirmed on basal cotyledonary explants of three zucchini cultivars cultured on MS medium, 1 mg/L BA, 0.25 mg/L PABA or 0.5 mg/L 2iP, 30 g/L sucrose and 8 g/L agar (pH 5.8) supplemented with 10 mg/L FA. Among 182 regenerated plants, 2 were tetraploid and the other diploid. We therefore believe that FA can serve as an alternative to traditional antimitotic treatments for genome doubling in *C. pepo* and perhaps other species of the this genus. However, further studies are needed to clarify the involvement of FA in genome doubling and its efficiency across other varieties and species.

Modern biotechnological approaches complement traditional breeding methods in developing new varieties. They can accelerate the process and can enable development of new traits. Our results demonstrate the possibility of homozygous line production through pollination with X-ray irradiated pollen and subsequent *in vitro* embryo rescue for styrian oil pumpkin and zucchini. Moreover, our results of *in vitro* culture of cotyledonary explants on FA supplemented media present a novel approach of polyploidization through adventitious regeneration. Furthermore, future experiments are planned to evaluate tolerance of obtained regenerants to *Fusarium*.

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Cucurbit Germplasm Collections at the North Central Regional Plant Introduction Station

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Additional index words. Cucumis, Cucurbita pepo, germplasm resources and conservation

Abstract. The North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa, USA is one of four primary Plant Introduction Stations in the National Plant Germplasm System (NPGS), and has responsibility for maintenance, regeneration, characterization, and distribution of the *Cucumis* and *Cucurbita pepo* collections in the NPGS. There are currently 4911 *Cucumis* (e.g. cucumber and melon) and 974 *Cucurbita pepo* (e.g. squash, pumpkin and gourd) accessions in the collections representing 42 taxa from 100 countries. The germplasm is maintained as seed and 4885 accessions are backed up at the National Center for Germplasm Resources Preservation in Fort Collins, CO. Characterization and evaluation data associated with the collection are available through the Germplasm Resources Information Network (GRIN) at http://www.ars-grin.gov/npgs/. Small seed samples are available free of charge to research and education entities world-wide when genetic diversity or genetic standards are a requirement. Collection diversity and management activities will be described.

The United States government, recognizing the importance of non-native plant species for food and agriculture, established four regional Plant Introduction Stations as part of the NPGS to ensure the preservation of plant materials brought into the country. Germplasm is acquired through NPGS-sponsored collection trips, exchange with other genebanks, and through donations from retired breeders or discontinued breeding programs. The NCRPIS has maintained the *Cucumis sativus* and *Cucurbita pepo* collections since the station was originally established in 1948. The *Cucumis* collection tripled in size in 1987 when 2068 accessions of *Cucumis melo* and 203 accessions of related wild *Cucumis* species were transferred from the Southern PI Station in Griffin, Georgia. The cucurbit collections experienced additional growth in 1987 as a result of a collection in Spain (474 *Cucumis* and 115 *Cucurbita pepo* accessions) sponsored in part by the International Board of Plant Genetic Resources (IBPGR) and the Food and Agriculture Organization (FAO); and in the mid- to late-1990s from USA-sponsored collection trips in India (638 accessions), China (104 accessions), and South Africa (57 wild *C. spp.* accessions). The most recent acquisitions were 73 melon accessions from a 2008 USA-sponsored collection trip in Turkmenistan.

Collection diversity. We currently conserve 42 taxa (Table 1) in the 4911 *Cucumis* and 974 *Cucurbita* accessions representing 100 countries (Table 2) with large numbers of accessions originating from Afghanistan (400), China (319), India (1057), Iran (379), Spain (589), and Turkey (700). The germplasm is classified as wild (8 %), cultivar or cultivated (25 %), or landrace (63 %) in nature with approximately four percent of the collection designated as undetermined improvement status. The cucurbits maintained at the NCRPIS are primarily annual but some species are perennial such as *Cucumis heptadactylus* (Fig. 1) which is also dioecious. Plant habit range from small to very large bush, semi-bush, and vines with an extreme diversity of fruits of highly variable sizes, shapes, and colors. The *Cucurbita* collection includes the hull-less seeded Styrian pumpkins used for oil production and confectionary purposes as well as the more commonly known vegetable garden and ornamental gourd type fruits. *Cucumis* fruits range from the spine-covered wild types to the commonly known cultivated dessert and vegetable types found in markets around the world (Fig. 2 and Fig. 3).

Regeneration and maintenance. Our station has a long history of a well-established cage and insect pollinator regeneration program (Clark et al. 1991; Ellis et al. 1981). Field regenerations are done in screened cages (Fig. 4), each supplied with an individual nucleus hive of honey bees. The insect pollinator program relied primarily on honey bees when it was initiated, but other bees such as alfalfa leaf cutting bees (ALC) and bumblebees have been added to address regeneration challenges encountered with the diverse germplasm maintained at the station. The small, stingless ALC (Fig. 5) have proven highly effective pollinators of *Cucumis* in small greenhouse cages, and are also used in field cages early in the season when few female flowers are present. Bumblebee colonies are used in *Cucurbita* cages when available.

Seeds of *Cucumis* and *Cucurbita pepo* are started in the greenhouse in early May, and all seedlings are tested by ELISA for seed-borne squash mosaic virus before transplanting into field cages in mid- to late May.

For *Cucumis*, eight hills (three to five plants each) are transplanted 60 cm apart in the center of a 2 × 6 m area over which a 2-m tall cage frame has been assembled. *Cucurbita pepo* seedlings are transplanted as eight hills (three to five plants each) into cages 4.5 × 12 × 1.5 m. The cage frames are immediately covered

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Table 1. Taxa maintained at the NCRPIS.

Taxon	Number of Accessions	Number of Available Accessions
Cucumis africanus	48	17
Cucumis anguria	48	39
Cucumis anguria var. anguria	10	5
Cucumis anguria var. longaculeatus	22	19
Cucumis asper	1	1
Cucumis dipsaceus	6	6
Cucumis ficifolius	7	7
Cucumis heptadactylus	1	1
Cucumis hirsutus	3	0
Cucumis meeusei	1	1
Cucumis melo	78	67
Cucumis melo subsp. *agrestis*	145	109
Cucumis melo subsp. *melo*	2865	1963
Cucumis melo var. *cantalupo*	17	8
Cucumis melo var. *chito*	1	1
Cucumis melo var. *conomon*	3	3
Cucumis melo var. *flexuosus*	47	31
Cucumis melo var. *inodorus*	3	3
Cucumis melo var. *texanus*	45	44
Cucumis metuliferus	47	46
Cucumis myriocarpus	20	9
Cucumis myriocarpus subsp. *leptodermis*	2	2
Cucumis myriocarpus subsp. *myriocarpus*	6	6
Cucumis prophetarum	3	2
Cucumis prophetarum subsp. *prophetarum*	1	1
Cucumis pustulatus	7	4
Cucumis sagittatus	4	3
Cucumis sativus	85	69
Cucumis sativus var. *hardwickii*	7	6
Cucumis sativus var. *sativus*	1291	1228
Cucumis sativus var. *sikkimensis*	2	2
Cucumis sativus var. *xishuangbannanesis*	1	0
Cucumis spp.	62	1
Cucumis subsericeus	1	1
Cucumis zambianus	12	11
Cucumis zeyheri	9	3
Cucurbita pepo	907	677
Cucurbita pepo subsp. fraterna	4	4
Cucurbita pepo subsp. ovifera	1	0
Cucurbita pepo subsp. pepo	3	1
Cucurbita pepo var. ozarkana	43	39
Cucurbita pepo var. texana	14	14

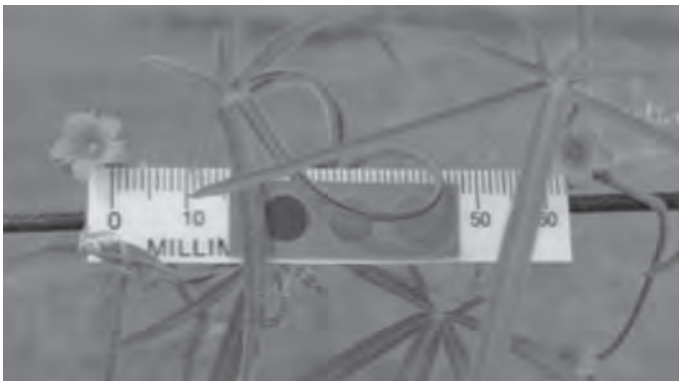

Fig. 1. *Cucumis heptadactylus* foliage and flower.

Fig. 2. *Cucumis zambianus* fruit.

with a lumite screen (mesh size 20 × 20) after transplanting, and plants are mulched with shredded paper to preserve moisture and reduce weed competition. The cages are necessary to facilitate controlled pollination. They provide an added benefit of protecting against bacterial wilt and squash mosaic virus infection by excluding cucumber beetle vectors. All seed increase cages are inspected for plant health multiple times during the growing season and fungicides are applied if needed to control diseases such as anthracnose and powdery mildew.

Insect pollinators are introduced into a cage when both male and female flowers are present.

A small queen-right colony of honey bees is placed inside each *Cucumis* cage as the vines grow and produce more flowers. In 2005, the field regenerations for *Cucurbita pepo* (squash, pumpkins, gourds) were transferred from open field growth with hand pollination to large cages. This eliminated much hand labor and the need for regular insecticide applications. Due to the large size of the cage and the dense canopy produced by the large vines, honey bee or bumblebee colonies are placed inside both ends

Fig. 3. *Cucumis melo* fruit diversity.

Table 2. NCRPIS Cucurbits country of origin.

Country	Accessions	Country	Accessions	Country	Accessions
Afghanistan	400	Georgia	11	Nigeria	4
Africa	2	Germany	12	Oman	10
Albania	10	Ghana	1	Pakistan	55
Algeria	4	Greece	29	Paraguay	1
Argentina	8	Guatemala	21	Peru	3
Armenia	4	Haiti	1	Philippines	5
Asia Minor	2	Hong Kong	4	Poland	32
Australia	7	Hungary	54	Portugal	1
Austria	3	India	1057	Puerto Rico	5
Belgium	3	Indonesia	5	Russian Fed.	51
Bhutan	6	Iran	379	Saudi Arabia	5
Bolivia	4	Iraq	12	Senegal	4
Botswana	15	Israel	42	Serbia	2
Brazil	8	Italy	3	South Africa	105
Bulgaria	2	Japan	101	Spain	589
Burundi	1	Kazakhstan	11	Sri Lanka	1
Canada	46	Kenya	5	Sweden	5
Chile	1	Korea, South	34	Switzerland	1
China	319	Lebanon	17	Syria	77
Colombia	3	Macedonia	112	Taiwan	17
Costa Rica	2	Malawi	2	Tajikistan	5
Cuba	2	Malaysia	2	Thailand	5
Cyprus	3	Maldives	18	Turkey	700
Czech Republic	16	Mali	2	Turkmenistan	77
Denmark	4	Mauritius	1	Ukraine	20
Dominican Repub.	1	Mexico	167	United Kingdom	15
Ecuador	5	Moldova	4	United States	323
Egypt	110	Morocco	6	Unknown	2
El Salvador	6	Myanmar	9	Uruguay	1
Ethiopia	17	Namibia	6	Uzbekistan	56
Finland	1	Nepal	14	Yemen	2
Former Serbia/Montenegro	184	Netherlands	62	Zambia	105
Former Soviet Union	50	New Zealand	3	Zimbabwe	99
France	32	Niger	1		

Fig. 4. *Cucumis* regeneration cage with honey bee colony.

Fig. 5. Alfalfa leaf cutting bees on *Cucumis* flower.

of *Cucurbita* cages. Insect pollinators remain in the field cages until early September. Fruits developing after early September generally do not mature before the first killing frost. In 2000, we developed small cages for greenhouse regenerations of *Cucumis* species. These cages are primarily used for seed increases of wild type species (e.g., *Cucumis anguria*, *C. heptadactylus, C. zambianus*), accessions with low seed numbers, and accessions that need a long growing season.

Multiple harvests are made as *Cucumis* fruits mature. Fruits are sliced, and seeds are extracted by hand and washed in a screened tray. Seeds are then floated in water to remove light, immature seeds and debris. *Cucurbita* fruits are harvested as they reach mature color or after a killing frost, and stored in a cold, dry room for one to three months to allow for after-ripening, which ensures a better quality seed. Fruits are split open with a hatchet, seeds are hand-extracted and washed in screened boxes to remove pulp. Cleaned *Cucumis* and *Cucurbita* seeds are dried in dryer carts at 30° to 34° C for 24 to 48 hours, and are then put through an air-screen cleaner to remove low-quality seeds and impurities. After germination tests, seeds are stored at 4° C and 28% RH.

When newly acquired germplasm is regenerated, specific characterization data are recorded to document an accession and the taxonomy is verified. We occasionally receive new germplasm that has been incorrectly identified and some that are identified only to genus. In one such instance, we found a previously undescribed species, *Cucumis zambianus* (Widrlechner et al., 2008), in the wild *Cucumis* collection. There are presently 63 accessions identified only as *Cucumis* spp. These are of interest to researchers for resistance evaluations, but they are often the most challenging in terms of seed production. Most of these are increased in the greenhouse.

Characterization and evaluation. Digital images and basic notes for taxonomic identification and accession characterization are taken from representative fruits of each accession before seeds are extracted. Data for approximately seventeen descriptors (primarily fruit descriptors) are recorded at harvest. These images and descriptor data are made available to researchers via the Germplasm Resources Information Network (GRIN) at www.ars-grin.gov. We collaborate with public and private researchers to (1) evaluate collections for disease and insect resistance, (2) to capture molecular characterization data, and (3) to obtain descriptor evaluation data. Evaluation data for pests and pathogen resistance and molecular characterization are often obtained through collaboration with public and private researchers who have specific expertise, interest, and resources to do the work.

Uses of germplasm. The types of seed requests received include breeding for novel traits; breeding for disease, insect, biotic and abiotic stress resistance; for genetic studies and molecular analysis; and for repatriation. The majority of the requests have been to identify new sources of resistance to pathogens (anthracnose, bacterial fruit blotch, cucurbit yellow stunting disorder virus, downy mildew, Fusarium wilt, gummy stem blight, powdery mildew, nematodes), and insects. One of the frequent themes for requests in recent years has been for nematode resistant germplasm. Several requests have focused on the nematode resistance reported in *Cucumis metuliferus* (Walters et al., 1993) and the potential use of this species and other wild species as rootstocks. Climate change is also playing a role in increased interest in germplasm, especially in regard to the wild crop relatives as potential sources of heat and drought resistance.

New sources of resistance to powdery mildew were identified in melon germplasm collected from India in 1992 (McCreight and Coffey, 2012). Similarly, an evaluation of 977 cucumber accessions for powdery mildew resistance (*Podosphaera xanthii*) identified 20 highly resistant accessions, with 18 of the 20 traceable to Asian backgrounds (Block and Reitsma, 2005). Host plant resistance to cucurbit yellow stunting disorder virus and feeding by sweet potato whitefly biotype B has been reported (McCreight et al., 2012) in melon accessions having more desirable fruit characteristics which may make them better donor parents for developing resistant dessert-type melons.

Distributions. The NPGS is a cooperative research organization and the germplasm collection, evaluation, and distribution systems are designed to serve research objectives. Available germplasm is freely distributed in small quantities to research and education entities when genetic diversity or genetic standards are required. NPGS germplasm is not distributed for home or personal use when those needs can be well-served by commercially-available varieties. The standard seed quantity for *Cucumis* and *Cucurbita pepo* is 50 seeds per accession. Requests can be submitted via Public GRIN at http://www.ars-grin.gov/npgs/index.html or by contacting the curator kathleen.reitsma@ars.usda.gov.

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Screening *Cucurbita pepo* Genetic Resources for Tolerance to Chilling Injury

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Additional index words. postharvest fruit quality, ethylene production, zucchini squash

Abstract. Tolerance to chilling injury (CI) is becoming ever more essential as a trait for export-oriented vegetable production requiring refrigerated transport and/or storage. With a view to incorporating this trait in the current breeding programs of zucchini, we have screened the *Cucurbita pepo* germplasm (groups zucchini, coccozelle and vegetable marrow) conserved in the Seed Bank of the University of Almería (BSUAL) for tolerance to chilling injury. The development of CI, fruit weight loss and ethylene production was assessed in the fruit of 13 landraces and 4 commercial hybrids stored over 14 days at 4 °C. The hybrids and most of the landraces were highly sensitive to cold storage, and at 7 days the damage to the fruits' surface had caused them to lose their market value. However, the CpCAL003 landrace incurred less CI and fruit weight loss, and so it was selected for analysis. Its tolerance to CI was confirmed in an additional trial the following year, and it is currently being studied to determine the inheritance of this trait. Fruit ethylene production was low at harvest but was induced in the fruits of all the analyzed accessions at 7 days of postharvest storage at 4 °C, after which it again fell to its lowest value after 14 days of storage. The level of this cold-induced ethylene at 7 days of postharvest storage varied among accessions, and it was found to be correlated with CI sensitivity, thus allowing it to be used as a marker to select CI tolerance in zucchini squash.

Chilling injury (CI) is a major physiological problem limiting the export and consumption of zucchini fruit. The postharvest storage of the fruit at 4ºC for a minimum of 7 days induces surface lesions or pitting, which reduces considerably the commercial value of the zucchini fruit in most of the currently used commercial cultivars (Carvajal et al, 2011; Megías et al, 2014).

The involvement of ethylene in CI is unclear. Many studies have indicated that ethylene or the ethylene inhibitor 1-MCP can either induce or alleviate CI symptoms in different fruits and vegetables (Sevillano et al., 2008). Nevertheless, ethylene is not always required for triggering CI. In kiwifruit, for example, CI symptoms occur before the production of ethylene. Moreover, although 1-MCP may reduce CI in many species, including persimmon, tangerine and loquat (Salvador et al., 2004 and 2006; Cai et al., 2006), in others like banana (Jiang et al., 2004) and nectarine (Dong et al., 2001) it is able to increase CI symptoms.

Zucchini is a physiologically immature fruit that produces low level of ethylene during its postharvest storage, but which is very susceptible to CI (Megías et al., 2014). Nevertheless, when the fruit is stored at 4 °C and then transferred to 20 °C for a minimum of 4h, ethylene is highly induced. This cold-induced ethylene is not necessary for triggering CI symptoms in zucchini squash, since they occur during the storage at 4 °C, before rewarming of the fruit and, therefore, before the burst of ethylene (Megías et al., 2014). We have found, however, that the level of cold-induced ethylene in zucchini is negatively correlated with CI. Therefore, the cold-induced ethylene is reduced in response to treatments that alleviate CI symptoms as well as in CI-tolerant cultivars.

The objective of this study was to identify genetic variability for the tolerance to CI in the immature fruit of *C. pepo*, and to determine the relationship between cold induced ethylene and the occurrence of CI.

Materials and Methods

We have studied the postharvest response of the immature fruit of 13 landraces and 4 commercial hybrids of *C. pepo* when stored at 4 °C for 14 days. All the landraces were from the Seed Bank of the University of Almería (BSUAL). The commercial hybrids 'Natura' and 'Sinatra', which were found to be more tolerant and sensitive, respectively, to CI in previous trials (Megías et al., 2014) were used as controls. The fruit used in this work was all harvested from plants growing in the same field trial under standard greenhouse conditions in Almeria, Spain. Fruits of uniform length (18-20 cm) were harvested and randomly divided in different lots before stored at 4 °C in controlled chambers for a total of 14 days. At days 7 and 14, 12 fruits per cultivar and storage period were transferred to room temperature (18 °C to 20 °C) for 6 hours before evaluating postharvest parameters and processing of the samples. For each variety, four replications of 3–4 fruits each were used for ethylene production, weight loss, and CI measurements. To assess CI, the fruit surface affected by pitting was used to classify each fruit as follows: 0 = no damage, 1 = 1 % to 5 % damage, 2 = 6 % to 15 % damage, 3 = 16% to

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25% damage, 4 = 26% to 50% damage, and 5 = more than 50% damage (Megías et al., 2014).

Ethylene production was determined at 0, 7 and 14 days of cold storage in twelve fruits for each time and storage temperature, i.e. four replicates of 3 fruits each (Megías et al., 2014). After storage time, fruits were incubated in sealed 10-l containers for 6 hours at room temperature. Gas samples were then taken from each container and their ethylene content determined three times in a Varian 3900 chromatograph fitted with a flame ionization detector (FID).

Results and Discussion

Our data is indicative of the sensitivity of the immature zucchini fruit to low storage temperatures, which clearly reduced its commercial value in most of the analyzed cultivars of *C. pepo* after few days of storage at 4 °C. In comparison with the rest of the cultivars, the hybrid `Sinatra´, which was used as control of CI sensitivity, showed one the highest CI index, while the hybrid 'Natura' used as control of CI tolerance, showed an intermediate CI index (Fig. 1A), indicating that some of our landraces in the trial had a higher tolerance to CI than 'Natura'.

After 14 days of storage at 4 °C the fruit of most of the cultivars showed an average CI index higher than 2.5, indicating that more than 30% of the fruit surface was damaged and therefore that the fruit had lost its marketable value (Fig. 1A). Only accessions CpCAL003 and CpCAL053 showed a CI index lower than the commercial hybrid 'Natura', used as control of CI tolerance (Fig. 1A). CpCAL003 showed a CI index below 2 after 14 days of storage at 4 °C and was found to be the most CI-tolerant cultivar in the assay (Fig. 1A). After 14 days of cold storage it was therefore difficult to establish differences among the different cultivars, except for CpCAL003.

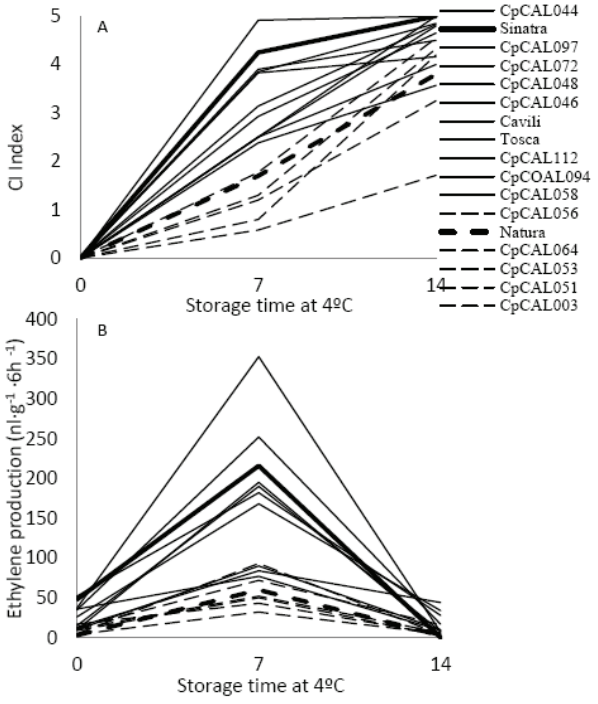

Fig. 1. Evolution of chilling injury index **(A)** and ethylene production **(B)** during the postharvest storage of fruit from 17 accessions of *C. pepo*. Fruit was stored for 7 and 14 days at 4 ºC and then transferred for 6 h at 20 ºC before analysis. The results represent the mean of four independent replicates for each sample. The accessions were classified based on their CI index at 7 days of storage. The solid lines represent cold-sensitive accessions, while the dashed lines indicate the cold-tolerant accessions. Thick lines within each group represent the commercial hybrids `Sinatra´ and `Natura´, which were used as controls of cold-sensitivity and cold-tolerance, respectively.

The different sensitivity of the accessions to cold storage was much more noticeable at 7 days (Fig. 1A). Based on the average CI index at 7 days of storage the 17 studied accessions were classified as cold-sensitive (CI > 2) or cold-tolerant (CI < 2). Four of the landraces, CpCAL64, CpCAL53, CpCAL51 and CpCAL003, showed an average CI index lower than that of the cold-tolerant hybrid 'Natura' (Fig. 1A). These accessions, and especially CpCAL003, could be used as new sources of tolerance to CI in squash breeding programs.

In addition to fruit surface lesions, represented in the CI index, the postharvest storage of zucchini fruit at 4 °C produced dehydration and fruit weight loss. Table 1 shows the reduction in weight of the fruit of 17 accessions of *C. pepo* after 7 and 14 days of storage at 4 °C. Fruit weight loss was more severe in those accessions that were classified as cold-sensitive on the basis of CI index, while those accessions classified as cold-tolerant had less weight loss (Table 1). Accordingly, we detected a positive correlation between the average weight loss of fruits and the average CI index at 7 days of cold storage in the 17 analyzed accessions (Fig. 2).

Cold storage induced the production of ethylene in all the accessions studied, although the level of ethylene production was markedly different among accessions (Fig. 1B). As observed previously (Carvajal et al., 2011; Megías et al., 2014) the highest ethylene production was found in the fruit stored for 7 days at 4 °C and then rewarmed at 20 °C for 6 hours (Fig. 1B). Results confirm previous data indicating that the sensitivity of the different zucchini accessions to CI is associated with the level of cold-induced ethylene (Megías et al., 2012, 2014). After 7 days

Table1. Weight loss (%) in the fruit of 17 accessions of *C. pepo* stored at 4°C for a total of 14 days.

	Weight loss	
Landrace	7 days	14 days
CPCAL003	2.63 ± 0.79	7.17 ± 2.58
NATURA	5.8 ± 1.6	9.7 ± 3.3
CPCAL051	4.15 ± 2.31	8.55 ± 4.78
CPCAL064	4.43 ± 2.63	12.67 ± 3.52
CPCAL094	4.44 ± 1.27	11.88 ± 2.10
CPCAL056	6.12 ± 3.37	11.32 ± 5.6
CPCAL053	6.71 ± 2.9	12.08 ± 5.54
CPCAL048	7.16 ± 3.27	14.65 ± 5.54
CPCAL046	7.51 ± 3.17	19.16 ± 3.61
CPCAL112	8.67 ± 3.15	17.61 ± 6.55
CPCAL097	9.05 ± 1.58	18.61 ± 3.35
CPCAL058	9.93 ± 5.27	19.87 ± 5.68
CPCAL044	10.76 ± 2.48	20.32 ± 3.15
TOSCA	7.14 ± 3.61	14.75 ± 4.21
CPCAL072	11.14 ± 3.11	20.19 ± 4.94
CAVILI	6.95 ± 3.04	14.83 ± 0.1
SINATRA	8.2 ± 1.3	14.9 ± 2.3

Values are the mean and standard deviation of 4 replicates of 3 fruits each.

of cold storage at 4 °C, the fruit of those accessions that were more sensitive to CI produced much more ethylene than the fruit of the cold-tolerant accessions (Fig. 1B). Only two accession that were classified as cold-sensitive produced an intermediate level of ethylene, similar to that produced by some of the cold-tolerant accessions (Fig. 1B). Nevertheless, the cold-induced ethylene in the cold-tolerant hybrid 'Natura' and in the identified most cold-tolerant accessions CpCAL53, CpCAL51 and CpCAL003, was very low (Fig. 1B). Moreover, we have found that the cold-induced ethylene in the fruit stored for 7 days was negatively correlated with CI index (Fig. 3).

Our data is in accordance with previous reports indicating that ethylene production is negatively correlated with cold tolerance in different genotypes of maize (Janowiak & Dorffling, 1995), cucumber (Cabrera et al., 1992), and mung bean (Collins et al., 1993). We have previously demonstrated that cold-induced ethylene is not responsible for the onset or the severity of CI symptoms in zucchini, but rather a cold induced response to CI (Megías et al., 2014). Nevertheless, the relationship found between cold induced ethylene and the occurrence of CI in cold-stored fruit can be used as a criterion to select cold-tolerant genotypes in squash breeding programs.

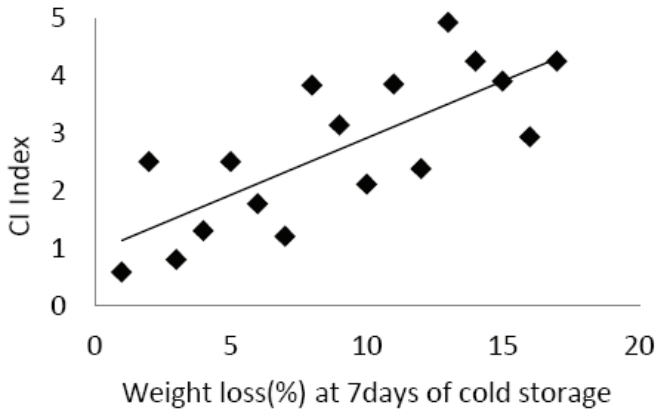

Fig. 2. Regression of weight loss (%) onto CI index at 7 days of cold storage at 4 °C in 17 accessions of *C. pepo*. The linear regression analysis (R = 0.70, *P* = 0.0019) indicates a correlation between variables with a significance level of 95%.

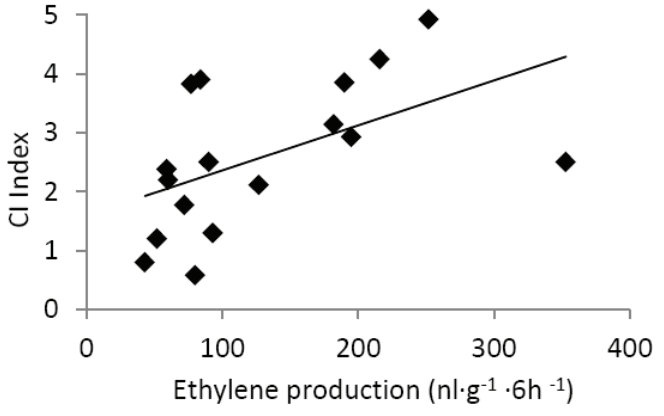

Fig. 3. Regression of ethylene production of the fruit of 17 accessions of *C. pepo* onto CI index after seven days at cold storage (4 °C). The linear regression analysis (R = 0.5239, *P* = 0.0309) indicates a correlation between variables with a significance level of 95%.

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Breeding for Parthenocarpy in Zucchini Squash

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Additional index words: *Cucurbita pepo*, andromonoecy, ethylene production, ethylene sensitivity

Abstract. The cultivation of zucchini under greenhouse winter conditions in Almería (Spain) reduces the number of male flowers and pollen, as well as the activity of natural pollinators. The removal of the synthetic auxin treatments currently used to set fruit in zucchini therefore requires the development of parthenocarpic varieties. We have explored 45 landraces of *C. pepo* with elongated fruits, morphotypes zucchini, vegetable marrow and cocozelle, to identify new sources of parthenocarpy for zucchini breeding programs. After two consecutive screening assays for both the percentage of parthenocarpic marketable fruits and parthenocarpic fruit growth rates, 5 accessions were selected. The parthenocarpy of some of these accessions differs from that of commercial hybrids in that it was not found to be associated with the syndrome of fruits with attached flowers, an undesirable trait in the current parthenocarpic hybrids. Moreover, we have observed that parthenocarpy is inversely correlated with ethylene production in the fruit. Whereas in non-parthenocarpic accessions unpollinated fruits produced a boost of ethylene at 3 days post-anthesis (DPA), concomitantly with fruit abortion and senescence, in parthenocarpic cultivars the fruits barely produced ethylene at 3DPA. Therefore, ethylene production in ovary/fruits at 3 DPA could be used as a marker to identify and select parthenocarpy in squash. Nevertheless, earlier evaluations of ethylene production and sensitivity in vegetative organs and in male flowers are not so well correlated with parthenocarpy in the analyzed cultivars.

Cucurbita pepo is a monoecious species with three consecutive sexual phases of development: a male phase, an alternant phase in which plant produces both male and female flowers, and a female phase (Peñaranda et al., 2007; Manzano et al., 2010; 2013). During the last phase of development plants only produce female flowers, which constitute a problem for pollination and fruit set in cultivations growing under winter conditions, which are those promoting the highest female flowering (Peñaranda et al., 2007; Manzano et al., 2010; 2013). Moreover, under these unfavorable environmental conditions the activity of pollinators is also restricted, which also limits pollination and fruit set. All these constraints have made partenocarpy a priority trait in the current breeding programs of zucchini squash for off-season greenhouse productions. It has been reported that the highest parthenocarpic production in *C. pepo* occurs in elongated morphotypes Zucchini and Cocozelle with dark green fruits (Nijs and Zanten, 1982; Om and Hong, 1989; Robinson and Reiners, 1999).

We found only three hybrids that are commercialized as parthenocarpic for greenhouse productions in Almería: 'Cavili', 'Partenon' and 'Argo'. The parthenocarpy of these cultivars is however associated with the undesirable syndrome of "fruits with attached flower". The floral organs in these cultivars remain attached to harvested fruit, and are removed manually by growers before commercialization. This causes a damage to the fruit, which makes it more susceptible to infection and rot during postharvest (Peñaranda et al., 2007). In a recent publication we have reported that the parthenocarpy of the cultivar 'Cavili' is associated with a lower ethylene production in the ovary and fruit before anthesis and during the days immediately after anthesis (Martínez et al., 2013; Martínez et al., 2014). The peak of ethylene in the unpollinated fruit at 3 days post anthesis (DPA) which is associated with flower abortion, is not only absent in the pollinated fruit, but also in the unpollinated fruit of the parthenocarpic cultivar 'Cavili' (Martínez et al., 2013).

In this paper we made a screening of traditional Spanish cultivars with elongated fruit to identify new sources of parthenocarpy for zucchini breeding programs, and compared ethylene production and sensitivity in the identified parthenocarpic and non-parthenocarpic accessions.

Materials and Methods

All analyzed accession derived from the core collection of the seeds banks at the University of Almería (BSUAL) and the Polytechnic University of Valencia (COMAV). The commercial hybrids 'Argo', 'Cavili', and 'Partenon' were used as positive control for parthenocarpy. In the first trial, 45 accessions of elongated morphotypes of *Cucurbita pepo* from the morphotypes Cocozelle, Zucchini and Vegetable Marrow, were evaluated. Ten plants of each accession were grown in the same greenhouse conditions, and the number of parthenocarpic fruits of commercial size were scored. From this first trial we selected 20 accessions.

The selected accessions were evaluated for parthenocarpic tendency in spring 2011. Length and width were measured at anthesis and at 3, 5, and 7 DPA. To study the relationship be-

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tween ethylene and parthenocarpy, 6 parthenocarpic and 6 non-parthenocarpic accessions were selected and monoecy stability, ethylene production and sensitivity measured.

Monoecy stability was studied using the andromonoecy index (AI) defined by Martínez et al. (2014). In some accessions of *C. pepo*, considered to have an unstable monoecy or a partial andromonoecy, high temperature stimulates the conversion of female into bisexual flowers in the plant. Since bisexual flowers show a variable degree of stamen development, they were scored from 0 to 3 according to their stamen development. Perfect female flowers were scored as 0, flowers with primordial stamens were classified as 1, flowers whose stamen have an intermediate size were scored as 2, whereas bisexual flowers with complete stamens development and able to produce pollen were scored as 3. The AI of each accession was calculated as the average bisexuality score from at least 10 plants with a minimum of 5 pistillate flowers per plant. Plants and accessions with an AI of 0 to 0.9 were considered monoecious, whereas those with an AI from 1 to 3 were considered partially andromonoecious (Martínez et al., 2014). The parthenocarpic potential of each accession was assessed by the longitudinal and transverse growth rate of at least 10 non-pollinated fruits from anthesis up to 7 DPA.

The ethylene sensitivity of the accession was assessed by the abscission time of ethylene-treated male flowers. Ten male flowers at 1-2 day before anthesis were harvested treated with 20 ppm of ethylene in hermetic containers for 2 days. The abscission time of each flower was scored in in 3 days post-treatment. The same number of male flowers were treated with air and maintained in the same conditions as control. Ethylene sensitivity was calculated as the percentage of reduction in abscission time caused by the ethylene treatment.

Ethylene production was assessed in vegetative organs and in young fruits. Ten young leaves of 2-3 cm length were removed from the plant and kept in sealed containers in dark at room temperature for 24 h. To determine ethylene production in fruits at 3 DPA, 3 fruits of the same age were harvested and kept in sealed containers for 6 h. Ethylene was measured in at least 3 replicates per accession, and 3 times per sample in a Varian 3900 gas chromatograph fitted with a flame ionization detector (FID)

Results and Discussion

The first screening was performed in winter 2009-2010. Under these conditions 20 accessions were selected for further analysis. The second screening was conducted with selected accessions in spring 2011. During the second screening, the longitudinal and diametrical growth rate of at least 10 unpollinated fruits of each accession was analyzed from anthesis up to 7 DPA, when a pollinated fruit has already reached marketable size (Table 1). Only 8 accessions of 20 were classified as parthenocarpic, since their fruits reached at least 16 cm in length before 7 DPA: 'E-27', 'CM-37, 'Partenon', 'Cavili', 'Argo', 'CpCAL112', 'V-185', 'PI261610'. At anthesis many of these accessions have flowers with increased ovarian size. However the parthenocarpic potential of the accession was clearly differentiated at 3 and 5 DPA (Table 1), wihich indicate a higher fruit growth rate in parthenocarpic accessions.

Our data confirms previous studies showing that elongated dark-skinned fruits have a greater tendency to parthenocarpy (Nijs and Zanten, 1982; Om and Hong, 1988; Robinson and Reiners, 1999). In fact, 7 of 8 identified partenocarpic cultivars had elongated fruits from the morphotypes Zucchini or Cocozelle,

Table 1. Mean fruit length in 20 accessions of *C. pepo* from anthesis up to 7DPA.

Parthenocarpic Cultivars				
Cultivar	Anthesis	3 DPA	5 DPA	7 DPA
'CM-37'	79.13	121.29	142.11	156.89
'PI261610'	83.66	130.07	159.61	171.79
'V-185'	89.17	147.73	179.55	201.70
'E-27'	84.80	122.64	155.52	176.64
'CpCAL112'	76.42	155.56	200.90	292.50
'ARGO'	104.17	191.22	269.89	338.88
'CAVILI'	74.18	141.44	204.27	249.30
'PARTENON'	90.80	171.02	245.78	294.51
Non-Parthenocarpic Cultivars				
Cultivar	Anthesis	3 DPA	5 DPA	7 DPA
'AFR-12'	66.90	91.24	106.73	112.98
'CA-154'	52.08	62.18	62.18	62.18
'GRECIA6'	65.00	96.33	92.99	94.26
'PASCUAL40'	57.03	79.86	91.84	93.50
'PI169462'	56.04	86.78	97.34	107.85
'S4'	77.86	104.66	104.66	104.66
'V-171'	55.80	71.84	72.90	74.44
'CpCAL003'	62.46	63.31	65.41	66.14
'CpCAL005'	59.97	68.80	69.29	69.29
'CpCAL044'	57.99	76.94	76.94	76.94
'CpCAL097'	65.90	75.25	86.26	93.79
'CpCAL110'	81.16	111.01	122.29	124.32

Values represent the mean of at least 10 fruits per accession.

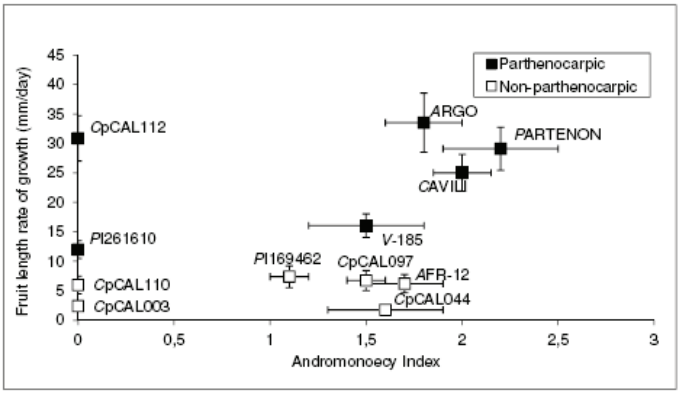

Fig. 1. Regression of Andromonoecy Index (AI) onto parthenocarpic fruit growth rate during the first 7 DPA in 6 parthenocarpic and 6 non-parthenocarpic accessions of *C. pepo*. Horizontal and vertical bars represent standard errors.

and only one, 'CM-37', belonged to the Vegetable Marrow group. All parthenocarpic accessions, with the exception of 'Cavili', have dark-green skin.

The relationship between parthenocarpy and ethylene was studied in 6 parthenocarpic and 6 non-parthenocarpic accessions. The parthenocarpic potential of each variety was compared with monoecy instability, which is a trait closely related with ethylene production (Martínez et al., 2014), as well as with ethylene production and sensitivity in different organs and plant development stages. Our results indicate that the pathenocarpy of the commercial hybrids 'Argo', 'Partenon' and 'Cavili', but also that of the accession 'V-185' was associated with monoecy instability or partial andromonoecy. Nevertheless, we have identi-

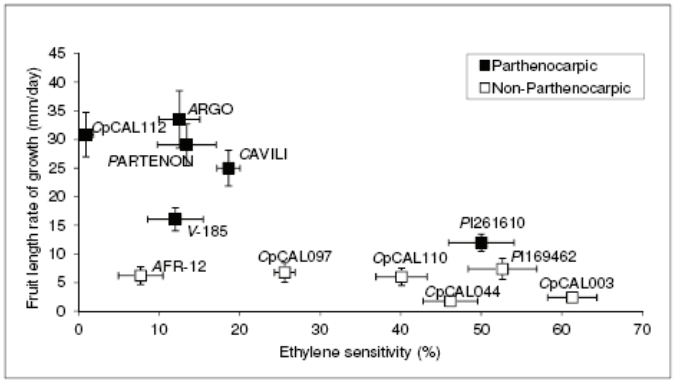

Fig. 2. Regression of ethylene sensitivity onto fruit length growth rate during the assay (7DPA) Lineal regression analysis (R = –0.699, *P* = 0.0113) point out the strong correlation between the two variables. Horizontal and vertical bars represent standard error for ethylene sensitivity and fruit growth rate, respectively, in each accession.

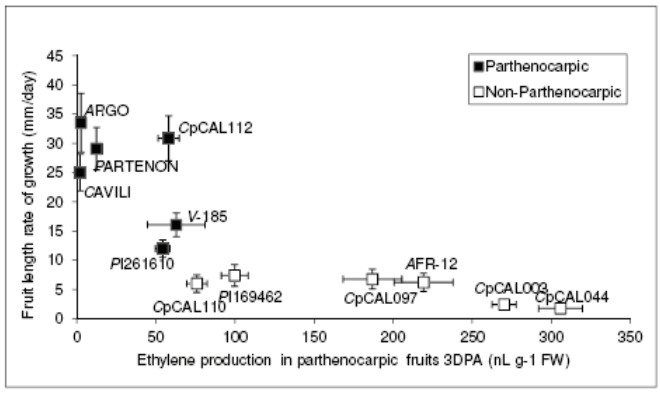

Fig. 4. Regression of ethylene production in unpollinated fruits at 3 DPA onto fruit length growth rate for the first 7 DPA in 6 parthenocarpic and 6 non-parthenocarpic accessions of *C. pepo*. The linear regression analysis (R = –0.799, *P* = 0.0018) indicates a strong negative correlation between variables. Horizontal and vertical bars represent standard errors.

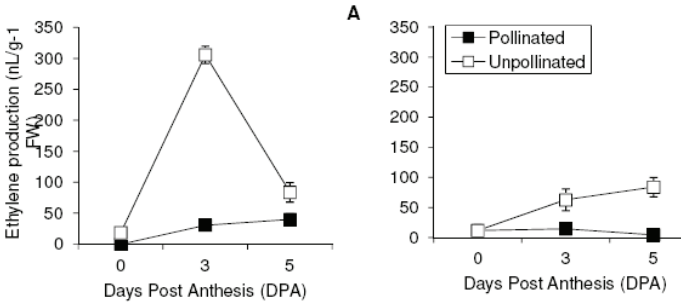

Fig. 3. Ethylene production in pollinated and unpollinated ovaries/fruits of two accessions of *C. pepo* that differ in their parthenocarpic potential. **(A)** 'CpCAL044', which has been classified as non-parthenocarpic, and **(B)** 'V-185', which has been classified as parthenocarpic. Bars represent standard errors (n = 3).

fied other parthenocarpic accessions ('CpCAL112', 'PI261610' and 'CM-37') with no monoecy instability (Fig. 1). These last sources of parthenocarpy will be of great interest for zucchini breeding programs.

The relation between parthenocarpy and ethylene sensitivity was analysed in male flowers. Although ethylene sensitivity was found to be inversely correlated with parthenocarpic fruit growth rate (R = –0.690; *P* = 0.0113), there were some accessions that did not fit that linear correlation (Fig. 2). In fact, the parthenocarpic accession 'PI261610' was found to be sensitive to ethylene, whereas the non-parthenocarpic accession 'AFR-12', showed low ethylene sensitivity (Fig. 2).

No correlation was found between parthenocarpy and ethylene production in leaves at early stages of plant development (data not shown). Nevertheless we have found that fruit ethylene production at 3 DPA is correlated with its parthenocarpic development. This was not only true for parthenocarpic cultivars with tendency to monoecy instability, as was previously reported for the 'Cavili' hybrid (Martínez et al., 2013), but also for those cultivars showing monoecy stability. Fig. 3 shows ethylene production in pollinated and unpollinated fruits of two contrasting accessions for parthenocarpy. As observed previously (Martínez et al., 2013), the absence of pollination induces an ethylene peak that precedes fruit abortion in the non-parthenocarpic accession, but not in the parthenocarpic accession (Fig. 3). We have evaluated the ethylene production in the fruit of 6 parthenocarpic and 6 non-parthenocarpic accessions at 3 DPA, finding a strong negative correlation (R = –0.799, *P* = 0.0018) with parthenocarpic fruit growth rate (Fig. 4). These results are in concordance with those obtained in *Arabidopsis*, where ethylene inhibitors were able to promote a higher fruit size (Carbonell-Bejerano et al., 2011), and in tomato, where gene expression studies indicated a downregulation of ethylene genes in response to natural or hormonal-induced parthenocarpy (Vriezen et al, 2008). Therefore, the lower ethylene production in the fruit during the days immediately after anthesis could be a good marker to identify and select parthenocarpic materials in *C. pepo*.

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Genetic Basis of Mitochondrial Sorting in Cucumber

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Additional index words. organellar genetics, sublimon, nuclear-cytoplasmic interactions

Abstract. Regeneration of cucumber (*Cucumis sativus*) from cell cultures produces plants with distinct mosaic (MSC) phenotypes, mis-shapen cotyledons and leaves, reduced fertility, and low seed germination. The MSC phenotypes are paternally transmitted and associated with deletions or under-representations of specific regions of their mitochondrial (mt) DNAs. A nuclear locus, Paternal Sorting of Mitochondria (*Psm*), conditions sorting to wild-type mitochondria in progenies when MSC plants are used as pollen parents. We mapped *Psm* to chromosome 3 of cucumber and identified a pentatricopeptide repeat (PPR) as a candidate gene controlling mitochondrial sorting in cucumber. This research demonstrates the usefulness of cucumber as a model plant to study nuclear-organellar interactions.

Cucumber is a unique model plant for organellar genetics because its three genomes show different modes of transmission [maternal for chloroplast, paternal for mitochondrial, and biparental for nuclear genes (Havey et al., 1998)] and its mitochondrial genome is very large at ~1.7 Mb (Alverson et al., 2011; Ward et al., 1981). Recombination among the repetitive regions in the cucumber mitochondrial DNA produce strongly mosaic (MSC) plants with deformed leaves, slow growth, and poor fertility (Bartoszewski et al., 2004; Malepszy et al., 1996). MSC is a valuable tool for studying mitochondrial transmission because the phenotype can be easily scored at the seedling stage (Lilly et al., 2001; Malepszy et al. 1996). We previously identified a nuclear locus (*Psm* for Paternal Sorting of Mitochondria) which preferentially sorts for rare wild-type mitochondrial DNAs transmitted paternally from MSC plants (Al-Faifi et al., 2008; Calderon et al.; 2012; Havey et al.; 2004). In this research, we produced a fine map of the genomic region carrying *Psm* towards the cloning of this unique locus and a better understanding of nuclear control of mitochondrial sorting in a higher plant.

Materials and Methods

A segregating F_2 family was produced from the cross of single plants from USDA Plant Introduction (PI) 401734 and *C. sativus* var. *hardwickii* (Csh) (Calderon et al., 2012). PI 401734 was used because it produced a relatively high frequency (>75%) of wild-type progenies when crossed as the female with MSC16. Csh produced a high frequency (>90%) of MSC progenies in crosses with MSC16. To score paternal sorting of mitochondria, each F_2 plant was crossed as the female with MSC16 and phenotypes of >50 testcross progenies were determined. Genomic DNA was extracted from the parental plants and F_2 progenies (Krysan, 2004). F_2 plants were genotyped for simple sequence repeats as described by (Ren et al., 2009). Genomic DNA of PI 401734 and Csh were re-sequenced using the Illumina platform. Reads from 401734 and Csh were aligned to the GY14 reference on Phytozome v 5.0 (http://www.phytozome.net/) using Integrated Genomics Viewer (version 2.3.32) and evaluated for single nucleotide polymorphisms (SNPs). Primers were designed for genotyping of SNPs across the *Psm* region using the Fluidigm system (Garcia-Mas et al., 2012). Goodness-of-fits to the expected segregations were calculated and detection of linkages in centimorgans (cM) was completed using MapManager (Manly et al., 2001). Quantitative trait analysis was completed using R (Broman and Sen, 1989).

Results and Discussion

Al-Faifi et al. (2008) previously mapped *Psm* to chromosome 3 of cucumber; however fine mapping was not possible due to relatively few polymorphisms across the *Psm* region in a relatively narrow cross between two cultivated cucumbers. Calderon et al. (2012) used the PI 401734 × Csh family to assign *Psm* to a 2.7 cM region between SSRs 21456 and 06011 on chromosome 3, corresponding to 459 kb of the genomic sequence. In order to identify more codominant markers for fine mapping of *Psm*, we used the Illumina platform to sequence the parental DNAs. After alignment to the GY14 reference, numerous SNPs were revealed among the previously mapped SSRs. In total, 45 SNPs were genotyped using the Fluidigm platform and F_2 DNAs from PI 401734 crossed with Csh. Re-analysis of mitochondrial sorting among the F_2 progenies revealed a highly significant (LOD 45) effect across a 171 kb region on chromosome 3. This region carries 29 putative genes of which two possess mitochondrially targeting sequences. One of the mitochondrially targeted genes

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is a pentatricopeptide repeat, a class of nuclear genes important in regulation and translation of mitochondrial genes.

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Genomic Resources for Bottle Gourd, *Lagenaria siceraria* (Mol.) Standl

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Additional index words. *Lagenaria siceraria,* RAD-Seq, shotgun, markers, synteny, population genomics

Abstract. **The bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] is among the first crops to be domesticated, and is an economically important cucurbit crop in many parts of the world. Recent work in our lab has shed some light on the genomics, population genetics and molecular breeding for this crop. Restriction site-associated DNA sequencing (RAD-Seq) of an F_2 population enabled the construction of a high-density genetic map comprising 2098 SNP loci, to which 922 scaffolds/contigs that were assembled from whole genome shotgun sequences were anchored. Macro-colinearity was revealed between the genomes of bottle gourd and its relatives, i.e. watermelon, cucumber and melon, whereas more complicated than expected structural variations were present between the bottle gourd and watermelon genomes. RAD-based population genomic analysis suggested two main subgene pools of the Chinese germplasm. LG7 was indicated to have undergone through strong selection as exhibiting elevated F_{ST} and LD strength. In addition to the genome-wide distributed SNPs, 892 InDel loci were identified from analyzing the RAD-Seq data to provide PCR-based markers that are favorable for breeders. PCR amplification with a set of the InDel markers showed a 94% amplification rate and an 80.5% polymorphism rate, suggesting a high fidelity of the bioinformatic pipeline we used. Taken together, these works provide valuable resources for bottle gourd genome research and breeding.**

Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] ($2n = 2x = 22$), also known as calabash, is a diploid belonging to the genus *Lagenaria* of the *Cucurbitaceae* family (Beevy and Kuriachan, 1996). Phylogenetically bottle gourd is close to many economically important cucurbit species including cucumber and melon that belong to the genus of *Cucumis*, as well as watermelon that belong to the genus *Citrullus*. Bottle gourd is grown for its fruit either being harvested young and used as a vegetable or harvested mature and used as a bottle, utensil, or pipe. Based on archaeological evidence, bottle gourd is presumed to have been domesticated in Africa (Decker-Walters et al., 2004; Whitaker, 1971), and might have dispersed to the New World by ocean currents or by human migration in pre-historic times (Erickson et al., 2005; Morimoto et al., 2005).

High genetic variability exists in bottle gourd, especially in fruit size and shape, which can be round, oblate, pyriform, elongated curvilinear, dipper, slender straight, tubby, snake-like and more. Thus far, very few genomic resources have been publically available for bottle gourd.

Materials and Methods

Plant materials included a 139-individual F_2 population derived from the cross of 'Hangzhou gourd' and 'J129', as well as a natural collection of 66 accessions. Restriction site-associated DNA sequencing (RAD-Seq) followed the standard procedures (Baird et al. 2008) using *Eco*RI to cut the gDNA. Raw sequence reads were clustered into tags using ustacks, and were further collapsed into clusters using cstacks. A Bayesian method was employed for accurate SNP genotype calling. An in house Perl script was used to scan for Insertion/Deletion (InDel) sites from RAD clusters between 'Hangzhou gourd' and 'J129'. The genetic linkage map based on SNP markers was constructed using JoinMap 4.0.

Genome shotgun sequencing was conducted following standard protocols. The sequence reads were assembled into scaffolds using SOAPdenovo (Li et al., 2010). The genome scaffolds were anchored to the genetic map by BLASTN search. To detect cross species synteny, each anchored scaffold was BLASTN searched against the genome sequences of cucumber (Huang et al., 2009), watermelon (Guo et al., 2013) and melon (Garcia-Mas et al., 2012) and the sequences were considered orthologous if sharing ≥ 90 % sequence identity with an e-value ≤ e^{-10}.

Observed heterozygosity (H_o) and pairwise population differentiation index (F_{ST}) were calculated using Arlequin 3.5.1.3. Candidate loci under selection were determined using an F_{ST}-based outlier test controlled for heterozygosity as implemented in Arlequin.

Results and Discussion

A SNP-based high density genetic map. RAD sequencing generated 334,167,874 and 209,743,655 reads with expected overhangs and barcodes from the F_2 and natural population, respectively. The majority of the loci was at a sequencing coverage between 29× and 89×. The mean coverage of polymorphic loci in the two datasets was 5.2× and 4.9×, respectively. Using a Bayesian method, we detected 3753 SNPs in the F_2 population, which was reduced to 2098 after filtering of loci with ≥ 45 % missing calls

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or severely skewed segregations (χ^2 test, $P = 0.05$). As for the natural population, 3226 bi-allelic SNP loci with a minor allele frequency (MAF) ≥ 1.5 % were obtained under the missing call rate cutoff of 20 %.

Regression model mapping with the 2098 quality SNPs produced a genetic map harboring 2084 loci. The mapped loci were distributed on 11 linkage groups (LGs) presumably corresponding to the 11 chromosomes of the haploid genome. The total length of the map was 1361 cM with an average marker spacing at a sub-cM level (0.6 cM) (Table 1).

Genome scaffolds anchored to the genetic map and the sequence-based cross species synteny. Whole genome shotgun sequencing generated 5.7 Gb DNA sequence data, which is equivalent of approximately 8.9× coverage of the 640 Mb genome. A total of 309,756 scaffolds and contigs (306 Mb) of the genome was obtained, of which 922 scaffolds were successfully anchored to the genetic map, and they were used to build the syntenic relationships across related cucurbit species.

In total, 433, 460 and 725 scaffolds found orthologs in the cucumber, melon and watermelon genomes, respectively, fitting the known phylogeny of cucurbits. Macro-collinearity was apparent between bottle gourd and each of the three genomes. Each bottle gourd LG matched one to three chromosomes of cucumber and melon, and up to four chromosomes of watermelon. LsLG8 and LsLG10 seemed to have undergone the least chromosome break/fusion events. In contrast, LsLG2 and LsLG6 had their syntenic segments in three or more chromosomes. We noted that even though watermelon is phylogenetically closest to bottle gourd and shares the same haploid chromosome number of 11, their syntenic relationship does not appear to be simple (Fig. 1).

Identification of a putative selection site associated with fruit shape via population genome scan. Based on genome-wide SNP genotypes, two main branches were suggested for the natural population. The larger branch (SubL) consists of 46 accessions, most of which have slender straight or elongated tubby fruits; the smaller branch (SubR) has 20 accessions that mostly have round or pyriform fruits. There is a seven-fold reduction of heterozygosity in SubR on LG7, which is a signal of selection (Fig. 2). Congruently, F_{ST} on LG7 was exceptionally high compared with other chromosomes. A closer look revealed a small region spanning ca. 1.4 cM on LG7 that contributed the most to the high F_{ST} (Fig. 2). Outlier test for SNP loci under selection suggested that *LX3405*, which resides in the high F_{ST} island, was under diversifying selection ($F_{ST} = 0.89$, $P < 0.05$).

Because the subdivision of the population is associated with fruit shape, we wondered whether the putative selection site is linked to any known fruit shape genes in tomato, a model for fruit shape research. Bridged by the bottle gourd-cucumber synteny, we found the fruit shape genes *SUN*, *FAS* and *LC* each was collinear to a region unrelated to LsLG7. *OVATE*, known to control elongated fruit growth in tomato and pepper (Liu et al., 2002; Tsaballa et al., 2011), however was found to reside in a region syntenic to the high F_{ST} island on LsLG7 (8.7 Mb next to the ortholog of *LX2975*, which is 0.4 cM apart from *LX3405*) (Fig. 3). These findings provide interesting clues for further elucidating fruit shape genes in bottle gourd.

Development and verification of InDel markers. In order to provide PCR-based markers that are more applicable in breeding, we scanned for InDel loci from RAD clusters between 'Hangzhou Gourd' and 'J129'. A total of 892 putative InDel loci were detected between the two genomes, among which 609 showed only a single bp length variation, 121 showed 2 bp, 92 showed 3 to 9 bp, and

Table 1. Summary of the 11 linkage groups.

LG	No. loci	Length (cM)	Mean marker space (cM)
1	320	108.3	0.3
2	300	177.5	0.6
3	235	149.7	0.6
4	210	102.2	0.5
5	178	190.1	1.1
6	176	100.2	0.6
7	165	75.5	0.5
8	162	111.9	0.7
9	139	115.8	0.8
10	115	115.7	1.0
11	84	114.5	1.4
Total	2084	1361.4	0.65

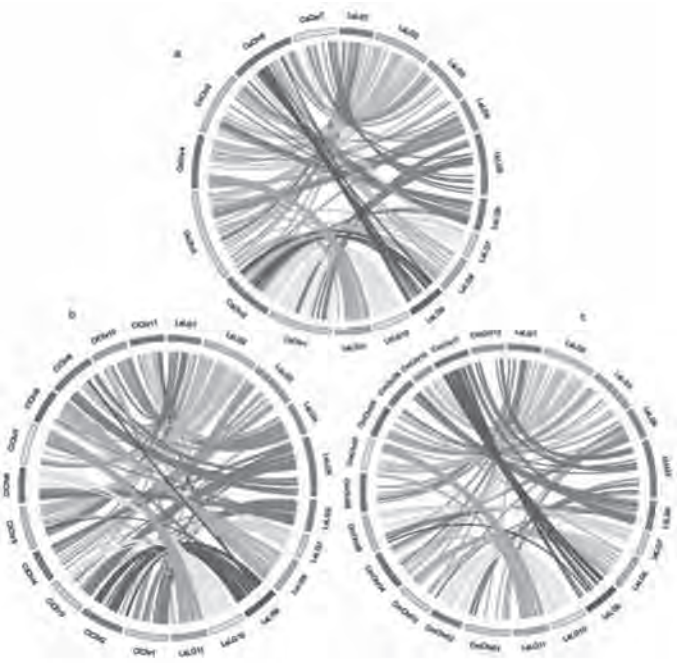

Fig. 1. Circos illustration of the genome synteny between bottle gourd and its relatives. **(a)** bottle gourd–cucumber; (**b)** bottle gourd–watermelon; and (**c)** bottle gourd–melon.

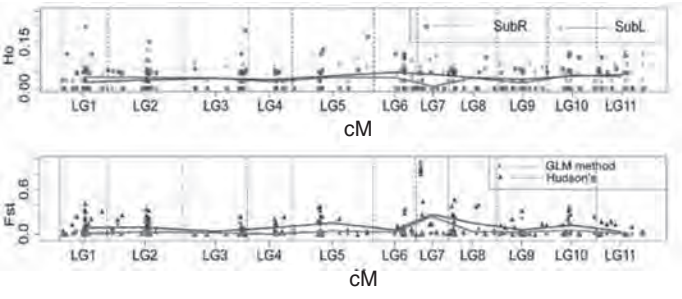

Fig. 2. H_o and the F_{ST} calculated with two different methods and plotted against genetic map position. A, observed heterozygosity (H_o) in SubL (in red) and SubR (in blue); B, population differentiation index (F_{ST}) between the two subgene.

70 showed ≥ 10 bp length variations. PCR primers were designed for the 92 loci with a putative InDel length ranging from 3 to 9bp to verify their fidelity and polymorphism. As a result, 87 (94%) out of the total 92 pairs of primer gave a successful amplification, 70 of them being able to reveal polymorphism between the two genomes (Fig. 4). Thus, the bioinformatic pipeline we used performed well in prediction of InDel loci. The InDel markers will provide convenient approaches for breeding practices including germplasm assessment, marker-assisted breeding and seed purity inspection.

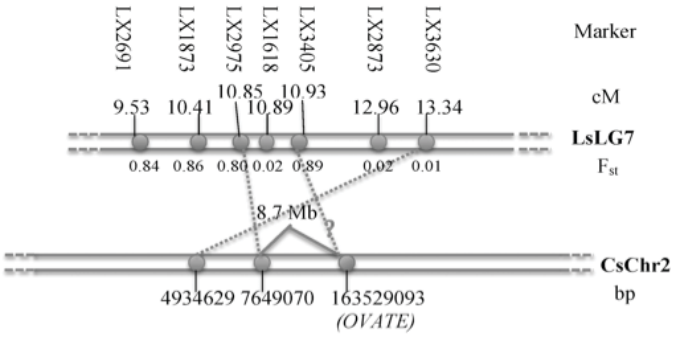

Fig. 3. Illustration of syntenic relationship between OVATE and the high F_{ST} island on LG7.

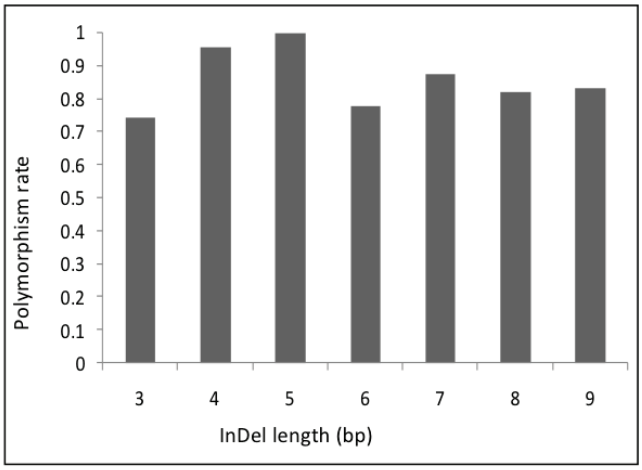

Fig. 4. Polymorphism rates of the PCR markers designed based on InDels with the length from 3 to 9 bp.

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An Assessment of Male Fitness of an Escaped Virus Resistant Transgene from Cultivated *Cucurbita pepo* during Introgession into Wild *Cucurbita pepo*

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Additional index words. cucumber beetles, male function, pollen, Texas gourd, virus resistant transgene, zucchini yellow mosaic virus

Abstract. Gene flow between cultivated and wild squash in the United States may result in the introduction of a virus resistant transgene (VRT) into wild populations. We have extensively studied the impact of the transgene as it introgresses into populations of wild squash (*Cucurbita pepo* ssp. *texana*, Texas gourd) and its impacts on non-target herbivores and the pathogens the herbivores transmit (the bacterium, *Erwinia tracheiphila*, and common mosaic viruses). In this study, we examine fitness through the male (pollen) function of transgenic introgressives in fields in which the virus was present throughout the growing season and fields in which the virus was present only at the end of the field season. We predicted that more seeds on susceptible plants would be sired by transgenic plants in the fields in which virus was present throughout the growing season, and that the proportion of seeds sired by transgenic plants would increase as virus spread through the fields. Our data support the first prediction but proportion of seeds sired did not increase as virus spread through the susceptible plants in the fields.

Gene exchange between crops and their wild relatives is common and difficult to contain (Ellstrand, 2003). Unlike most traits of cultivated species, there are concerns that transgenes conferring resistance to herbivores or pathogens could enhance the fitness and weediness of wild species and/or have indirect impacts on non-target species such as pollinators, herbivores, predators, soil fauna, and other plants in the community (e.g., Ellstrand, 2003; Pilson and Prenderville, 2004). The introgression of resistance transgenes into populations of native species could also modify the genetic diversity of these populations (Ellstrand, 2003) and it has the potential to alter the species composition of the community should these species become more competitive/aggressive (e.g., Pilson and Prenderville, 2004; Fuchs and Gonsalves, 2007).

Gene flow from cultivated to free-living taxa of *Cucurbita* is common and well-documented (e.g., Kirkpatrick and Wilson, 1988). Relative to wild taxa of *Cucurbita*, cultivated varieties tend to have larger seeds, shorter internodes, and larger fruits with a soft pericarp and sweet (not bitter) flesh. Normally, these traits are a selective disadvantage in the wild and tend to be rapidly purged from wild populations. However, in 1994, the United States Department of Agriculture (USDA) deregulated a transgenic yellow crookneck squash (Asgrow, ZW-20) that was engineered to express a dual coat protein (CP) construct of watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV) that conferred resistance to those viruses (USDA, 1994). In 1996, a second transgenic cultivar (CZW-3) with CP-based resistance to WMV, ZYMV, and cucumber mosaic virus (CMV) was deregulated (USDA, 1996). In both transgenic cultivars a marker gene, neomycin phosphotransferase II (*nptII*) was co-transferred. By the late 1990s, several cultivars with the transgene were developed, marketed, and grown commercially throughout the United States but especially in the Southeastern United States, where it greatly reduced pesticide use and increased yield (Fuchs and Gonsalves, 2007). There is now concern that the virus resistant transgene (VRT) will escape and introgress into wild populations with potentially adverse effects on the environment.

Since 2006, we have extensively studied the interactions among *Cucurbita pepo* ssp. *texana* (the Texas gourd), transgenic introgressives with the Texas gourd, the primary herbivores (cucumber beetles and aphids) and the pathogens the herbivores transmit (the bacterium, *Erwinia tracheiphila*, and common mosaic viruses) (Sasu et al., 2009; Harth et al., 2012). In this study, we examine the number of seeds sired by transgenic introgressives on susceptible plants (non-transgenic introgressives and the Texas gourd) growing in experimental fields in which ZYMV was introduced either early or late into the fields. We predicted that the proportion of seeds sired would be greater in the fields with early virus introduction. Moreover, we predicted that the proportion of seeds sired by transgenic plants in the fields without virus would be constant across sampling dates while the proportion of seeds sired by transgenic plants in the fields with virus would increase as the incidence of ZYMV infection increases during the growing season.

Acknowledgements. We thank T. Omeis for greenhouse assistance and Bob Oberheim and his staff for use of the Penn State Agricultural Experiment Station at Rock Springs, Pennsylvania. This project is supported by the Biotechnology Risk Assessment Program competitive grant number 2009-33120-20093 from the USDA - National Institute of Food and Agriculture.

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Materials and Methods

The wild gourd, *Cucurbita pepo* ssp. *texana* (Cucurbitaceae) is an annual monoecious vine with indeterminate growth and reproduction. It is native to Texas and to states along the Mississippi River from Southern Illinois southward (sometimes called ssp. *ozarkana*). It is completely inter-fertile with the cultivated pumpkins and squashes (*C. pepo* ssp. *pepo* and ssp. *ovifera*) and several annual *Cucurbita* taxa from Mexico (*C. pepo* ssp. *fraterna*, *C. moschata*, *C. ficifolia*, *C. agyrosperma ssp sororia and ssp agyrosperma*) (e.g., Arriaga et al., 2006). After a period of vegetative growth (5–7 nodes), wild gourds produce one large yellow flower (either male or female) in the axil of each leaf. The flowers last for only one morning and are pollinated by bees, especially squash bees. The fruits of the Texas gourd are round to oval with a volume of 175–450 ml and typically contain 150-300 seeds that weigh 20–40 mg (Winsor et al., 2000).

The leaves and other organs of the wild gourd produce cucurbitacins (oxygenated tetracyclic triterpenes). Cucurbitacins are among the bitterest compounds known (Metcalf and Rhodes, 1990). However, cucumber beetles (*Diabrotica* spp. and *Acalymma* spp.) are adapted to feed on cucurbitacins in the leaves of *Cucurbita* species and are found throughout the native ranges of *Cucurbita* species (Robinson and Decker-Walters, 1997). The cucumber beetles chemically modify the cucurbitacins, use them for their own protection, and the males also transfer some of the modified cucurbitacins to the females in their seminal fluid which is used to chemically protect the eggs (Ferguson et al., 1985; Metcalf and Rhodes, 1990). Cucumber beetles are attracted to cucurbitacins in the foliage of *Cucurbita* and, when flowers are present, it has been shown that floral volatiles not only attract bees (the pollinators), but also cucumber beetles over relatively large distances (e.g., Lampman and Metcalf, 1988). These beetles cause a characteristic pattern of holes (1–1.5 cm in diameter) in the portions of the leaves serviced by the smallest veins and beetle damage has been shown to substantially reduce yield in cultivated cucurbits and reproductive output in the wild gourd (e.g., Du et al., 2008; Stephenson et al., 2004). After feeding on the leaves, the beetles aggregate in the flowers to mate.

The full impact of herbivory by cucumber beetles on wild gourds, however, also includes increased exposure to a pathogen, *Erwinia tracheiphila* (Enterobacteriaceae), the causative agent of bacterial wilt disease. Cucumber beetles are the only known vector of *Erwinia* which overwinters in their guts. Transmission occurs when fecal pellets containing *Erwinia* land on leaf wounds at the sites of feeding damage (Fleischer et al., 1999). Recently, we have shown that there is a second, and perhaps more common, mode of transmission (Sasu et al., 2010). When the beetles aggregate in the flowers to mate their fecal pellets containing *Erwinia* fall onto or near the floral nectaries and the bacteria enters *via* the nectaries. Once inside the plant the bacteria proliferate in the xylem where they secrete a mucilaginous, exopolysaccharide matrix that cuts off the water supply resulting in wilting. Wilt symptoms typically develop 10–15 days after infection and the disease is nearly always fatal once symptoms appear (Stephenson et al., 2004). Bacterial wilt disease is the most economically important disease of cultivated cucurbits (cucumbers, melons, pumpkins and squash) in the Eastern United States (Fleischer et al., 1999).

Several generalist aphids are known to infest *Cucurbita*. These aphids also vector the four most common viral diseases of cucurbits (cucumber mosaic virus [CMV], papaya ringspot virus [PRSV], watermelon mosaic virus [WMV], and zucchini yellow mosaic virus [ZYMV]). ZYMV and WMV are the two most common viral diseases of cucurbits at our field sites in Central Pennsylvania. Both are single-stranded, positive-sense RNA viruses of the family Potyviridae and are transmitted *via* aphids in a non-persistent manner. Transmission occurs when the stylet of the aphid probes a new plant (analogous to a dirty needle). These viral diseases produce symptoms that include blisters, necrotic lesions, branches with short internodes and small highly serrate leaves, and other leaf deformities.

Over the years, we performed a series of large, field scale, experimental studies of the Texas gourd pathosystem. These studies revealed that: (a) viral diseases have no impact on survivorship during the growing season, but decrease male and female flower number, fruit number and in vitro pollen tube growth; (b) large plants, measured as absolute size, or flower number, have a higher incidence of wilt disease than smaller, viral infected plants; (c) more beetles congregate per flower in the flowers of healthy (non-viral infected) plants than in the flowers of viral infected plants; (d) there is significantly less beetle damage on virus infected plants than on non-diseased plants; (e) herbivory by cucumber beetles reduces reproductive output through both the male and female functions; (f) pollen from viral diseased plants and from beetle damaged plants sired significantly fewer seeds in competition with pollen from healthy plants (that is, herbivory and viral diseases reduce both the quantity AND quality of pollen); (g) the adverse effects of beetle herbivory and viral infections are greater for fruit production than for male flower production, indicating that the impact of environmental stress can differentially affect the two sexual functions; (h) both resistance and tolerance to cucumber beetles varies with ontogeny; (i) there is heritable variation in the ontogeny of resistance and tolerance to cucumber beetles; and (j) the foliage of wilt diseased plants attracts more cucumber beetles than the foliage of healthy plants and this attraction is mediated by volatile organic compounds; and (k) the flowers of healthy plants attract more cucumber beetles than the flowers of virus or wilt diseased plants (Stephenson et al., 2004; Hayes et al., 2004; Avila-Sakar and Stephenson, 2006; Ferrari et al., 2006; Du et al., 2008).

In the Liberator III transgenic cultivar, the VRT is hemizygous and, importantly, the *NPTII* gene conferring resistance to neomycin has not been deactivated and is still tightly linked to the coat protein genes of the three viruses. Consequently, we were able to introgress the transgene into 20 families of Texas gourds (using the wild gourd as the recurrent parent) because the presence of the NPTII protein in hybrid progeny can be detected using DAS ELISA (kits available from Agdia Inc. Elkhart, IN, USA).

As part of a larger study of the effects of the VRT on fitness, we germinated seeds in a greenhouse and in early May 2013, and, in late May, we transplanted 18 Texas gourd plants, 9 BC8 transgenic plants (tBC8) and 9 BC8 non-transgenic plants (ntBC8) from each of 5 families into each of four 0.4ha fields (180 total plants per field, 25% were transgenic) at the the Pennsylvania State University Agriculture Experiment Station at Rock Springs, PA. Two fields were adjacent and located > 1 km from two fields that were also adjacent. No insecticides were used on any of the four fields. In one pair of adjacent fields, 60 of the susceptible plants (40 Texas gourds and 20 ntBC8) were hand inoculated with ZYMV prior to transplanting using the carborundum rub method (see Simmons et al., 2013). No inoculated plants were transplanted into the remaining two fields. Over the course of the growing season, we recorded the presence of virus infection symptoms on all plants in all fields on a weekly basis. A sample

of plants exhibiting symptoms for the first time each week were tested using ZYMV immunostrips (Agdia Inc., Elkhard, IN, USA) to confirm the field diagnosis.

In order to determine if the probability that pollen from a transgenic plant will sire a seed on a susceptible plant changes as the incidence of ZYMV infection increases in the fields, we marked 10 female flowers on susceptible plants growing in each field on three dates between July 1 and August 15. At the end of the growing season, we harvested the marked fruits; removed and cleaned their seeds; and stored them. In April and early May 2013, we pooled the seeds produced from the fields with ZYMV inoculation and separately from the non-inoculated fields on each of the three dates; germinated random samples in the greenhouse; harvested leaf tissue from the seedlings; and scored the seedlings for the presence of the transgene (NPTII protein) using DAS-ELISA tests (as above). At this time, we have scored a total of 760 seedlings from the fields with and without ZYMV inoculation across the three time periods (early flowers, mid-season flowers, late season flowers.

Results

The transgene effectively deterred viral infections in the tBC8 plants in the virus inoculated fields although a few plants exhibited mild symptoms and tested positive for zucchini yellow mosaic virus in the ZYMV inoculated fields in late August. Figure 1 shows the incidence of virus infection among susceptible plants on the fields with and without ZYMV inoculation. In the non-inoculated (but unsprayed with insecticide) fields, the first plants with symptoms of ZYMV infection occurred in early August. By mid-August, however, only a few of the susceptible plants had symptoms of ZYMV in the non-inoculated fields.

In both the inoculated and the non-inoculated fields we scored 380 seedlings for the presence of the transgene. We found that the proportion of seeds sired by the transgene was not independent of the field type (chi-square = 12.6; df = 2; $0.001 < P < 0.005$). More seeds were sired by the transgene (32%) on the inoculated fields than on the non-inoculated fields (21%). However, we also found that the proportion of seeds sired by pollen with the transgene did not increase in the inoculated fields as the number of plants with virus symptoms increased (i.e., from early, to mid, to late in the flowering season). In fact, the proportion of the seeds sired by the transgene in the inoculated fields decreased significantly over the growing season (44 % early, 32 % mid, 27 % late; chi-square = 8.77; df = 3; $0.01 < P < 0.05$).

Discussion

Because the transgenic introgressives are hemizygous for the transgene, only half of the pollen grains will carry the transgene. Because 25% of the plants in each field were tBC8, we would expect that 12.5% of the seeds produced on the susceptible plants would be transgenic if the flowers from all plant types (Texas gourd, ntBC8, and tBC8) were equally attractive to pollinators and if all plant types produced the same number of male flowers, the same amount of pollen per flower, and if all pollen had equal competitive ability following germination on a stigma. Because ZYMV infection has been shown to decrease male flower production (e.g., Stephenson et al., 2004; Sasu et al., 2009) on the Texas gourd, we predicted that the proportion of transgenic progeny on susceptible plants would be greater on the inoculated fields than on the non-inoculated fields. Our data

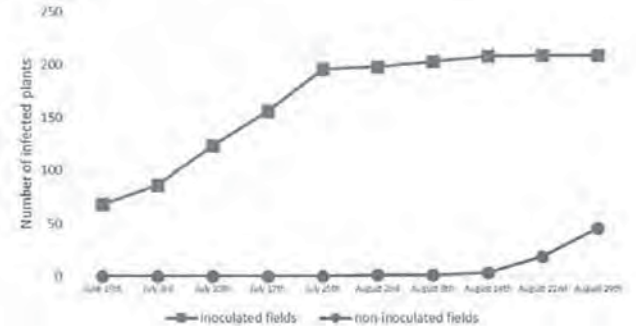

Fig. 1. Incidence of virus infection among susceptible plants on the fields with and without ZYMV inoculation.

clearly support this prediction. However, we also predicted that the proportion of transgenic progeny on susceptible plants in the inoculated fields would increase as the incidence of ZYMV increased during the growing season. Our findings clearly do not support this prediction and, in fact, we found that the proportion of seeds sired by the transgenic plants decreased significantly as the incidence of plants with viral symptoms increased! Clearly, there are additional factors that are important. Previous studies (Sasu et al., 2009) have shown that transgenic Texas gourd plants suffer greater exposure to *Erwinia tracheiphila* (the causative agent of bacterial wilt disease) and a greater incidence of the deadly bacterial wilt disease when viral diseases are present in the same population. Our ongoing research also suggests that ZYMV infected plants are more resistant to powdery mildew than healthy plants (non-virus infected, mostly transgenic plants by mid and late in the flowering season) and that powdery mildew adversely affects male flower production. Both of these diseases were also present in our fields and may account for the decline in the number of seeds sired by the transgenic plants over the course of the growing season.

Although the transgenic plants sired more seeds on the inoculated fields than on the non-inoculated fields, we are baffled by the fact that the transgenic plants sired significantly more seeds (21 %) than the expected 12.5% (chi-square = 25.4; df = 1; $P << 0.001$) on the non-inoculated fields suggesting that the transgenic plants have some advantage in terms of pollen production and/or pollen competitive ability. Because we used BC8 plants (with the Texas gourd as the recurrent parent), the advantages of the cultivar genes should have been mostly bred out of these plants. Moreover, one-third of the susceptible plants were the ntBC8 siblings of the tBC8 plants. An understanding of these findings awaits further experimentation.

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Comparative Analysis of Gene Expression Networks Underlying Winter Squash Fruit Quality

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Additional index words. *Cucurbita pepo*, acorn squash, carotenoids, dry matter, flavor

Abstract. Fruit quality in winter squash (*Cucurbita* spp.) is dependent on the combined processes of sugar, starch, and carotenoid metabolism, with higher levels of sugar, starch, and carotenoids providing enhanced flavor, texture, and nutrient content respectively. In order to investigate the genetic basis of these traits, we sequenced the fruit and seed transcriptome of a high-quality acorn squash, 'Sweet REBA,' and an oilseed pumpkin with a poor quality pericarp, 'Lady Godiva,' (both *Cucurbita pepo*) at five developmental time points ranging from 5–40 days after pollination. The acorn squash had a higher percent soluble solids and a higher percent dry matter, reflecting a higher sugar and starch content. The oilseed pumpkin had a greater overall carotenoid content, with both lutein and beta-carotene as the major carotenoids. In contrast, the acorn squash had lower overall levels of carotenoids, with beta-carotene as the major carotenoid. A bioinformatics analysis of the transcriptome identified the squash homologs of the candidate genes involved in sugar, starch, and carotenoid biosynthesis underlying these contrasts. Known rate-limiting genes in each of these networks were differentially expressed, consistent with their predicted involvement in their associated phenotype. This analysis will be the first study to describe the global molecular processes and gene expression networks underlying fruit quality in squash.

Fruit quality in winter squash (*Cucurbita* spp.) is influenced by sugar, starch, and carotenoid content. Sugar content determines the sweetness of winter squash and is positively correlated with consumer perception of squash flavor (Cumarasamy et al., 2002). Percent dry matter, largely determined by starch content, is positively correlated with texture (Corrigan et al., 2001). Carotenoid content controls fruit color and nutritional value and the primary squash carotenoids are β-carotene and lutein (Azevedo-Meleiro and Rodriguez-Amaya, 2007). Fruit quality as a whole is determined by the interaction of these factors, which are all quantitative traits with a complex inheritance (Irving et al., 1997; Lu and Li, 2008).

A deeper knowledge of the genetic basis of squash fruit quality will permit more efficient improvement of quality through breeding. The objective of this study was to perform transcriptome sequencing and fruit quality analyses to compare the biosynthetic networks underlying differences in fruit quality phenotypes throughout development and between contrasting cultivars.

Materials and Methods

Two contrasting *Cucurbita pepo* cultivars were used for fruit quality and RNA-seq analysis, 'Lady Godiva,' an oilseed pumpkin with poor eating quality, and 'Sweet Reba,' an acorn squash with high eating quality. Self-pollinated fruits were collected at five time points. Fruit mesocarp and seeds were flash-frozen in liquid nitrogen for RNA extraction and carotenoid analysis. In addition, mesocarp samples were taken for dry matter and soluble solids analysis.

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Mesocarp and seed mRNA from each time point were extracted using a Qiagen RNeasy kit and sequenced in three biological replicates, each consisting of two pooled samples from different fruit. Library preparation, sequencing, and bioinformatics were performed by the Center for Genomics and Bioinformatics at Indiana University. An Illumina TruSeq Sample Prep Kit was used for library preparation and barcoded samples were sequenced on two lanes of an Illumina HiSeq 2000. Read quality and adapter trimming were performed using Trimmomatic (Trimmomatic). The trimmed paired reads and singleton reads were normalized using Trinity's in silico normalization utility, normalize_by_kmer_coverage.pl (Grabherr et al., 2011). Following normalization, Velvet (Zerbino and Birney, 2008) and Oases (Schulz et al., 2012) were used to assemble the transcriptome. After assembly, reads were mapped to the transcriptome using Bowtie 2 (Langmead and Salzberg, 2012). Mapped reads per transcript were then counted and RPKM (reads per kilobase per million) was subsequently calculated. For further analysis, the longest unigene was chosen to represent each isogroup. Fruit quality gene homologs were identified using BLAST (Altschul et al., 1997).

Percent soluble solids was measured using a refractometer and percent dry matter was calculated through comparison of wet and dry weights of mesocarp samples. Mesocarp β-carotene and lutein content were measured using high-performance liquid chromatography using the protocol described in Van Eck et al. (2010).

Results and Discussion

Self-pollinated fruit were collected from 'Lady Godiva' and 'Sweet Reba' at 5, 10, 15, 20, and 40 days after pollination (DAP) and fruit quality factors were measured on the fruit mesocarp at each time point. The percentage of soluble solids was higher

in ‘Sweet REBA’ at all time points (Fig. 1A). In both cultivars, percent soluble solids stayed between 4 and 6 percent until 40 DAP, where it rose to over 9 percent in ‘Sweet REBA.’ Percent dry matter was also higher in ‘Sweet REBA’ at all time points (Fig. 1B). Both cultivars had similar changes in dry matter throughout development. Because both percent soluble solids and percent dry matter are correlated with fruit quality in winter squash (Corrigan et al., 2001; Cumarasamy et al., 2002), these results are consistent with the categorization of ‘Sweet REBA’ as high-quality and ‘Lady Godiva’ as low-quality.

The primary two carotenoids in the fruit mesocarp were lutein and β-carotene. ‘Lady Godiva’ had a higher lutein content, accumulating more than 5 μg/g fresh weight by 40 DAP, while ‘Sweet REBA’ accumulated very little lutein (Fig. 1C). Both ‘Lady Godiva’ and ‘Sweet REBA’ accumulated β-carotene, with ‘Sweet REBA’ accumulating approximately twice as much as ‘Lady Godiva’ (Fig. 1D). When both are considered, ‘Lady Godiva’ accumulated more total carotenoids than ‘Sweet REBA.’

Fruit mesocarp and seed RNA was extracted, sequenced, and assembled into a fruit and seed transcriptome consisting of 51,978 unigenes. Gene expression throughout fruit development was estimated by calculating the RPKM values for the unigenes. Squash homologs for fruit quality genes were identified based on the unigene annotations and by blasting with known fruit quality gene sequences.

In the carotenoid pathway, one homolog for DOXP synthase and two for phytoene synthase were identified. Both of these are known to be rate-limiting for total carotenoid synthesis (reviewed in Hirschberg, 2001). The expression patterns of these genes were similar in the two cultivars throughout fruit development, but were much higher in ‘Lady Godiva’ (Fig. 2, A–C), which is consistent with its higher carotenoid content. In addition, lycopene ε-cyclase and lycopene β-cyclase homologs were identified. These two genes are located where the carotenoid biosynthesis pathway diverges, with lutein and β-carotene on opposite sides of the branch point (Lu and Li, 2008). Lycopene ε-cyclase had a higher expression in ‘Lady Godiva,’ consistent with its higher lutein content, while lycopene β-cyclase had a higher expression in ‘Sweet REBA,’ consistent with its higher β-carotene content (Fig. 2, D–E).

The homologs of known sucrose and starch rate-limiting genes were also identified. Sucrose-phosphate synthase and sucrose-phosphate phosphatase had similar expression patterns (Fig. 3, A–B), with a higher expression in ‘Sweet REBA’ in all time points except 15 DAP. ADP-glucose pyrophosphorylase (large subunit) and AATPT (amyloplastidial ATP/ADP translocator) had fairly similar expression patterns and also had a higher expression in ‘Sweet REBA’ (Fig. 3, C–D). This differential expression of these known rate-limiting genes (Geigenberger et al., 2004; Stitt and Zeeman, 2012) is consistent with the higher percent soluble solids (related to sucrose content) and percent dry matter (related to starch content) of ‘Sweet REBA.’

By measuring fruit quality and examining the expression patterns of genes known to be involved in fruit quality, we have confirmed that the same biosynthesis genes influence squash carotenoid, sugar, and starch levels in squash as in other plants. This work will form the foundation of a deeper analysis of the regulatory networks that control the expression of these biosynthetic genes.

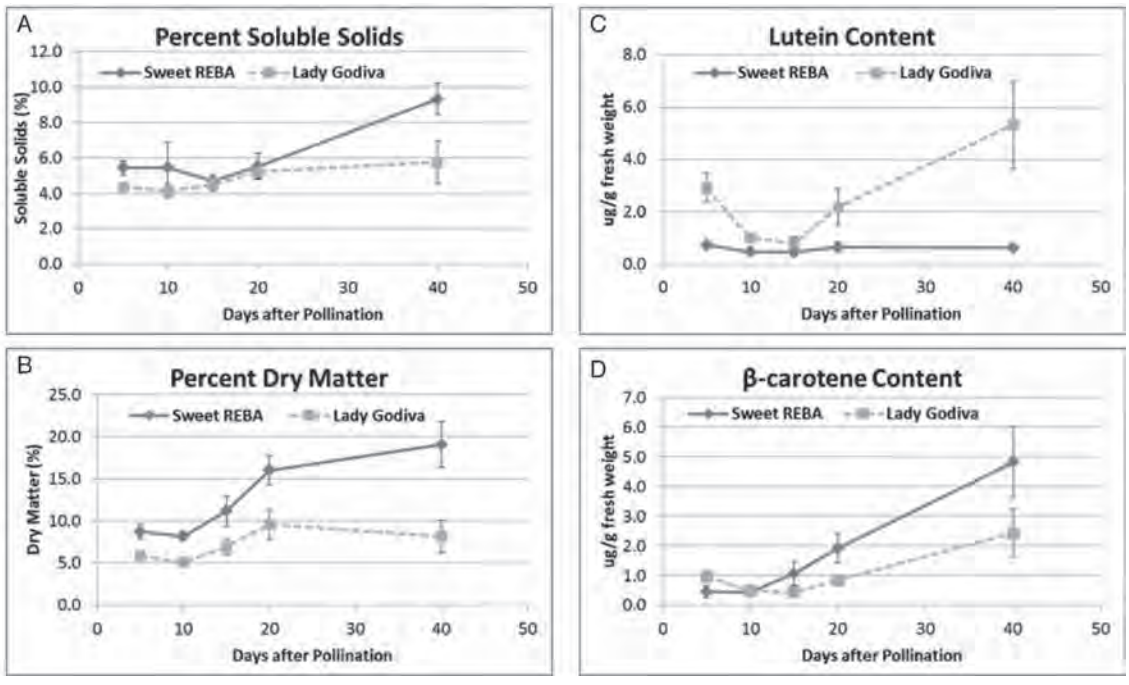

Fig. 1. Fruit quality factors of ‘Sweet REBA’ and ‘Lady Godiva’ squash throughout fruit development. Self-pollinated fruit were collected at 5, 10, 15, 20, and 40 days after pollination. All measurements were performed on fruit mesocarp and are averages of 6–9 fruit. Error bars indicate the standard deviation. **(A)** Percentage of soluble solids, measured using a refractometer. **(B)** Percentage of dry matter, measured through comparison of wet and dry weights. **(C)** Lutein, measured using HPLC. **(D)** β-carotene, measured using HPLC.

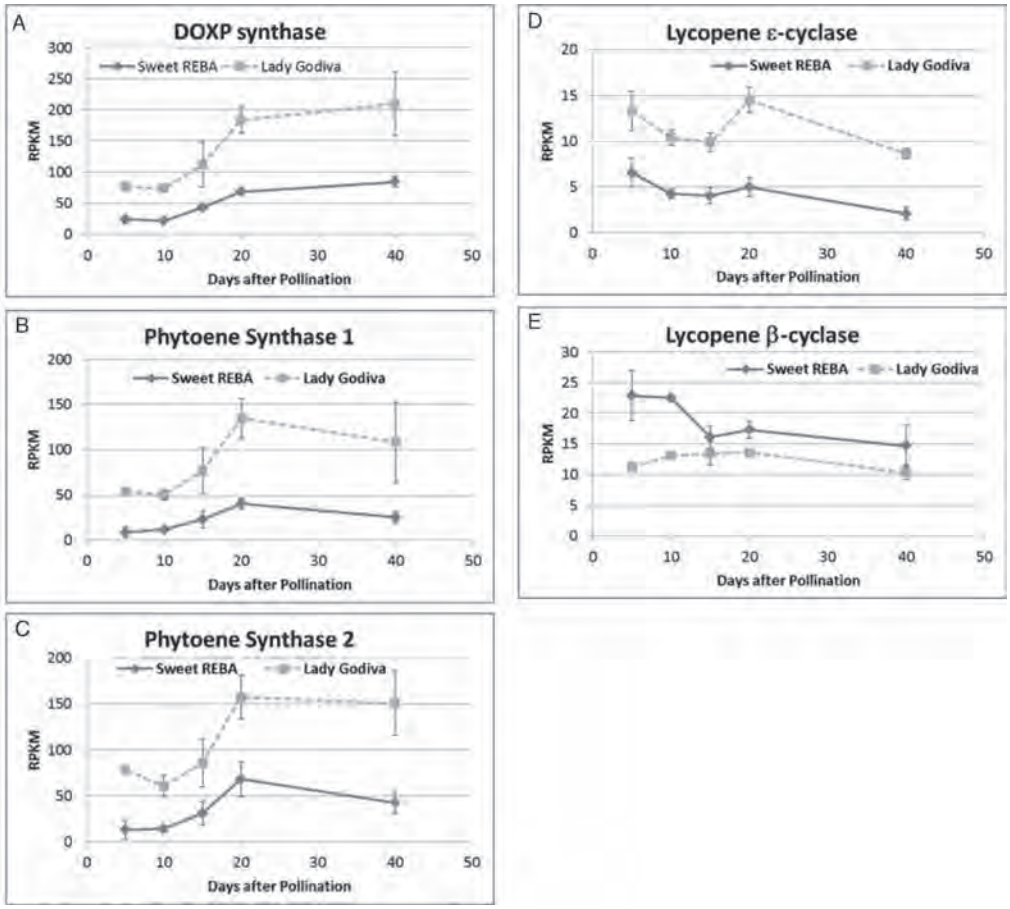

Fig. 2. Gene expression of carotenoid pathway rate-limiting and branch point enzymes. Graphs display the gene expression of the only identified homologs of these carotenoid pathway rate-limiting and branch point genes. RPKM gene expression values of both genotypes are displayed over the five developmental time points, averaged from three biological replicates. Error bars indicate the standard deviation.

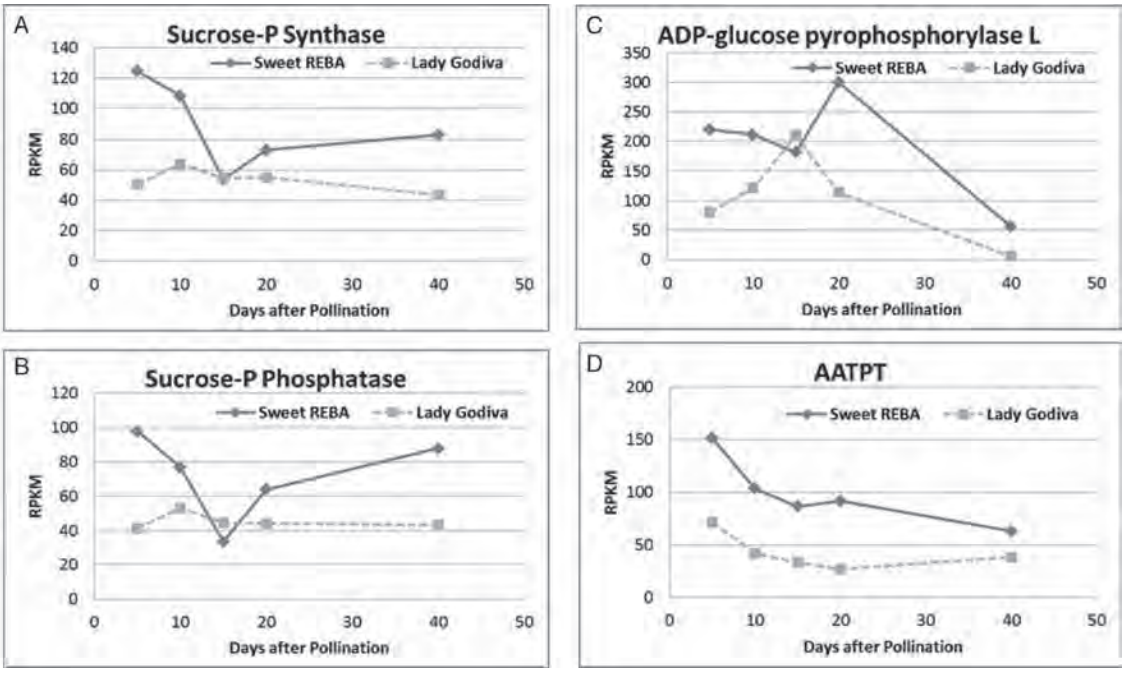

Fig. 3. Sucrose and starch synthesis gene expression. Multiple homologs were identified for each sucrose **(A–B)** and starch **(C–D)** synthesis gene. Graphs display the gene expression of the unigene with the highest expression level. RPKM gene expression values of both genotypes are displayed over the five developmental time points, averaged from three biological replicates. Error bars indicate the standard deviation.

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Genetic Factors Associated with Seed Oil Percentage and Fatty Acid Composition of Watermelon Seed

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Additional index words. egusi, kernel percentage, seed size, palmitic, stearic, oleic, linoleic

Abstract. Seed oil percentage (SOP) and fatty acid composition of watermelon (*Citrullus lanatus*) seeds are important traits in Africa where the seeds of the egusi watermelon provide a significant source of nutrition and income. To elucidate the nutritive value of watermelon seed, a study was carried out to identify the genetic factors associated with SOP and fatty acid composition. Seeds from an F_2 population developed from a cross between an egusi type (PI 560023) and Strain II (PI 279261) were phenotyped for SOP, kernel percentage (KP), seed size traits, palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids. The egusi locus on chromosome (Chr.) 6 explained 83% of the variation in SOP in the population. KP and seed size traits showed significant positive and negative correlations ($P < 0.05$) with SOP respectively and mapped on Chr. 1, 5 and 6. A total of 8 QTL were associated with fatty acid composition with a QTL for oleic and linoleic acid co-localizing on Chr. 6. Eighty genes involved in fatty biosynthesis were identified from the functionally annotated genes on the watermelon draft genome. Seven genes encoding acyl-(acyl-carrier-protein) (ACP) desaturase, fatty acyl-ACP thioesterase B, fatty acid elongase and omega-3-fatty acid desaturase enzymes were found within or in close proximity to the QTL identified for fatty acids in this study.

Watermelon is an economically important member of the Cucurbitaceae family cultivated mainly for its edible flesh (Robinson and Decker-Walters, 1997). The seeds of watermelon also provide an important source of nutrition and income in some parts of the world including Iran (Baboli and Kordi, 2010), Africa (Al-Khalifa, 1996) China (Zhang, 1996), and Israel (Edelstein and Nerson, 2002). The egusi watermelon (*Citrullus lanatus* ssp. *mucosospermus* var. *egusi*), is popularly cultivated for its edible seeds in Africa (Gusmini et al., 2004). Characterized by a thick fleshy pericarp, the seed of the egusi watermelon is highly nutritious with a high seed oil percentage (SOP) and protein content (Gusmini et al., 2004; Jarret and Levy, 2012). Therefore, egusi seeds play a crucial role in supplementing the nutrients of the staple carbohydrate foods of the poor, who cannot afford animal-derived protein foods (Achu et al., 2005). The seeds are manually dehulled to separate the kernels from the seed coat and eaten raw, roasted, made into soup or processed into cooking oil (Al-Khalifa, 1996). To facilitate marker-asssisted breeding for oil quality and quantity traits in watermelon seed, it is of interest to breeders to understand the genetic factors associated with SOP and fatty acid compositon. Therefore several studies were carried out in an effort to elucidate the genetic factors associated with these traits in watermelon.

Materials and Methods

An F_2 population (n = 142) was developed from a cross between Strain II of the Japanese cultivar Yamato-cream (PI 279261; normal seed type) and an egusi type from Nigeria (PI 560023). Single nucleotide polymorphism assays were performed on the parents and progeny using an Illumina's GoldenGate SNP array and BeadStudio software (Illumina, San Diego, CA) as described in Sandlin et al. (2012). A genetic map was developed that included 357 SNP markers spanning 14 linkage groups (LGs) with an average gap of 4.2 cM between markers (Sandlin et al., 2012).

SOP measurements for the parental lines, F_1, and progeny were determined using nuclear magnetic resonance [NMR (MiniSpec MQ20, NMR Analyzer, Bruker Optics Inc., Billerica, MA)] (Prothro et al., 2012). The kernel percentage (KP) was measured as the weight of 15 manually dehulled seed as a percentage of the weight of the intact seed and seed size was measured as the weight of 100 seeds (100SWT), the average seed length (SL) and seed width (SWD) of 5 randomly chosen seeds from each line (Meru and McGregor, 2013). The 15 seeds from each F_2 plant and parental lines manually dehulled to determine KP were used for fatty acid analysis (Meru and McGregor, 2014). Fatty acid methyl esters were prepared using the standard method for analyses of fatty acid composition in fats and oils recommended by the American Oil Chemist's Society (Ce 1-62; American Oil Chemist's Society, 2009) and separated on a GC column in a gas chromatograph. Pearson correlations were calculated to determine the relationship between traits using JMP (SAS Institute Inc., Cary, NC).

QTL for SOP were detected by multiple interval mapping while those for seed size traits, KP and fatty acid composition were detected by composite interval mapping in WinQTL Cartographer version 2.5 (Wang et al., 2011) where the statistical significance of a QTL was determined by likelihood-odds (LOD) thresholds set by 1000 permutations ($\alpha = 0.05$) (Churchill and Doerge, 1994). Fatty acid biosynthesis genes in watermelon were determined by comparing annotated genes in the watermelon draft genome (Guo et al., 2013) with genes involved in fatty acid biosynthesis for other crops (Li et al., 2010). Markers flanking the 1.5-LOD [~95% confidence interval] interval for all the QTL were identified on the linkage map and their corresponding positions on the watermelon physical map (Guo et al., 2013) determined. The regions between the flanking markers were then inspected for genes in the fatty acid biosynthesis pathway.

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Results and Discussion

The range of SOP in watermelon seed reported for the population (17.8 % to 37.8 %; Prothro et al., 2012) and that reported by Jarret and Levy (2012) (14.8 % to 43.5 %) indicate a wide variation in SOP among watermelon accessions. There is a clear delineation in SOP between the normal and the egusi seed types with the latter having a higher SOP (Prothro et al., 2012). Four QTL controlling SOP in watermelon seed have been mapped on Chr. 2 and 6 (Prothro et al., 2012). The *egusi* locus that controls the thick fleshy pericarp in the egusi seed mapped to the same region with the main QTL for SOP (R^2 ~84%; Table 1) on Chr. 6. The co-localization of these two traits indicate that the high SOP observed in egusi seed is due to the *egusi* locus which explains the delineation in SOP between the normal and the egusi seed types.

Variation in SOP within either normal (n =100) and egusi (n = 42) seed type has been reported in several studies (Jarret and Levy, 2012; Prothro et al., 2012) and points to additional genetic factors contributing to SOP. Previous studies have suggested that seed size and KP play a role in SOP (Jarret and Levy, 2012; Prothro et al., 2012). 100SWT, SL and SWD were found to be negatively correlated with SOP in both normal and egusi seed type but KP was positively correlated with SOP (Meru and McGregor, 2013). The QTL for seed size and KP in normal and egusi were mapped on Chr. 1, 5 and 6 (Table 1) (Meru and McGregor, 2013). It is important however to note that the QTL for KP in normal and egusi seeds seem to be controlled by different loci. Breeding for improved SOP through the indirect selection of seed size traits and KP can be implemented in watermelon as is the case in sunflower (Tang et al., 2006).

Linoleic acid was the predominant fatty acid in watermelon seed (Meru and McGregor, 2014) and was significantly ($P < 0.05$) negatively correlated to palmitic (–0.37), stearic (–0.21) and oleic (–0.92) acids (Meru and McGregor, 2014). Therefore, breeding for simultaneous improvement in linoleic acid, palmitic, stearic and oleic may present a challenge given this relationship between the fatty acids. Eight QTL associated with fatty acid composition were mapped on Chr. 2, 3, 5, 6, 7, and 8 (Table 1) (Meru and McGregor, 2014). Seven of these QTL explain more than 10 % of the phenotypic variation (Table 1) in the fatty acids and present possible targets for marker-assisted breeding. Seven genes encoding acyl-(acyl-carrier-protein) (ACP) desaturase, fatty acyl-ACP thioesterase B, fatty acid elongase and omega-3-fatty acid desaturase enzymes were found within or in close proximity to the QTL identified for fatty acids in this study (Meru and McGregor, 2014, in press). Further study is required to determine the functional contribution of the candidate genes to variation in fatty acids so as to develop functional markers.

Through the study of functionally annotated genes in the watermelon draft genome (Guo et al., 2013), a total of 80 genes that are involved in fatty acid biosynthesis in watermelon were identified (Meru and McGregor, 2014). These genes encode enzymes that modulate the ratio of saturated and unsaturated fatty acids thus determine the end use of a particular vegetable oil. Among these, five genes potentially encoding fatty acyl-ACP thioesterase-B, 12 genes potentially encoding acyl-ACP desaturases and four

Table 1. Positions (cM) and the corresponding 1.0-likelihood-odds (LOD) support interval associated with seed oil percentage (SOP) (Prothro et al., 2012), kernel percentage (KP), 100 seed weight (100SWT), seed length (SL) (Meru and McGregor, 2013) and fatty acid composition in the Strain II (PI 279261) x Egusi (PI 560023) F_2 watermelon population (Meru and McGregor, 2014).

Trait	LG[z]	Position (cM)	Chr[y]	LOD[x]	R^2 (%)[w]	Additive effect[v]	Dominance effect[v]
SOP	2	42.6	6	1.90	–5.95	0.009	0.009
SOP	2	57.7	6	18.94	83.88	–0.048	–0.072
SOP	2	81.0	6	4.50	9.26	–0.002	0.003
SOP	9B	86.2	2	3.48	1.31	0.006	0.014
KP[u]	2	36.6	6	5.60	22.30	0.020	–0.010
100SWT[u]	2	38.6	6	17.40	62.90	–3.730	–1.010
SL[u]	2	33.6	6	23.10	60.80	–1.740	–0.340
	6	88.0	5	4.20	7.90	–0.800	–0.180
SWD[u]	2	36.0	6	13.60	34.40	–0.880	–0.220
KP[t]	7	102.2	1	5.83	33.80	1.250	–6.830
Palmitic	9B	106.7	2	3.67	7.55	0.200	–0.440
Palmitic	11B	44.9	3	10.70	24.73	0.670	0.350
Palmitic	6	188.0	5	4.94	12.67	–0.450	–0.120
Stearic	8	7.9	7	3.85	10.17	–0.850	–0.820
Oleic	9B	3.0	2	5.13	10.67	–1.650	0.040
Oleic	2	36.6	6	6.43	17.86	–1.690	–2.060
Oleic	4	17.6	8	6.46	13.48	1.780	–0.630
Linoleic	2	36.6	6	5.88	21.46	1.590	2.090

[z] Linkage group in Sandlin et al. (2012).
[y] Chromosome location in the draft watermelon genome sequence (Guo et al., 2013).
[x] Log_{10} likelihood ratio.
[w] Phenotypic variation explained.
[v] Negative values indicate that the effect is contributed by the allele from the egusi parent (PI 560023).
[u] Trait mapped in normal seed individuals
[t] Trait mapped in egusi seed individuals

genes potentially encoding omega-6 fatty acid desaturase-2 were identified. These fatty acid genes are similar in number to those reported for peanut (*Arachis hypogaea*) (Yin et al., 2013) and Jatropha (*Jatropha curcas*) (Natarajan and Parani, 2011).

The variation in SOP and fatty acid composition reported for watermelon genebank accessions by Jarret and Levy (2012) indicate that conventional breeding can be used to develop watermelon cultivars of superior oil quality (Meru and McGregor, 2014).

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Identification of Quantitative Trait Loci (QTL) in Watermelon (*Citrullus lanatus*)

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Additional index words: SNP, genotype-by-sequencing, fruit, seed, flower, fatty acid

Abstract. In the past, watermelon (*Citrullus lanatus*) lagged behind other important cucurbits like melon and cucumber when it came to genomic tools. However, recent advances in watermelon genomics enabled us to identify quantitative trait loci (QTL) associated with several important traits, including fruit traits (size, shape, rind thickness and brix), seed traits (size, oil and fatty acid composition) and flower traits (sex expression and flowering time). The draft watermelon genome sequence made it possible to identify candidate genes in the regions of interest. For example, flowering time was found to be oligogenic with a major, stable, co-localized QTL on chromosome 3 responsible for ~50% of the observed phenotypic variation. Within this region, homologues of the *flowering locus T* (*Cla009504)* and *tempranillo 1* (*Cla000855)* genes were identified as candidate genes. The advances in watermelon genomics now need to be translated into practical, validated markers for marker assisted selection to expedite watermelon breeding. In addition, the next wave of trait associations needs to tackle more complex traits like disease resistance and drought tolerance. This will require the development of reliable, high throughput phenotyping methods since the availability of basic genomic tools is no longer a limiting factor in trait associations for watermelon.

Watermelon was responsible for approximately 9.6% of the total world vegetable tonnage produced in 2009 (Food and Agriculture Organization of the United Nations, 2011). In the US, watermelon production acreage was 132,600 acres in 2010 with a value of ~$500 million. Consumption of watermelon in the US has increased by 37% from 1980 levels to ~7.2 kg per capita, mainly due to the popularity of seedless cultivars (Wehner, 2008).

The genus *Citrullus* (2n = 2x = 22) includes four species; *C. lanatus, C. rehmii*, *C. ecirrhosus* Cogn., and *C. colocynthis* (Robinson and Decker-Walters, 1997). Two different systems are currently used in scientific literature for the infra-specific classification of *C. lanatus*. In this manuscript we will use the classification as described by Robinson and Decker-Walters (1997) and used by the USDA germplasm collection, where, *C. lanatus* is divided into *C. lanatus* var. *lanatus,* which includes the elite cultivars, *C. lanatus* var. *citroides (*citron types) and *C. lanatus* subsp. *mucosospermus* (egusi types) (Fursa, 1972).

Historically the application of marker assisted selection (MAS) in watermelon breeding programs has been limited by a lack of high-throughput DNA markers and genetic mapping information (Levi et al., 2004; Levi et al., 2006). Due to the limited observed marker polymorphisms within *C. lanatus*, and particularly among elite watermelon cultivars (Guo et al., 2013; Levi et al., 2001), early mapping studies focused on inter-specific or inter-subspecific crosses, where marker distortion is common. However the development of single nucleotide polymorphism (SNP) technology (Henry, 2008) and genotyping-by-sequencing makes high throughput mapping possible and enables comparative mapping across several populations and mapping of elite × elite populations.

Breeding efforts in watermelon have largely concentrated on fruit quality and morphological characteristics. These characteristics include fruit size and shape, sugar content (Brix), flesh color, and rind patterns (Wehner, 2008). Due to the preference for seedless fruit, US watermelon production is dependent on synchronized flowering of diploid pollenizers and triploid watermelon cultivars for fruit production. This makes flowering time an important emerging trait, since monoecious watermelon is grown as a dioecious crop under this system. A number of seed traits are also important in watermelon breeding. Seed size is important because watermelon breeders aim to develop hybrid cultivars with large seed for planting (especially where direct seeding is used), but that will produce fruit with small seed. In areas of the world where seed is eaten, seed oil percentage and seed protein content are important traits. In West Africa the egusi watermelon is popular due to its large, flat seeds that are can contain up to 50 % oil (Achu et al., 2005) and 28 % protein (Achu et al., 2005; Bankole et al., 2005).

Our goals were to use bi-parental populations and SNP markers to develop genetic maps representing the primary watermelon gene pool and to use these resources to identify QTL associated with horticulturally important traits in the crop.

Materials and Methods

Four bi-parental mapping populations, representing the watermelon gene pool were developed: 'Klondike Black Seeded' × 'New Hampshire Midget' (K×N) F_6 recombinant inbred line (RIL) population, Strain II × Egusi (S×E) F_2 population, ZWRM50 × Citroides (Z×C) F_2 population (Sandlin et al., 2012) and 'Sugar Baby' × 'Calhoun Gray' (SB×CALG) F_2 population. SNP genotyping for the first three populations were carried out using the Illumina's GoldenGate_SNP array and BeadStudio_software

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Table 1. Genomic regions in watermelon draft genome sequence (Guo et al., 2013) associated with QTL in the 'Klondike Black Seeded' × 'New Hampshire Midget' (K×N), Strain II × PI 560023 (S×E) and ZWRM50 × PI 244019 (Z×C) watermelon populations.

Trait	Population†	QTL name‡	Chr	Physical position (Mbp)	Reference
Andromonoecious	Z×C	*Qand3*^{M}	3	25.11–26.73	Prothro et al. (2013)
Andromonoecious	Z×C	*Qher6*	6	3.77–5.04	Prothro et al. (2013)
Male Flowers (%)	Z×C	*Qmal1*	1	17.71–23.89	Prothro et al. (2013)
100 seed weight	K×N; Z×C	*Q100swt2-1*^{I}	2	27.19–28.85	Prothro et al. (2012a)
100 seed weight	Z×C	*Q100swt2-2*^{I}	2	28.24–31.96	Prothro et al. (2012a)
100 seed weight	K×N; S×E; Z×C	*Q100swt6*^{M}	6	4.58–6.44	Prothro et al. (2012a); Meru & McGregor (2013)
100 seed weight	K×N	*Q100swt8*	8	20.56–23.41	Prothro et al. (2012a)
° Brix	K×N^{L}	*Qbrx1*	1	31.42–32.48	Sandlin et al. (2012)
° Brix	S×E	*Qbrx2-1*^{I}	2	15.4–19.15	Sandlin et al. (2012)
° Brix	K×N	*Qbrx2-2*^{I}	2	28.24–29.34	Sandlin et al. (2012)
° Brix	K×N	*Qbrx7*	7	30.2–31.21	Sandlin et al. (2012)
Days to female flower	K×N^{Y}	*Qdff3-1*^{M}	3	12.8–15.66	McGregor et al. (2014)
Days to female flower	K×N	*Qdff3-2*	3	26.95–28.03	McGregor et al. (2014)
Days to female flower	K×N	*Qdff11*	11	7.78–10.3	McGregor et al. (2014)
Days to male flower	K×N	*Qdmf2*	2	28.85–30.18	McGregor et al. (2014)
Days to male flower	K×N^{Y}	*Qdmf3-1*^{M}	3	12.8–15.66	McGregor et al. (2014)
Days to male flower	K×N	*Qdmf3-2*	3	24.78–25.98	McGregor et al. (2014)
Egusi (*eg*) locus	S×E	*eg*	6	6.75–11.03	Prothro et al. (2012b)
Female Flowers (%)	Z×C	*Qfem10*	10	21.43–23.42	Prothro et al. (2013)
Fruit length	S×E	*Qfl2-1*	2	15.4–19.15	Sandlin et al. (2012)
Fruit length	K×N^{L}; S×E; Z×C	*Qfl2-2*^{I}	2	27.19–29.63	Sandlin et al. (2012)
Fruit length	K×N^{L}; Z×C	*Qfl3*^{M}	3	25.98–26.95	Sandlin et al. (2012)
Fruit length	S×E	*Qfl5*	5	6.23–6.98	Sandlin et al. (2012)
Fruit length	Z×C	*Qfl10*	10	26.93–27.48	Sandlin et al. (2012)
Female-Male interval	K×N	*Qfmi2*^{I}	2	28.24–29.34	McGregor et al. (2014)
Female-Male interval	K×N	*Qfmi3*^{I}	3	10.15–20.69	McGregor et al. (2014)
Fruit shape index	K×N	*Qfsi2*	2	29.34–31.96	Sandlin et al. (2012)
Fruit shape index	K×N^{L}; Z×C	*Qfsi3*^{M}	3	26.95–25.98	Sandlin et al. (2012)
Fruit shape index	K×N	*Qfsi10*	10	11.79–14.46	Sandlin et al. (2012)
Fruit width	S×E	*Qfwd2-1*^{I}	2	15.4–20.27	Sandlin et al. (2012)
Fruit width	K×N^{L}; S×E; Z×C	*Qfwd2-2*^{M}	2	28.24–30.25	Sandlin et al. (2012)
Fruit width	K×N^{L}	*Qfwd3*	3	28.03–26.95	Sandlin et al. (2012)
Fruit width	Z×C	*Qfwd4*	4	15.96–22.13	Sandlin et al. (2012)

(continued)

(Illumina, San Diego, CA, USA) as described in Sandlin et al. (2012). The SBxCALG population was genotyped using genotype-by sequencing at the Institute for Genomic Diversity (Cornell University Ithaca, NY) (Elshire et al., 2011) and the watermelon draft genome was used as reference (Guo et al., 2013).

Linkage maps were constructed using JoinMap version 4 (Van Ooijen, 2006) and composite interval mapping (CIM; Model 6 and walk speed of 1 cM (Zeng, 1994; Zeng et al., 1999) or multiple interval mapping (MIM) (Kao et al., 1999) in WinQTL Cartographer version 2.5 (Wang et al., 2011) was used to identified QTL associated with fruit (Sandlin et al., 2012), seed (Meru and McGregor, 2013; Meru and McGregor, 2014; Prothro et al., 2012a; Prothro et al., 2012b) and flower traits (McGregor et al., 2014; Prothro et al., 2013). QTL were considered minor if R^2 was less than 10%, intermediate if R^2 was between 10% and 25% and major if R^2 was more than 25%.

Results and Discussion

Sixty two QTL (Table 1) were associated with the traits of interest in the bi-parental populations (McGregor et al., 2014; Meru and McGregor, 2013; Meru and McGregor, 2014; Prothro et al., 2013; Prothro et al., 2012a; Prothro et al., 2012b; Sandlin et al., 2012). These included major QTL for the andromonoecious trait (*Qand3*^{M}), fruit size and shape (*Qfsi3*^{M}, *Qfwd2-2*^{M}, *Qfwt2-2*^{M}), rind thickness (*Qrth2-1*^{M}), days to flowering (*Qdff3-1*^{M}; *Qdmf3-1*^{M}), seed size (*Q100swt6*^{M}, *Qsl6*^{M}, *Qswd6*^{M}), seed kernel percentage (*Qker*1^{M}) and seed oil percentage (*Qsop6-2*^{M}). Eleven of the QTL were detected in more than one genetic background (population) and a number of QTL were identified at multiple locations or years in the KxN RIL population Table 1). QTL that are stable across genetic backgrounds and/or environments are highly desirable are targets for markers assisted selection. The location of the egusi (*eg*) locus was identified on chromosome 6 and this region was associated with a major QTL for seed oil percentage (*Qsop6-2*^{M}) in the SxE population (Prothro et al., 2012b).

The availability of the watermelon draft genome (Guo et al., 2013) made it possible to identify candidate genes in the regions of interest for some of the traits of interest. For the major QTL associated with the andromonoecious trait (*Qand*3^{M}), *Cla011230,* a homologue of the 1-aminocyclopropane-1-carboxylic acid synthase (ACS) genes causing the andromonoecious trait in melon and cucumber (Boualem et al., 2008; Boualem et al., 2009) were identified in the region of interest (Prothro et al., 2013). Two candidate genes, a homologue of the *flowering locus T* (*Cla009504)* and *tempranillo 1* (*Cla000855)* genes associated with flowering

Table 1 (continued). Genomic regions in watermelon draft genome sequence (Guo et al., 2013) associated with QTL in the 'Klondike Black Seeded' × 'New Hampshire Midget' (K×N), Strain II × PI 560023 (S×E) and ZWRM50 × PI 244019 (Z×C) watermelon populations.

Trait	Population†	QTL name‡	Chr	Physical position (Mbp)	Reference
Fruit weight	S×E; Z×C	*Qfwt2-1*^{I}	2	13.3–23.46	Sandlin et al. (2012)
Fruit weight	K×N; SxE	*Qfwt2-2*^{M}	2	28.24–30.56	Sandlin et al. (2012)
Fruit weight	S×E	*Qfwt5-1*	5	4.75–6.98	Sandlin et al. (2012)
Fruit weight	K×N	*Qfwt5-2*	5	25.42–26.2	Sandlin et al. (2012)
Fruit weight	Z×C	*Qfwt3*	3	20.69–10.15	Sandlin et al. (2012)
Seed Kernel	S×E	*Qker1*^{M}	1	11.22–14.38	Meru & McGregor (2013)
Seed Kernel	S×E	*Qker6*^{I}	6	3.77–6.44	Meru & McGregor (2013)
Linoleic acid	S×E	*Qlin6*^{I}	6	3.77–6.44	Meru & McGregor (2014)
Oleic acid	S×E	*Qole2*^{I}	2	15.4–19.15	Meru & McGregor (2014)
Oleic acid	S×E	*Qole6*^{I}	6	3.77–6.44	Meru & McGregor (2014)
Oleic acid	S×E	*Qole8*^{I}	8	14.33–17.39	Meru & McGregor (2014)
Palmitic acid	S×E	*Qpal2*	2	29.63–31.13	Meru & McGregor (2014)
Palmitic acid	S×E	*Qpal3*^{I}	3	0.33–3.36	Meru & McGregor (2014)
Palmitic acid	S×E	*Qpal5*^{I}	5	26.2–29.07	Meru & McGregor (2014)
Rind thickness	K×N^{L}; S×E	*Qrth2-1*^{M}	2	28.24–30.25	Sandlin et al. (2012)
Rind thickness	K×N	*Qrth5*	5	26.67–30.03	Sandlin et al. (2012)
Rind thickness	S×E	*Qrth6*^{I}	6	4.58–6.75	Sandlin et al. (2012)
Seed length	Z×C	*Qsl2*^{I}	2	29.63–31.96	Prothro et al. (2012a)
Seed length	Z×C	*Qsl3*	3	5.07–10.15	Prothro et al. (2012a)
Seed length	K×N; S×E; Z×C	*Qsl6*^{M}	6	4.58–6.44	Prothro et al. (2012a); Meru & McGregor (2013)
Seed length	K×N	*Qsl8*	8	20.56–23.41	Prothro et al. (2012a)
Seed length	S×E	*Qsl5*	5	8.81–9.43	Meru & McGregor (2013)
Seed oil percentage	S×E	*Qsop2*	2	27.52–29.67	Prothro et al. (2012b)
Seed oil percentage	S×E	*Qsop6-1*	6	4.58–6.75	Prothro et al. (2012b)
Seed oil percentage	S×E	*Qsop6-2*^{M}	6	6.75–11.03	Prothro et al. (2012b)
Seed oil percentage	S×E	*Qsop6-3*	6	11.03–12.65	Prothro et al. (2012b)
Stearic acid	S×E	*Qste7*^{M}	7	29.41–30.82	Meru & McGregor (2014)
Seed Width	Z×C	*Qswd2*^{M}	2	28.24–31.96	Prothro et al. (2012a)
Seed Width	K×N; S×E; Z×C	*Qswd6*^{M}	6	4.58–6.44	Prothro et al. (2012a); Meru & McGregor (2013)

† QTL detected in multiple locations (L) or years (Y) in the same population

‡ QTL were considered intermediate (I) if R^2 was between 10% and 25% and major (M) if R^2 was more than 25%.

time in other species were identified in the chromosomal region associated with days to flowering (*Qdff3-1*^{M}; *Qdmf3-1*^{M}) (McGregor et al., 2014).

Despite the advances in watermelon genomic tools of the past few years, the application of markers for marker assisted selection in breeding programs still lag behind. One limitation of the three initial bi-parental genetic maps used for QTL mapping was the relatively small number of markers on the maps (KxN = 378, SxE = 357 and ZxC = 338) and the relatively high cost per data point. We therefore used genotype-by-sequencing for simultaneous SNP discovery and genotyping (Elshire et al., 2011) in the SBxCALG F_2 population. Despite the low diversity among elite watermelon cultivars, 1,195 polymorphic SNP markers were segregating in this population (G. Meru, unpublished data), confirming that genotyping can be carried out quickly and relatively cheaply and is no longer a limiting factor in translational genomic studies for watermelon.

In the future, more complex traits like disease resistance and drought tolerance should be tackled. As is true with many crops, the limiting factor in finding association between genomic regions in watermelon is no longer genotyping, but phenotyping. Phenotyping watermelon and other cucurbits is specifically challenging due to their horizontal vine growth and the relatively large area required per plant. As phenotyping in many row crops are moving toward high throughput methods, cucurbit researchers will have to come up with innovative, novel ideas on how to adapt and apply this phenotyping revolution in modern plant breeding to these crops.

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Genotyping-by-Sequencing Yields Dense Marker Datasets Effective for Genetic Characterization of Squash

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Additional index words. *Cucurbita pepo*, GBS, SNP, gene mapping

Abstract. In recent years, the use of next-generation sequencing (NGS) technologies to generate high-density single nucleotide polymorphism (SNP) markers has greatly expanded the capacity in many crop species for trait mapping, germplasm characterization, and marker-assisted and genomic selection. Genotyping-by-sequencing (GBS) is a method that uses NGS to identify SNPs in a reduced representation library generated through restriction enzyme digestion. Advantages of GBS over other marker systems include its low cost, high-throughput nature, lack of ascertainment bias, and adaptability to all populations of interest. In the squash species *Cucurbita pepo*, we report the use of GBS to generate thousands of SNP markers for application in population characterization and mapping of traits important to growers and consumers.

Historically, molecular marker datasets for *Cucurbita* have covered the genome at low-density. These datasets have been successfully used to characterize genetic diversity and develop genetic maps (Gong et al., 2012; Zraidi et al., 2007; Gong et al., 2008; Esteras et al., 2012). The capacity of low-density datasets, however, to elucidate the molecular basis of important phenotypes is limited (Beissinger, 2013). To date, no genes in *Cucurbita* spp. have been isolated through map-based, forward genetics approaches. Additionally, few markers have been used for marker-assisted selection (MAS) in squash, even though the development of markers, especially those linked to Mendelian genes conferring disease resistance, have been ranked a top priority by breeders and growers (VBI, personal communication).

In many crop species, next-generation sequencing technologies (NGS) have facilitated the development of high-density marker datasets based on SNPs, which are abundant in all plant genomes (Chen et al., 2011). While whole genome sequencing remains expensive, de novo discovery of SNP markers via direct sequencing of genomic fragments in reduced representation libraries (RRLs) allows researchers to saturate the genome with markers at relatively low cost (Davey et al., 2011). Genotyping-by-sequencing (GBS), one method of RRL sequencing, has been widely used for genetic analysis of crop species in recent years, and for some cucurbits, including watermelon and squash, GBS has been used to generate thousands of SNPs (Reddy, 2014; Mazourek, unpublished data). GBS requires restriction enzyme digestion of DNA from individual plant samples, ligation of sample-specific barcode adaptors, amplification of ligated fragments, multiplexing of samples, sequencing, and downstream SNP calling and filtering (Elshire et al., 2011). Advantages of GBS include the ability to adjust genomic coverage and genome region specificity through enzyme selection, cost-efficiency through sample multiplexing, and the elimination of SNP ascertainment bias, a common obstacle associated with other genotyping methods, e.g., SNP arrays.

In this study, we report the use of GBS to generate SNP markers in *Cucurbita pepo*, with applications in trait mapping and germplasm characterization.

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Materials and Methods

Germplasm for this study consisted of two populations. The first comprised a diverse panel of over 90 *Cucurbita pepo* cultivars and breeding lines representing the cocozelle, pumpkin, vegetable marrow, zucchini, acorn, crookneck, scallop, and straightneck morphotypes of *C. pepo* subsp. *ovifera* and *C. pepo* subsp. *pepo,* in addition to several accessions of *Cucurbita okeechobeensis* subsp. *martinezii* and *Cucurbita moschata*. The second population was a *C. pepo* zucchini biparental mapping population consisting of 80 F_2 individuals. Genotypes were grown in the field near Ithaca, NY, and scored for disease resistance and fruit traits. Phenotypes for the diverse panel were scored on a plot basis, and phenotypes for the biparental population were scored on a per-plant basis.

For genotyping, DNA was isolated from each genotype using the Qiagen DNeasy Plate Extraction kits. 96-plex GBS library preparation followed the protocol by Elshire et al. 2011 using the ApekI restriction enzyme. Libraries were sequenced on an Illumina HiSeq 2000. SNPs were called using pipelines implemented in the 'Stacks' and 'Tassel' softwares, and custom scripts were developed to filter data based on sample call rate, minor allele frequency, and genotype segregation distortion. Traits of interest were mapped using R/qtl and TASSEL softwares.

Results and Discussion

For all *C. pepo* genotypes, thousands of SNP markers were identified using GBS with the ApekI restriction enzyme. Fewer SNPs were identified in the F_2 than in the diversity panel, but this was expected considering that zucchini accessions are generally characterized by limited genetic diversity (Gong et al., 2012). The amount of missing data for polymorphic loci was low, e.g., less than 20 %, suggesting that multiplexing could be increased in *Cucurbita pepo* to increase cost efficiency with few negative effects.

Trait mapping using GBS-derived SNPs revealed associations with loci important to growers, and sequences from these SNPs can be used in gene fine-mapping. The continued refinement of the GBS protocol to optimally generate data for a broad range of *Cucurbita* populations, including various species, subspecies, and morphotypes, may help to unlock the genetic basis of many important traits. Markers for and within genes contributing to these traits hold much potential for *Cucurbita* crop improvement.

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Proteomic Analysis of Cucumber Secretome During Cucumber-Downy Mildew Interactions

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Additional index words. *Cucumis sativus*. L, *Pseudoperonospora cubensis*, apoplastic proteins, proteomics; MALDI-TOF/TOF

Abstract: The phytopathogenic oomycete *Pseudoperonospora cubensis* has caused serious economic losses to cucumber worldwide. The extracellular space (ECS) of the cucumber leaf serves as a front line of defense against the invading *Ps. cubensis* oomycete pathogen where recognition and defense may take place. To identify and characterize the *in vivo* proteins secreted into the ECS in the plant-oomycete interaction, 2-leaf-stage seedlings of the resistant "NW088" and susceptible "CCMC" inbred cucumber lines were inoculated with *Ps. Cubensis* . Soluble apoplast proteins of leaves from treated and control seedlings were extracted 1, 2 and 4 days after inoculation and analyzed with gel-based proteomics approaches. A total of 48 protein spots with reproducible differences were observed and excised for characterization with MALDI-TOF/TOF MS, of which 45 were successfully identified. Among them, 21 were bioinformatically predicted to be apoplastic proteins that are involved mainly in the carbohydrate metabolism, stress and defense responses, and energy metabolism. These data increase our knowledge of pathogen resistance mechanisms against downy mildew and are useful in future studies to investigate the role of apoplastic proteins in host resistance.

Downy mildew (DM) is one of the most common and destructive foliar diseases in cucumber (Cohen et al., 2003; Call, 2008; Savory et al., 2011; Adhikari et al., 2012). The phytopathogenic oomycete *Pseudoperonospora cubensis* (*P. Cubensis*), the causative agent of cucumber downy mildew, is an obligate, biotrophic pathogen (Adhikari et al., 2012). In China, its frequent occurrence can result in 10–30% yield loss in cucumber production. In particular, cucumbers are now growing year round in plastic or glass houses creating very favorable environments for DM development and easy maintenance of the pathogen. No effective resistance to this pathogen has been deployed in cucumber production (Adhikari et al.,2012), and the resistance mechanisms of DM resistance are not well understood.

During the course of cucumber-*P.cubensis* interactions, the extracellular space (ECS) serves as the front line of defense against the invading *P.cubensis* pathogen where recognition and defense take place (Pechanova et al., 2010). The fluid moving in ECS is often referred to as the apoplastic fluid (APF) which provides a means of delivering molecules and facilitates intercellular communications (Delaunois et al., 2013). The components and function of proteins in APF during *P. cubensis* infection are largely unknown. Recent advances in plant proteomics technology (Kim et al., 2013) have made high throughput analysis of the secretory proteins (secretome) feasible. The in planta secretome has been described in *Medicago truncatula* (Soares et al., 2007), *Nicotiana tabacum* (Delannoy et al., 2008), Arabidopsis (Casasoli et al., 2008), rice (Song et al., 2011) and the grapevine (Delaunois et al., 2013) which provide a basic understanding of protein composition in APF of these species under natural or abiotic stress conditions. There is no report on the analysis of secretome of plant-oomycete interaction. In this study, we conducted comparative analysis of the proteins secreted into the ECS in DM resistant and susceptible lines. Individual proteins were identified and characterized, and their potential roles in host defense against DM pathogen were discussed.

Materials and Methods

Ps. cubensis pathogen and cucumber materials. Cucumber plants were grown from highly resistant NW088 and susceptible inbred line CCMC against *P. cubensis.* DM pathogen (*P. cubensis*) was isolated from diseased leaves of cucumber plants in the greenhouse.

Infection of cucumber seedlings with P. cubensis pathogen. When two true leaves were fully expanded, the seedlings of NW088 and CCMC were spray-inoculated, until run-off, with a concentration of 1×10^5 sporangia/ml. The plants were incubated for 24h at 100% relative humidity in the dark. The leaves of both mock (sprayed with ddH_2O) and *P. cubensis* inoculated samples were harvested at 0, 1, 2 and 4 days post inoculation (dpi) and processed immediately for the isolation of secreted proteins.

Extraction of secreted proteins from leaves. A vacuum infiltration procedure was used for APF extraction. The leaves were cut off from the petiole and carefully washed with distilled water, and then the intact leaves were fully submerged in deionized water, followed by vacuum infiltration. The APF was collected by centrifugation at 1000×g for 10 min at 4℃ and stored at –80℃.

Purity assessment of isolated secreted proteins. To evaluate intracellular protein contamination of apoplastic, malate dehydrogenase (MDH) activity, a known cytoplasmic biomarker, was assayed in the prepared secretory protein fractions and total proteins (Song et al., 2011). Three independent assays were used for MDH analysis.

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2-D gel electrophoresis. Protein concentration was determined using the Protein Assay Kit II, BSA standard (BioRad, Germany) according to the manufacturer's instructions. For IEF, the PROTEAN IEF system (Bio-Rad, Munich, Germany) was used with 3–10 non-linear (NL) pH gradient strips (IPG strips, Bio-Rad, USA). The 2-D gels were colloidal CBB-stained. In all cases at least three biological replicates for 2-DE were carried out for data collection.

Image acquisition and statistical analysis. The stained 2-D gels were scanned and analyzed with the PDQuest ™ 8.0 software (BioRad). Only those spots that showed statistically significant differences in intensity as calculated using Student's test ($P < 0.05$) were selected, digested with trypsin (Promega, sequencing grade), and subjected to mass spectrometry analysis. *MALDI-TOF-MS analysis and database searching.* The samples were analyzed with a 5800 MALDI-TOF/TOF Proteomics Analyzer (AB SCIEX，USA). All peptide mass fingerprints (PMFs) acquired from MALDI -TOF/TOF mass spectral data were searched against cucumber protein database (http://cmb.bnu.edu.cn/Cucumis_sativus_v20）using MASCOT (Version 2.3, http://www.matrixscience.com/).

Bioinformatic analysis of identified in vivo secretory proteins. Several bioinformatics tools were used to identify secretory protein species including Signal P, MHMM server v.2.0 and Target P (Agrawal et al., 2010). Those containing signal peptides, but lacking transmembrane domains and cellular organelle retention signals were classified as secreted proteins. Secretome P was used to predict non-classical secreted proteins. Identified proteins were placed into different categories according to their involvement in the biological process using gene ontology tools (http //www agbase.sstate.edu/) and UniProtKB (http://www. uniprot.org/).

Results and Discussion

Assessment for intracellular contamination in apoplast extracts. To assess the level of cytoplasmic contamination, the MDH activity assay was carried out. The percentage of MDH activity in *P. cubensis*-infected apoplastic proteins was 4.3% to 5.7% and 4.2% to 5.2% of that in total proteins in resistant (NW088) and susceptible (CCMC) cucumber lines (Table 1), respectively, which was well below 10% that was considered acceptable for proteomic analysis (Song et al., 2011)**.** This result indicated that, while contamination of the apoplast extracts with intracellular proteins cannot be completely excluded, it could be kept at a reasonably low level. Therefore prepared secreted protein fraction can be used for subsequent analysis.

Identification of apoplast proteins in response to P. cubensis infection. A comprehensive APF proteome analysis of *P. cubensis*-susceptible and -resistant genotypes was conducted in leaf samples at 0, 1, 2 and 4 dpi to elucidate qualitative and quantitative changes following *P. cubensis*-infection. A specific criterion was applied to the identified proteins for the selection of differentially abundant spots. A protein was considered differentially expressed if its level (protein spot intensity) showed at least ± 1.5-fold changes (i.e., ratio ≥ 1.5 or ≤ 0.67) in all three biological replicates at one time point between NW088 and CCMC. This resulted in 48 protein spots showing consistent expression patterns. These protein spots were excised from the gels for identification by MALDI-TOF/TOF MS, and results from 45 spots were successfully obtained representing 39 proteins.

Based on the domain characteristics (Song et al., 2011), 21 proteins were predicted to be apoplastic proteins, among which 9 each possessed a signal peptide, and 16 each had no signal peptide. Among the 25 apoplastic protein spots, 19 spots representing 16 proteins showed differences in the *P. cubensis*-resistant cucumber group ($NW088_{1dpi}$, $NW088_{2dpi}$, $NW088_{4dpi}$/$NW088_{CK}$). Of these, 12 spots were up-regulated and 6 spots were down-regulated at all three time points after *P. cubensis* inoculation. In the *P. cubensis*-susceptible cucumber group ($CCMC_{1dpi}$, $CCMC_{2dpi}$, $CCMC_{4dpi}$/$CCMC_{CK}$), 18 protein spots representing 15 proteins showed changes in intensity. Of these, 12 spots were up-regulated and 6 spots were down-regulated at all three time points after *P. cubensis* inoculation.

To determine apoplastic proteins vary under specific *P. cubensis*-infected time points, a Venn diagram analysis of the number of differentially expressed apoplastic protein spots after *P. cubensis* inoculation was conducted. The apoplastic proteins with significant fold changes showed an overlap of 9 proteins which were differentially regulated irrespective of types of plant-pathogen interaction after *P. cubensis* inoculation (Fig. 1). The large number of changes in apoplastic proteins may suggest that these proteins play important roles in defense responses of cucumber against *P. cubensis* infection.

Biological processes and molecular functions of differentially expressed secreted proteins. According to their involvement in the biological processes and gene ontology tools, the proteins with significant fold changes between *P. cubensis*-resistant and susceptible cucumber lines could be functionally classified into nine and eight groups, respectively. Proteins involved in carbohydrate metabolic process made up the largest proportion (36.8 % and 33.3 %, respectively), followed by the stress and defense response group (21.1 % and 16.7 %, respectively), and energy metabolism (15.8 % and 16.7 %, respectively).

Table 1. Activities of the malate dehydrogenase (MDH).

Protein samples	Protein content （mg/ml）	Enzyme activity (U/ml)	Relative enzyme activity (U/mg)	MDH activity ratio (apoplast/total %)
Total protein (NW088)	0.36 ± 0.04	90.26 ± 5.98	250.72 ± 9.87	–
Apoplast protein Control	0.28 ± 0.03	3.02 ± 0.04	10.79 ± 1.34	4.3
1dpi	0.27 ± 0.04	3.41 ± 0.03	12.63 ± 1.98	5.0
2dpi	0.28 ± 0.07	4.03 ± 0.07	14.39 ± 2.79	5.7
4dpi	0.29 ± 0.05	3.86 ± 0.06	13.31 ± 2.65	5.3
Total protein (CCMC)	0.34 ± 0.06	94.02 ± 6.04	276.53 ± 8.96	–
Apoplast protein Control	0.27 ± 0.05	3.24 ± 0.02	12.00 ± 0.03	4.3
1dpi	0.30 ± 0.06	3.46 ± 0.05	11.53 ± 0.05	4.2
2dpi	0.28 ± 0.06	3.98 ± 0.05	14.22 ± 0.08	5.1
4dpi	0.28 ± 0.07	4.06 ± 0.07	14.50 ± 0.07	5.2

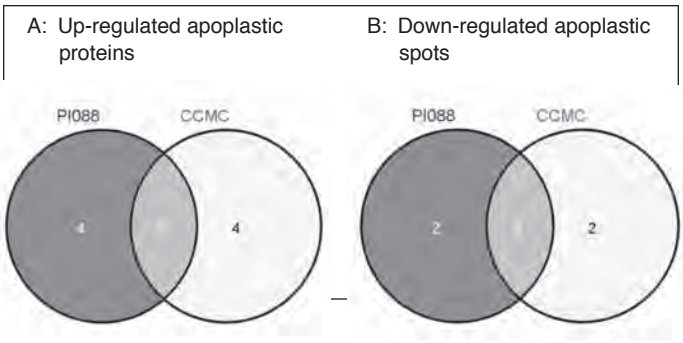

Fig. 1. Venn diagram analysis of abundance changed apoplastic proteins under *P. Cubensis* stress. The number of up- or down-regulated proteins in resistant (NW088)and susceptible (CCMC) cucumber lines are shown in the different sections.

To obtain a functional overview of all identified apoplastic proteins, we analyzed their putative functions using the GO database (http://www.geneontology.org/). The proteins involved in different biological processes, biological metabolism appeared as the most abundant functional category, such as carbohydrate metabolic process, glycolsis, oxidation-reduction process, lipid metabolic process, glucose metabolic process.

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Using Genomic Data for Enhancing Disease and Pest Resistance in Watermelon Cultivars

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Abstract. There is a continuous need to enhance watermelon cultivars for disease and pest resistance. The U.S. Plant Introductions (PIs) of Citrullus spp. are a valuable source for enhancing disease and pest resistance in watermelon cultivars. The watermelon genome sequence project brings new opportunities in discovery of disease or pest resistance gene sequences. The recent international watermelon genome sequencing project identified at least 79 gene sequences that were preferentially selected, while other gene sequences, among them several disease-resistance genes, that may have been lost during domestication. It has been shown that gene loci that show extremely low or high levels of genetic differentiation are often implied to be subject to natural selection, either acting on the locus itself or on a closely linked locus. To elucidate the underlying process of watermelon evolution and domestication we designed PCR primer pairs for 49 gene sequences that are not found in the cultivated red-sweet watermelon but exit in *C. lanatus* var. *citroides*, and have been designated as "novel" or "lost" gene sequences. Using the primer pairs we conducted PCR experiments with genotypes representing the different Citrullus species. Population structure and principle component analyses elucidated genetic relationships and existence of the "novel" gene sequences in PIs representing *C. lanatus* var. *citroides*, *C. lanatus* var. *lanatus*, *C. colocynthis*, *C. ecirhosus*, and *C. rehmii* and in watermelon cultivars (*C. lanatus* var. *lanatus*). The genomic data in this study should be useful in future breeding programs aiming to enhance disease resistance in watermelon.

A narrow genetic base exists among watermelon cultivars (Levi et al., 2001 [a or b]). The recent sequencing projects of the watermelon genome (Guo et al., 2013) further underscore the narrow genetic base, displaying a low number of single nucleotide polymorphism (SNP) markers (an average of one SNP for every 1,430 bp) between the sequenced genomes of the American heirloom "Charleston Gray" (elongated fruit with light green-gray rind and a pink flesh) and the elite Chinese watermelon line "97103" (small globular fruit with dark rind and red flesh). On the other hand, wide genetic diversity exists between watermelon cultivars and United States Plant Introductions (PIs) of *Citrullus* species and subspecies, including PIs of the desert watermelon *Citrullus colocynthis* (L.) that exist in the deserts of northern Africa, the Mediterranean region and south and central Asia, and the *C. lanatus* (Thunb.) Matsum et Nakai, which is indigenous to the arid sandy regions of southern Africa (Bates and Robinson, 1995). This species includes *C. lanatus* subsp. *lanatus* (*CLL*) (also known as *C. lanatus* subsp. *vulgaris* that represent the sweet-dessert watermelon group) (*CLV*) and *C. lanatus* subsp. *lanatus* var. *lanatus* (often reported as "*C. lanatus* var. *citroides*") (*CLC*) that is considered a group of ancient cultigens derived from the 'Tsamma' melon that thrive in southern Africa (Wehner, 2008).

The watermelon genome sequencing project (Guo et al., 2013) also revealed that the *CLC* contains a large number of gene sequences, among them gene sequences associated with disease or pest resistance that may have been altered or lost during the many years of domestication of the sweet watermelon. This gene sequences were designated as "novel" gene sequences.

The objective of this study was to survey the "novel" gene sequences in PIs representing the different *Citrullus* spp. and determine if they were lost during the domestication of the sweet-dessert watermelon or if they had undergone through changes in early stages of the *Citrullus* spp. evolution. Also, to examine the possibility of incorporating the "lost" resistant gene sequences into the genome of watermelon cultivars.

To elucidate the process of watermelon evolution and domestication we designed PCR primers for these "lost" gene sequences and conducted PCR experiments with genotypes representing the different *Citrullus* species and subspecies. Here, we used PCR primers and gene sequencing data to survey the "novel" gene

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sequences among PIs representing the different *Citrullus* spp., including *C. lanatus* var. *citroides*, *C. lanatus* var. *lanatus, C. colocynthis*, *C. ecirhosus*, and *C. rehmii* and among watermelon cultivars.

Material and Methods

*Plant material and isolation of DNA***.** Fourty-eight PIs and cultivars were selected for analysis. Seedlings of these PIs were grown in the greenhouse at 26 °C/20 °C (day/night temperatures). Young leaves were collected from 3 two-week-old plants representing each PI or cultivar and stored at –80 °C for later DNA isolation. The DNA was isolated from the frozen leaves using the method described by Levi and Thomas (1999).

PCR amplification and analysis using primer pairs: Primer pairs were designed for each of the 49 novel gene sequences and were used in PCR experiments as described by Levi et al. (2010).

Gene sequencing data analysis. Using the PCR primer pairs designed for each of the novel genes, gene sequences were cloned from several PIs representing the different *Citrullus spp*. The gene sequence fragments were analyzed using a CEQ 8800 DNA Genetic Analysis System (Beckman Coulter, Fullerton, CA). For visualization of DNA fragments on the CEQ 8800, the forward primers were labeled with one of three WellRED dye labels (D2,

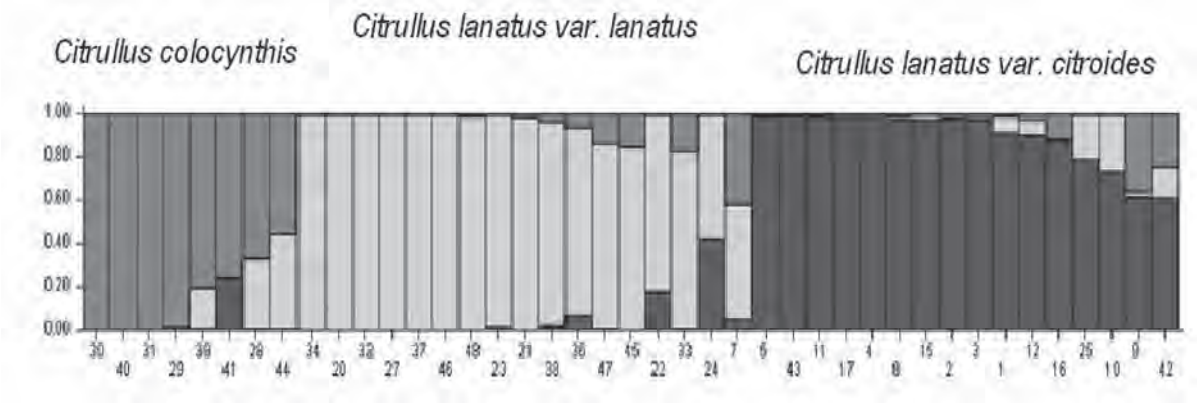

Fig. 1. Cluster analyses performed in STRUCTURE (Pritchard et al., 2000) based on 89 DNA fragments produced by primers specific to the "novel" gene sequences. Each vertical bar represents an individual PI or cultivar while colors indicate the different gene loci clusters detected for the three main *Citrullus* groups.

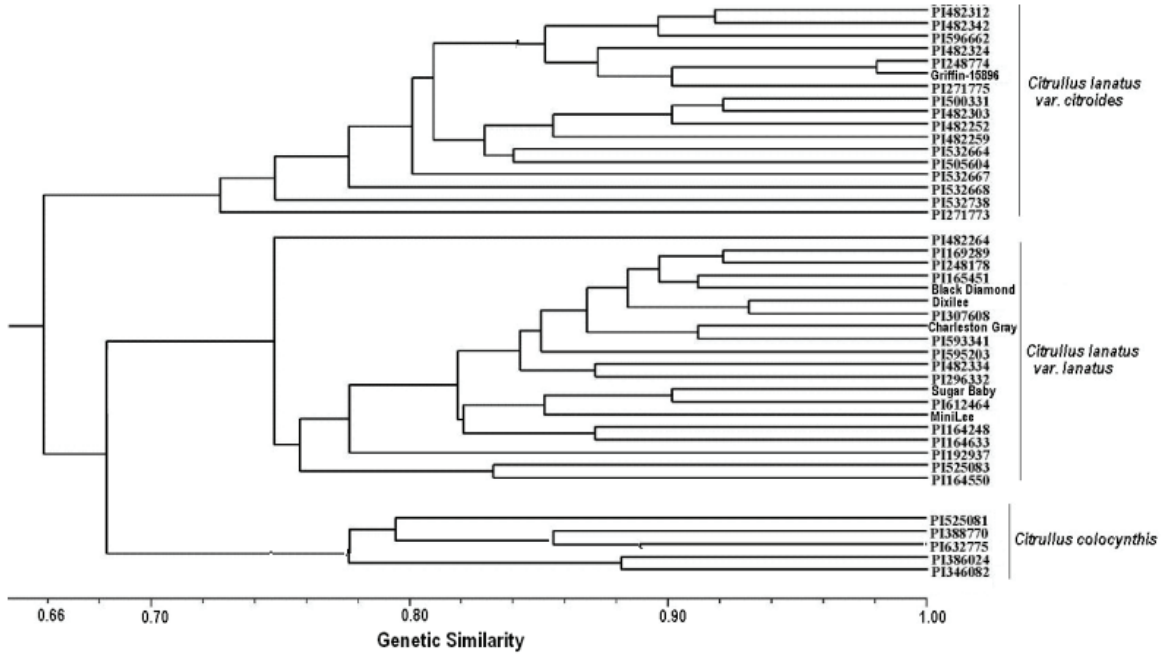

Fig. 2. UPGMA dendrogram of U.S. Plant Introductions (PIs) of *Citrullus* spp. and watermelon cultivars using 89 DNA fragments produced by primers specific to the "novel" gene sequences. The dendogram displays three distinct clusters and indicates closer genetic relationships between *C. lanatus var. lanatus* and *C. colocynthis* compared with the *C. lanatus var. citroides* group.

D3, or D4; Proligo, Boulder, CO) as previously described for SRAP markers (Levi et al., 2009).

Marker data collection and analysis. The gene sequence fragments were scored based on their presence or absence using the built-in fragment analysis software (provided with the Beckman CEQ-8800 system). A similarity matrix for the data was generated using the Nei-Li similarity index (Nei and Li, 1979). A dendrogram (Fig. 2) was created based on the un-weighted pair group method with arithmetic average (UPGMA) using the SAHN module in NTSYS-PC version 2.02j (Rohlf, 1998). Bootstrap support for clusters was conducted in FreeTree using 5000 permuted datasets (Pavlicek et al. 1999). A cophenetic matrix was generated from the dendrogram by using the COPH module in NTSYS-PC. A population structure analysis procedure for clarifying genotypic ambiguity (Falush et al., 2007) was used (Fig. 3), by means of the computer program "Structure version 2.2" (Developed by Falush et al., 2007), which is available on the worldwide web at http://pritch.bsd.uchicago.edu/structure.html.

Results and Discussion

The results in this study indicate that the desert watermelon *C. colocynthis* and *C. lanatus* var. *lanatu*s are of common decent, sharing a considerable number of gene sequences that distinguish them from the *C. lanatus* var. *citroides* genotypes (Fig. 1). The population structure analysis (Fig. 2) indicates that several of the gene sequences unique to *C. lanatus* var. *citroides* genotypes may have been lost or altered during early stages of differentiation of *C. lanatus* var. *lanatu*s and *C. colocynthis* from *C. lanatus* var. *citroides*. Several of the highly specific PCR primer pairs that were designed for the "novel" gene sequences in *C. lanatus* var. *citroides* amplified polymorphic fragments among the *C. lanatus* var. *lanatu*s and *C. colocynthis* genotypes, indicating that these gene sequences do exist, but have been altered substantially in these two *Citrullus* species. Furthermore, the sweet-red watermelon cultivars share a considerable number of homologous gene sequences with *C. lanatus* var. *lanatu*s genotypes that have undesirable fruit quality (white and un-sweet flesh) (Fig. 1), indicating that the "novel" gene sequences were altered in *C. lanatus* var. *lanatus* due to natural selection or any other genetic pressure prior to domestication of the sweet-red watermelon. The differentiation of the *Citrullus* groups in this study based on the "novel" genes is highly consistent with that using HFO-TAG or SNP markers (Levi et al., 2012). The accessions designated as *C. ecirhosus* (Grif. 16945) and *C. rehmii* (Grif. 16135) were clustered together with the *C. colocynthis* group, indicating that they might the same species.

At present, we have been constructing genetic populations and have been using primer pairs to incorporate the lost gene sequences into the genome of watermelon cultivars with the idea that incorporating the "novel" NBS-LRR gene sequence into the sweet-desert watermelon genome may confer higher resistance to diseases and pests of this important cucurbit crop.

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Genome-wide Characterization and Tissue-specific Expression of Calcium-dependent Protein Kinase Gene Family in *Cucumis sativus*

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Additional index words. calcium-dependent protein kinases, cucumber, gene family, gene expression

Abstract. **In higher plants, calcium is a ubiquitous second messenger in signal transduction pathway. The plant-specific calcium-dependent protein kinases (CDPKs) play important roles in regulating downstream components of calcium signaling. In this study, genome-wide analysis of the cucumber draft genome sequences identified 25 *CsCDPK* gene homologs. As compared with other plant species with genomes sequenced such as *Arabidopsis,* rice and poplar, cucumber genome contains a smaller number of *CsCDPK* family members. Quantitative real-time PCR examination in multiple tissues indicated that all 25 *CsCDPK* genes expressed in at least one tissue, suggesting that these *CsCDPKs* take part in many cellular processes. Our genomic and bioinformatic analyses provide an important foundation for further functional dissection of the *CsCDPK* gene family in the cucumber genome.**

Plants consistently encounter various environmental challenges, including fluctuations of temperature, waterlogging, drought, and high salinity (Xiong et al., 2002). In response to these stresses, plants have developed a series of survival mechanisms. Among them, calcium (Ca^{2+}) plays an essential role in various environmental signaling transduction pathways (Tuteja and Mahajan, 2007). Transient changes in Ca^{2+} concentration in the cytoplasm in response to extrinsic and intrinsic cues are sensed and decoded by an array of calcium sensors, viz., calmodulins, calmodulin-like proteins, calcineurin B-like proteins and calcium-dependent protein kinases (CDPKs) (McCormack et al., 2005). CDPKs have been identified throughout the plant kingdom and in some protozoans but not in animals. All CDPK proteins possess five domains: the variable N-terminal domain, protein kinase domain, autoinhibitory junction domain, calmodulin-like domain (CLD), and the C-terminal domain. The N-terminal domain often bares myristoylation or palmitoylation sites that are believed to be associated with subcellular targeting. The protein kinase domain is the catalytic domain with an ATP binding site, which is followed by the autoinhibitory junction domain and the calmodulin-like domain that contains EF hands for binding to calcium. The N- and C-terminal domains are variable, differing in their length and amino acid composition among various CDPK proteins, which has been suggested that these variable domains determine the specific functions of individual CDPKs (McCormack et al., 2005; Harper et al., 1994). According to the recent research, all C-terminal regions to the catalytic domain work together for activation. CDPK are the best characterized Ca^{2+} sensors in plants, which have been identified and investigated in many plants. Several studies have demonstrated the presence of 31, 34, 35, 20 and 30 CDPK genes in the genomes of rice, *Arabidopsis*, maize, wheat and poplar, respectively (Ma et al., 2013; Li et al., 2008; Zuo et al., 2013; Heabak et al., 2003).

Cucumber (*Cucumis sativus* L) is an agriculturally and economically important crop all over the world ranking 4th in quantity of world vegetable production after tomato, cabbage and onion (FAO STAT 2011, http://faostat.fao.org). In addition, cucumber is also serves as a model system for studies on plant vascular biology and sex determination (Liu et al., 2013). The availability of complete genome sequence of cucumber has enabled us to do genome-wide analysis to help undertake functional characterization of all the genes belonging to multigene families. In this study, genome-wide identification of *CsCDPKs* was performed, and the sequence phylogenies were analyzed to reveal relationships among different members of *CsCDPKs*. Their expression patterns in different tissues were also investigated.

Materials and Methods

Sequences of *Arabidopsis*, and rice *CDPK* were obtained from the Arabidopsis Information Resource (TAIR, http://www.Arabidopsis.org/, release 10.0), and rice genome annotation database (http://rice.plantbiology.msu.edu/, release 5.0), respectively. The cucumber protein, cDNA and genome DNA databases were obtained from ICuGI (http://www.icugi.org, Version 1). Gene prediction was performed with the hidden Markov model (HMM) program (eddy, 1998). Hidden Markov models were produced using kinase and EF hand domains from *Arabidopsis* and rice CDPK protein by using HMMER 2.1.1 software package (http://www.hmmer.wust.edu). Manual re-annotation was also performed using onlineweb server FGENESH (http://linux1.softberry.com/berry.phtml). All putative candidates were manually verified with the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/) to confirm the presence of the protein kinase domain and the CaM domain. Multiple alignments of amino acid sequences were performed by Clustal X (Version 1.83) program. The unrooted phylogenetic trees were constructed with MEGA5.0 using the Neighbor-Joining (NJ) method and the bootstrap test was carried out with 1,000 replicates.

For gene expression study, cucumber plants were grown in the experimental farm of the Department of Horticulture, Yangzhou University. Cucumber seeds of the variety 'Zaoer-N' were grown in 2.5-cm diameter pots containing peat, vermiculite, and

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perlite (3:1:1, v/v) in a greenhouse with 28 °C day/ 18 °C night temperature and 70-85% relative humidity. At different growth stages, root, stem, true leaf, tendril, male flower, and mature fruit tissues were sampled and flash frozen in liquid nitrogen for tissue-specific expression analysis. Total RNA of the cucumber roots was isolated using RNAiso Plus (Takara, China). Dried RNA samples were dissolved in DEPC-treated water and quantified for a concentration of 1,000 µg/mL using Biophotometer Plus (Expander, Germany). RNA was reverse-transcribed using a Takara PrimeScript® RT reagent kit with gDNA eraser according to the manufacturer's specifications. RT-PCR was performed using a RealMasterMix (SYBR Green) kit (TIANGEN, China) following manufacturer's protocols. SYBR Green PCR cycling was performed on an iQ™ 5 Multicolor real-time PCR detection system (Bio-RAD, USA) in 20 µL reactions. The PCR primers were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA). Primer sequence information is presented in Table 1. Three biological replicates of the stress and control treatments were used for real-time PCR. The analysis of relative mRNA expression data was performed using the $2^{-\triangle\triangle Ct}$ method. Each expression profile was independently verified in 3 technical replicates performed under identical conditions.

Results and Discussions

This research identified 26 genes in the cucumber genome as possible members of the *CsCDPK* family of which one gene *Csa025386* was eliminated because of its similarity with another gene *Csa014084*. The remaining 25 genes were designated as *CsCDPK1–CsCDPK25* according to the proposed nomenclature for CDPK genes (Table 1). The results showed that 23 genes could be mapped on chromosomes 1 to 7, while two genes (*CsCDPK3* and *CsCDPK25*) did mapped on any position in the cucumber genome. *CsCDPKs* distribution were uneven among the seven cucumber chromosomes, however the results revealed that *CsCDPKs* were found in all chromosomes. The number genes identified in CDPK family in cucumber were smaller, may due the absence of the recent whole-genome duplication in cucumber genome (Huang e*t al.*, 2009), therefore may have led to the decrease of *CsCDPKs* number in cucumber. However, *Arabidopsis*, *poplar* and *Oryza* have undergone duplication events recently, which might have led to the increased the number of the CDPK family in their genome (Li et al., 2012).

In order to compare *CsCDPKs* with *Arabidopsis* and *Oryza*, an NJ phylogenetic tree was generated using full-length protein sequences of *CsCDPKs*, *AtCDPKs* and *OsCDPKs* proteins (Fig. 1). Similar to that in rice and *Arabidopsis*, the 25 *CsCDPKs* were also divided into four distinct groups (I to IV) of which groups I, II, and III were further divided into two subgroups (a and b). Seven *Arabidopsis*, nine *Oryza* and four *CsCDPKs* (*CsCDPK2*, *4*, *7*, and *9*) belonged to group Ia; Three *Arabidopsis*, two *Oryza* and two *CsCDPKs* (*CsCDPK1*, *3*) belonged to group Ib. Ten *Arabidopsis*, two *Oryza*, and two *CsCDPKs* (*CsCDPK8*, and *23*) belonged to group IIa; Three *Arabidopsis*, six *Oryza*, and two *CsCDPKs* (*CsCDPK5*, and *15*) belonged to group IIb. One *Arabidopsis*, three *Oryza*, and one *CsCDPK* (*CsCDPK10*) belonged to group IIIa. Seven *Arabidopsis*, five *Oryza*, and five *CsCDPKs* (*CsCDPK12*, *13*, *14*, *20*, and *25*) belonged to group IIIb. Two *Arabidopsis*, four *Oryza*, and nine *CsCDPKs* (*CsCDPK6*, *16*, *22*, *11*, *24*, *19*, *21*, *18*, and *17*) belonged to group IV. The results of phylogenetic analysis of the predicted CsCDPK protein sequences revealed that there were no equal representations of cucumber, *Arabidopsis*, and *Oryza* proteins in the four subgroups (Fig.1). In CDPK family, cucumber recorded the highest CDPK IV genes

Table1. CDPK gene family members in the cucumber genome.

CsCDPK	Gene	Chromosome	Location	No. of EF hand	M.W. (KDa)
1	Csa017282	Chr2	13413251 - 13416483	4	56.3
2	Csa002575	Chr3	26379049 - 26382276	4	74.3
3	Csa019908	Scaffold000143	136312 - 147152	4	56.3
4	Csa014084	Chr7	6028294 - 6033396	4	63.3
5	Csa016123	Chr4	5476283 - 5480430	4	59.2
6	Csa000481	Chr6	23496059 - 23500408	4	64.6
7	Csa001056	Chr5	23965632 - 23968627	4	63.9
8	Csa002018	Chr3	31372914 - 31377011	4	60.2
9	Csa002499	Chr3	26446394 - 26451015	4	65.2
10	Csa002986	Chr4	579654 - 584797	4	60
11	Csa003171	Chr7	278234 - 284080	1	62.2
12	Csa003756	Chr1	4988834 - 4993347	4	62.1
13	Csa004928	Chr3	23864680 - 23867789	4	59.4
14	Csa006484	Chr1	20814389 - 20817934	4	59.7
15	Csa007804	Chr6	18200679 - 18203381	4	59.8
16	Csa008536	Chr6	5813020 - 5822106	1	75.8
17	Csa009020	Chr6	3691916 - 3702430	3	63.4
18	Csa009254	Chr2	6625278 - 6629571	1	64.1
19	Csa010127	Chr6	21038726 - 21043639	1	81.3
20	Csa011913	Chr6	18461292 - 18468490	4	59.5
21	Csa013313	Chr4	15687837 - 15693368	1	64
22	Csa015347	Chr5	19118535 - 19121429	1	62.6
23	Csa017005	Chr3	3559906 - 3563799	4	58.6
24	Csa018149	Chr2	13070036 - 13073942	1	69.4
25	Csa021911	Scaffold000257	15571 - 18857	4	60.4

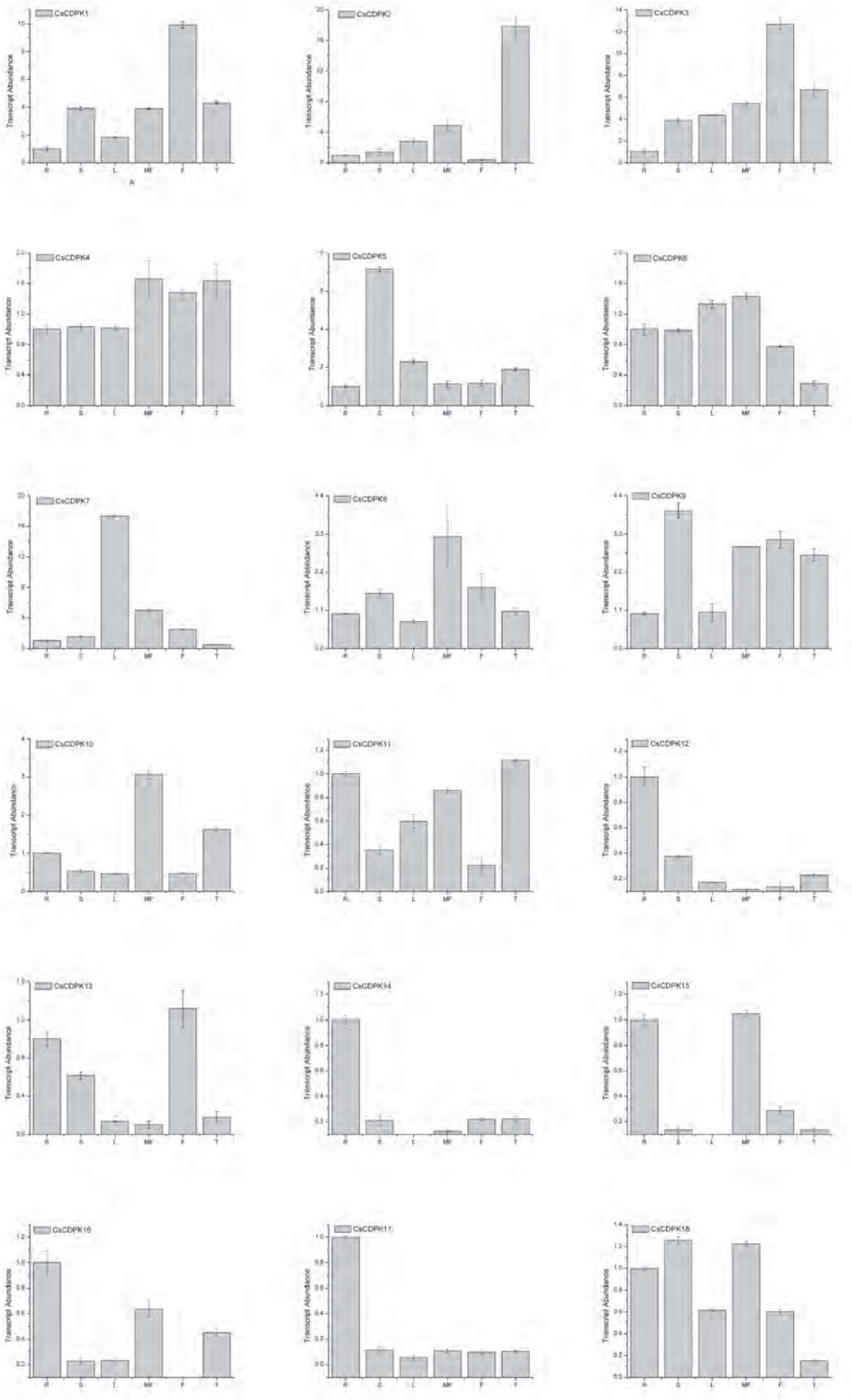
CsCDPK1
CsCDPK2
CsCDPK3
CsCDPK4
CsCDPK5
CsCDPK6
CsCDPK7
CsCDPK8
CsCDPK9
CsCDPK10
CsCDPK11
CsCDPK12
CsCDPK13
CsCDPK14
CsCDPK15
CsCDPK16
CsCDPK17
CsCDPK18
Transcript Abundance
R
S
L
MF
F
T

Fig. 1.

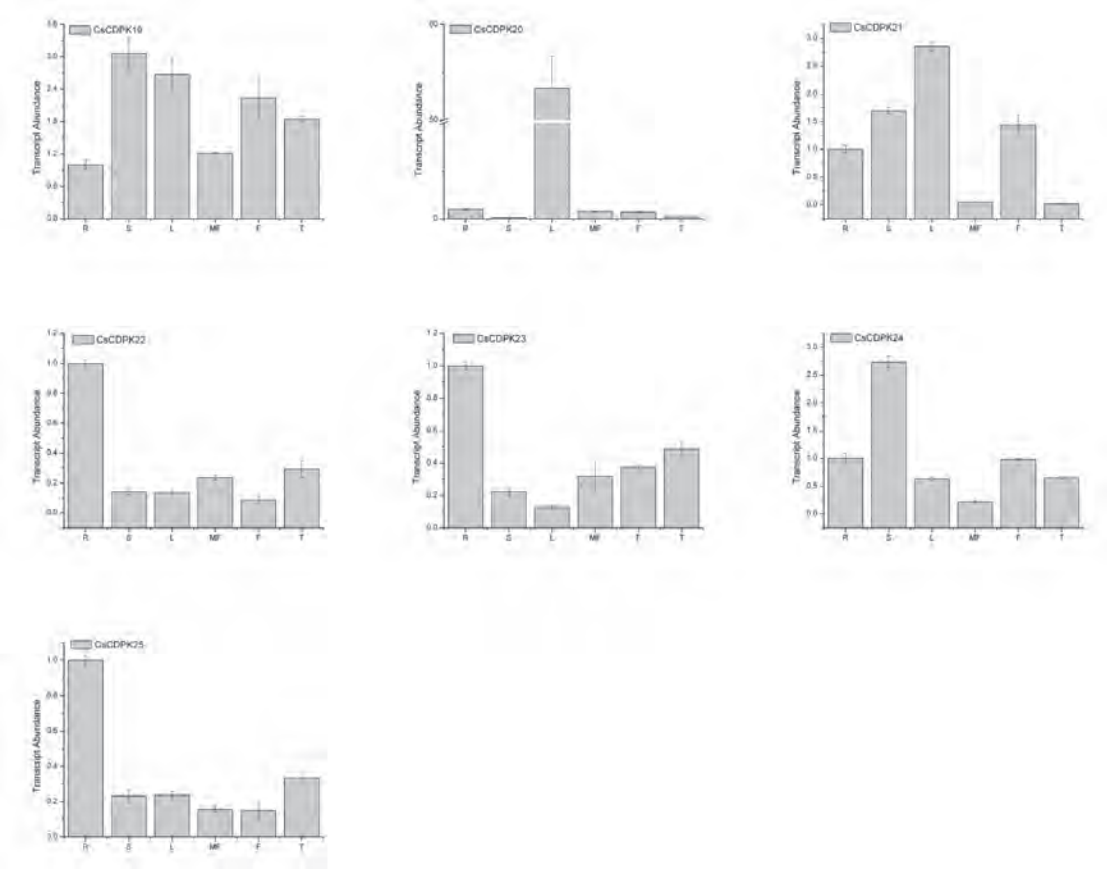

Fig. 2. Tissue-specific expression patterns of CsCDPK genes. Relative transcript abundances of CsCDPK genes were examined by qRT-PCR. The X axis is the tissues of cucumber. From left to right: R root, S stem, L leaf, MF male flower, F fruits, T tendrils, respectively. The cucumber β-actin gene (GenBank AB010922) was performed as an internal control.

while *Oryza* and *Arabidopsis* consisted of the least subfamilies which could be attributed to the expansion of cucumber-specific branch in group IV or the members lost during the evolutionary processes in *Oryza* and *Arabidopsis*.

To gain insight into the functions of *CsCDPKs* in different tissues, qRT-PCR were used to examine the expression patterns for all cucumber CDPK genes in the roots (R), stems (S), leaves (L), male flowers (MF), fruits (F) and tendrils (T). The expression of the 25 *CsCDPKs* revealed different patterns of tissue-specific expressions (Fig. 2). *CsCDPK12*, *CsCDPK14*, *CsCDPK16*, *CsCDPK17*, *CsCDPK22*, *CsCDPK23*, and *CsCDPK25* predominantly expressed in roots. *CsCDPK2* and *CsCDPK11* predominantly expressed in tendrils. *CsCDPK8*, *CsCDPK20*, and *CsCDPK21* predominantly expressed in leaves. *CsCDPK3* and *CsCDPK13* predominantly expressed in fruits. *CsCDPK5*, *CsCDPK7*, *CsCDPK9*, *CsCDPK18*, *CsCDPK19* and *CsCDPK24* predominantly expressed in stems. *CsCDPK4*, *CsCDPK6*, *CsCDPK8*, *CsCDPK10*, and *CsCDPK15* predominantly expressed in male flowers. Our results showed that the 25 *CsCDPKs* showed tissue-specific expression patterns, which may suggest distinct functions of these kinases in different tissues (Fig. 2).

For cucumber, practically no functional studies of CDPK genes have been reported. Therefore in-depth studies of *CsCDPKs* are needed to understand the molecular mechanisms of stress responses in cucumber.

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Nutraceutical Potential of Icebox Watermelon Fruit During Its Development and Ripening

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Additional index words. lycopene, sucrose phosphate synthase, sugars, total soluble solids

Abstract. **Nowadays, consumption of plant derived nutraceuticals is strongly recommended due to their nutritional and therapeutic potential and safety. Watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai.] is a rich source of nutraceuticals which are essential for optimal health. The aim of the present study was to evaluate the nutraceutical profile of the icebox cultivar of watermelon (cv Suman) during its development and ripening. The process of ripening influenced the accumulation of sugars (reducing and non-reducing) and the level of total soluble solids in the fruit. During the course of maturation and ripening, the total chlorophyll of the outer mesocarpic tissue undergoes degradation concomitantly with the higher accumulation of lycopene. Phenolics (phenols, polyphenols) show fluctuations in their levels during development, but they get accumulated maximum in the ripened stage. A consistent increment in the activity of sucrose phosphate synthase was noticed till the pre-ripened stage, but eventually a 7 fold decline in this enzyme activity was observed. Likewise, the activity level of neutral invertase was high in the young fruit, but subsequently this enzyme activity also diminished. In contrast, polyphenol oxidase and catalase, exhibited varied levels of their activities during development and ripening. Based on the results of the current study, it can be concluded that watermelon fruit quality is influenced by the ripening associated processes. Thus it is envisaged that the outcome of this work would help in exploiting the nutraceutical profile of these types of cultivars of watermelon for dietary supplemental products for health promotion.**

Watermelon (C*itrullus lanatus* (Thunb.) Matsum. & Nakai), which belongs to the family Cucurbitaceae, is a warm-season crop (Rosnah et al., 2010). The first icebox cultivar of watermelon was introduced in the United States almost 50 years ago, but these types of cultivars have been commercialized recently. The interesting characteristics of the icebox watermelon are their small size and also they offer farmers a means of producing high quality watermelons locally. Fruit ripening involves physiological, biochemical changes like synthesis and degradation, carbohydrate metabolism, and generation of secondary metabolic compounds as well as structural changes, such as cell wall hydrolysis, pigment which influence fruit appearance, texture, flavor and aroma and moreover the process of ripening is a genetically programmed process (Mworia et al., 2012).

Vegetables and fruits or horticultural crops in general represent the best examples of edible plant harvest having functional food properties with a potential to develop nutritional ingredients or supplements indeed causing a change in the perception of horticultural crops and products. Fruits such as berries, grapes (*Vitis vinifera* L.), watermelon, pomegranate (*Punica granatum*) etc., are having such kind of nutraceutical potential (Mahima et al., 2013). Therefore, in view of this, the present study was aimed to provide a comprehensive view of bioactive compounds that influence the nutraceutical potential of the icebox cultivar of watermelon. Furthermore, this study was warranted to evaluate the quality attributes along with their enzymatic profile of watermelon during its sequential stages of development and ripening.

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Materials and Methods

The watermelon fruit (cv. Suman) was collected from Dehgam region of Gujarat, India at its five sequential stages of development and ripening, viz: young, pre-mature, mature, pre-ripened and ripened. After measuring the physical parameters such as weight, length and diameter of the collected fruit, they were subjected for their biochemical analyses. The total soluble solid (TSS) content was determined by Association of Official Analytical Chemists (AOAC, 1994) method. Quantitative estimation of sugars (reducing and non-reducing) and phenolics were determined as per the methods described by Menon and Rao (2012a) and for evaluating the sugar metabolizing enzymes like sucrose phosphate synthase (SPS), neutral invertase (NI) and the antioxidant enzyme polyphenol oxidase (PPO), the procedure followed was described earlier by Menon and Rao (2012b). The evaluation of pigments such as total chlorophyll (TC) and lycopene and catalase (CAT) was performed as per the methods by Wang et al. (2005) and Wang et al. (2004), respectively.

Results and Discussion

The soluble solid level generally reflects the level of soluble sugars and is considered as a basic parameter in the evaluation of fruit quality as well as marketability. Total soluble solids in the watermelon fruit was 4.4 % in the initial stage of development, increased gradually and reached during maturation to the level of 8.6 %. The highest content of TSS was found to be in the ripe fruit. i.e. 12.03 % which indicates the sweetness of this icebox cultivar (Fig. 1A). Similar trend of TSS content was observed during the development and ripening of "Makdimon" cultivar

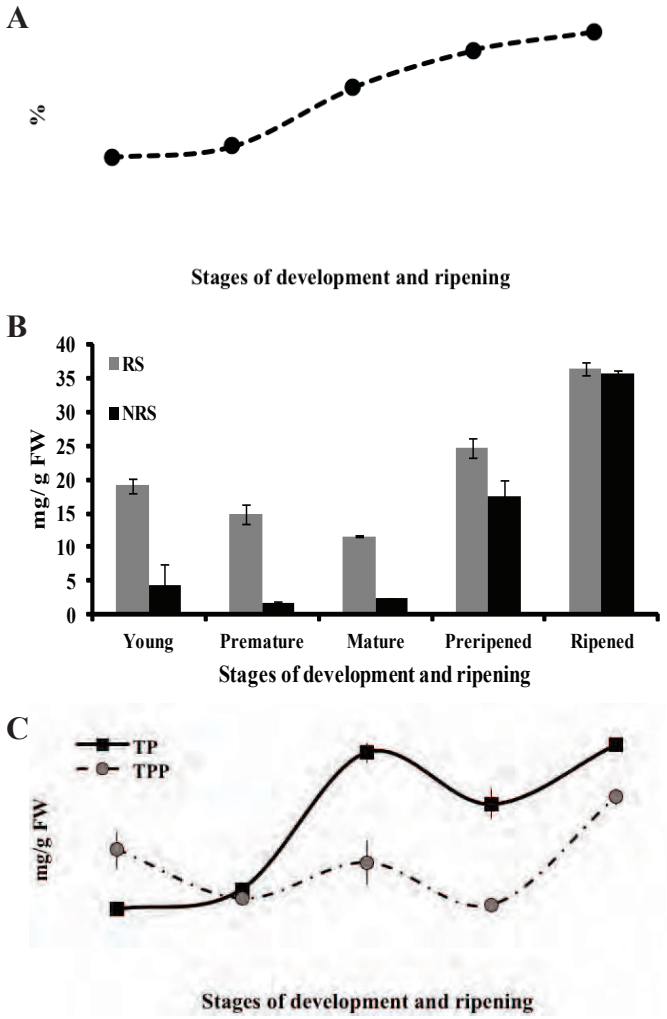

Fig. 1. Changes in the amount of total soluble solids (TSS) (%) (**A**), reducing sugars (RS), non-reducing sugars (NRS), (**B**) and total phenols (TP) and total polyphenols (TPP), and (**C**) in watermelon (cv. Suman) fruit during its development and ripening (mg/FW).

of muskmelon (*Cucumis melo* L.) fruit (Lingle and Dunlap, 1987). According to this study, the TSS levels are low in the early developmental stages of melon, while it rapidly increased during ripening which may be due to an increase in sucrose, with decreased concentrations of glucose or fructose.

The concentration of reducing sugars (RS) was 19.09 in the young 'Suman' fruit and 14.86 in its pre-mature fruit, values expressed as mg/g FW. During maturation, the values of RS decreased to 11.63 mg/g FW. The concentration of RS reached to 24.70 mg/g FW when the fruit attained pre-ripened stage and appeared to attain a maximum level of it at the fully ripe stage (Fig. 1 (B) confirming the earlier studies in melons by Hubbard et al. (1989), Lester (2008) and Menon and Rao (2014). In case of non-reducing sugars (NRS), at early stages of development, an increased amount occurred in the fruit, whereas its level declined during maturation. However, a 7 fold increment in the concentration of NRS was observed during ripening which was significant ($P < 0.05$). Oliveira et al. (2005) reported that during ripening, sugars always increased as a result of biosynthetic process or by polysaccharide degradation.

Phenolic compounds are believed to account for a major part of the antioxidant activity in plants. The concentration of total phenols (TP) was higher during the development and maturation, but suddenly dropped in its level as the fruit attained the pre-ripened stage. However, the maximum accumulation of TP was noticed in the ripe fruit of watermelon as displayed in Fig. 1 (C). The amount of total polyphenols (TPP) fluctuated in 'Suman' during its development, but a significant accumulation of it appeared in the ripened stage. Salandanan et al. (2009) quantified different pattern of total phenolics in organically and conventionally grown melons and explained that the level of phenolics was influenced by yearly environmental effects and the production system which further influences the antioxidant properties of melon cultivars. The main factors influencing the presence of polyphenols in plant foods are environmental conditions, genetic factors, degree of ripeness, variety etc., (Melo et al., 2006).

SPS, the sugar synthesizing enzyme, exhibited a consistent rise in its level during the development and ripening of 'Suman'. A significant ($P < 0.05$) activity of SPS was noticed in the pre-ripened stage of 'Suman'. Higher activity of NI was observed in the early stages of development and decreased in the intermediate stages with a rapid rise during ripening (Table 1). Similar levels of SPS activity were reported in other icebox cultivars of watermelon (Soumya and Rao, 2014) and muskmelon (Menon and Rao, 2012a). According to Hubbard et al. (1989), the increased activity of SPS may be due to that sucrose get synthesized from alternate substrates other than those derived from sucrose hydrolysis, while Lester et al. (2001) opined that dominance of SPS activity coincides with sucrose accumulation during ripening Some studies have described the role of NI as a sugar cleaving enzyme, but our study is in agreement with the findings of Burger and Schaffer (2007) in muskmelon which clearly indicated that the increased activity of NI characterizes sucrose accumulation during ripening process.

The amount of TC determined in the 'Suman' cultivar showed its maximum and significant ($P < 0.05$) accumulation in the young stage. The level of TC got degraded abruptly with the progression of maturity and ripening, as displayed in Fig. 2. The concentration of lycopene got fluctuated during the fruit development of 'Suman'. A low rate of the amount of lycopene was noticed in the young stage which declined abruptly in the successive stage. A dramatic increase in its quantity was noticed with the onset of maturity followed by a significant level in the ripened stage of the fruit. Dragovic-Uzelac (2007) supposed the accumulation of lycopene as to enhance carotenoid biosynthesis or by the catalytic activity of phytoene synthase of the first step of the formation of carotenoids. Perkins-Veazie et al. (2006) opined that a wide range in lycopene content exists among watermelon germplasm and that watermelon cultivars with very high lycopene contents are available.

The activity level of PPO was higher in the early stages of fruit development. During maturation, PPO activity got declined, but a significant ($P < 0.05$) level of it was obtained in the ripe fruit of watermelon as presented in Table 1. The activity of PPO was found to be higher in the beginning of the melon development and during the final process of its ripening. The increased activity of PPO in the initial stages of development can be related to acceleration of the metabolism of fruits (Chisari et al., 2009). The increased activity of PPO may be due to the higher metabolic activity during the developmental stages of melon fruit.

The activity of the CAT enzyme had increased gradually during fruit development with a significant ($P < 0.05$) level of it in the pre-ripened stage. Earlier studies in melons also reported similar

Table 1. Specific activities of sucrose phosphate synthase (SPS), neutral invertase (NI) (µmol/h/mg protein), polyphenol oxidase (PPO) and catalase (CAT) (Units/mg protein) of watermelon (cv. Suman) during its development and ripening.

Stages	SPS	NI	PPO	CAT
Young	0.0555 ± 0.01	0.006 ± 0.002	0.004 ± 0.001	0.0002 ± 0.0001
Pre-mature	0.0546 ± 0.01	0.005 ± 0.001	0.010 ± 0.004	0.0005 ± 0.0004
Mature	0.226 ± 0.07	0.003 ± 0.002	0.001 ± 0.0005	0.0004 ± 0.0002
Pre-ripened	0.665 ± 0.10	0.017 ± 0.001	0.006 ± 0.002	0.005 ± 0. 0.0004
Ripened	0.096 ± 0.05	0.001 ± 0.00008	0.012 ± 0.002	0.001 ± 0.0006

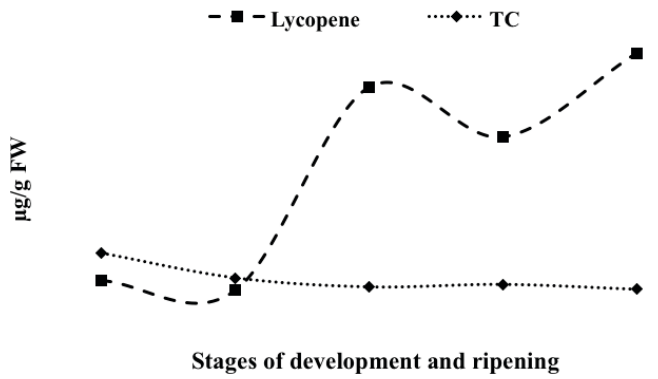

Fig. 2. Changes in the amount of lycopene and total chlorophyll (TC) in watermelon (cv Suman) fruit during its development and ripening (µg/g FW).

trend of CAT enzyme activity (Ben-Amor et al., 1999; Menon and Rao, 2012a). According to Masia (1998), there is a strong correlation between the surge of cellular oxidants at the onset of ripening with an elevated activity of CAT enzyme.

Conclusion

The present study reveals that a significant accumulation of nutraceuticals (antioxidants, phenolics) occurs in the ripe icebox watermelon fruit. Nutraceuticals provide health as well as the medical benefits such as prevention of diseases. The current investigation highlighted the nutraceutical potential of icebox cultivar fruit 'suman' based on the abundance of its antioxidants, other quality attributes like TSS, sugars and also their related enzymes during ripening process. It is envisaged that these findings may help in future to formulate nutraceutical or dietary supplements from watermelons.

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A QTL Controlling Male Flower Truss in Melon (*Cucumis melo* L.)

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Additional index words. candidate gene, flowering, recombinant inbred lines, molecular marker.

Abstract. A population of recombinant inbred lines (RIL) derived from a cross between the melon genotype TGR-1551 that exhibits male flowers grouped in multiflower trusses, and the Spanish melon cultivar, 'Bola de Oro', that exhibits 1-2 male flowers/node, has been evaluated for presence or absence of male trusses in 2012 and 2013. The quantitative trait loci (QTL) analyses detected one major QTL on linkage group (LG) VI in a region poorly saturated of our RIL map. The QTL was associated with the SSR marker ECM-81, and flanked by GCM303 and ECM133. A gene with high homology to the tomato gene Single Flower Truss (SFT) is located in this melon genomic area making it thus a candidate to be the responsible for the character.

The Cucurbitaceae family is a fascinating group of flowering plants from the morphological and embryological point of view (Singh and Dathan, 1998). The plant node is complex in all the taxa bearing a tendril, a vegetative shoot and a floral shoot. The floral shoot is represented in *Cucumis* spp. by a pro-bract with male or female flowers. Both, male and female flowers follow different flowering patterns according to the species, and the ratio male/female flower varies in function of several environmental characters. In cucumber, multiple bisexual flowers per node are observed in hermaphroditic lines, but single-pistillate flowers are common to monoecious and gynoecious lines. Multi- pistillate flowering in this species is controlled by a single recessive gene (multi-pistillate, *mp*) (Nandgaonkar and Baker, 1981). In melons, male flowers are usually single but they could also appear in groups of 1-2 to 5 per node in main and secondary stems, whereas female and perfect flowers are single and appear usually on secondary and tertiary stems. Also, a multiple flowering pattern in melon (2 to 5 pistillate flowers at every node) was observed in a single plant of PI414723, a monoecious cultivar from India (Khan et. al., 1987). However, and although male flowers could be observed in small groups, its distribution in inflorescences had never been described so far. Although it could be arguable, this pattern of male flowering in which staminate flowers are successively open along an inflorescence can suppose an advantage for melons by providing more pollen during longer periods and then, giving more chances for pollination of female flowers so improving melon yield.

The inflorescence represents a modified shoot system which functions to bear flowers. In determinate inflorescences, the main shoot stops producing additional lateral branches with flowers or bracts and the entire inflorescence apex ends in a terminal flower. In inflorescences of indeterminate type, the inflorescence apex continues to grow and produces either flowers or inflorescences on its flanks. The indeterminate form of inflorescence architecture seen in many flowering plants was independently derived from determinate inflorescence structures several times during evolution (Stebbins, 1974). Several genes in different species have been described as associated with the inflorescence identity in apical and axillary meristems. CENTRORADIALIS (CEN) of *Antirrhinum* (Bradley et al, 1996) and TERMINAL FLOWER 1 (TFL1) in *Arabidopsis* (Shannon and Meeks-Wagner, 1993) encode orthologous genes that are important for the maintenance of indeterminate inflorescence growth (Bradley et al., 1997). During flowering, both genes are required to maintain inflorescence identity of the shoot apical meristem while flower meristems are produced in its flanks. Mutations of these genes convert the indeterminate inflorescence of these plants to determinate inflorescences bearing a terminal flower (Shannon and Meeks-Wagner, 1991; Bradley et al., 1996). By contrast, the homologous SELF PRUNING (SP) gene in tomato, which together with, e.g. CEN and TFL1 or the tobacco CET genes, define the CEN-like gene family (Pnueli et al., 1998, 2001), might have a different function because the sp mutation does not alter inflorescence structure, but rather changes whole plant architecture (Thouet et al., 2008). The expression of CEN-like genes in axillary meristems seems to be the rule in all plant species examined so far as it has been reported in *Lotus japonicus*, *Impatiens balsamina*, ryegrass, tobacco and *Arabidopsis* (Amaya et al., 1999; Ratcliffe et al., 1999; Jensen et al., 2001; Ordidge et al., 2005; Guo et al., 2006; Conti and Bradley, 2007). Flowering is synonymous of stem apical meristem termination and another CEN-like gene, FLOWERING LOCUS T (FT) in *Arabidopsis* or its homologue SINGLE FLOWER TRUSS (SFT) in tomato (Lifschitz et al., 2006) is also important to control flower identity in meristems and inflorescence structure.

We observed that the Zimbabwean melon line TGR-1551 bears male flowers in indeterminate inflorescences (Fig. 1). This character has been examined and evaluated in a RIL population

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Fig. 1. Male flowering pattern in melon cultivar 'Bola de Oro' (BO), showing 1–2 flowers per node, and TGR-1551 (TGR), presenting male flower trusses.

which derives from a cross of TGR-1551 with the Spanish melon cultivar 'Bola de Oro' that bears single or at most two male, ungrouped flowers per node. In this paper we aimed to localize the genomic region(s) in which localize(s) the gene(s) associated with the presence of male trusses in melon and, taking advantage of recent melon genome sequencing and annotation (García-Mas et al., 2012) to hypothesize on candidate gene(s) controlling the trait.

Materials and Methods

A population of 114 RIL ($F_{7:8}$), originated from a cross between the melon genotype TGR-1551 and the cultivar 'Bola de Oro' by the single-seed descent method, as well as a linkage map developed from it, were available for QTL analyses. The linkage map was obtained with 223 molecular loci, most of them (99.5%) codominant (SSR and EST-SSR), by using JoinMap® 4.0 software (VanOoijen, 2006) with a minimum LOD score of 4.0, a recombination frequency value of 0.3, and *P*-values > 0.01 for marker linkage analysis. These loci were distributed among 22 linkage groups, and then regrouped into 12, according to the molecular marker order, and designed following Périn (2002). The linkage map spanned a total distance of 992,82cM with an average of 4,6cM between markers.

Since differences in male flower trusses were observed in the RIL population derived from TGR-1551 (bearing male flowers in indeterminate inflorescences) and the Spanish melon cultivar 'Bola de Oro' (which bears 1–2 male flowers per node), this population was evaluated for the presence/absence of staminate flower trusses in 2012 and 2013. Evaluations were carried out on plants grown under greenhouse, in bare soil, with drip irrigation. At least three plants per RIL were considered and the character was recorded as 0, when plants had not male flower trusses and 1, when plants showed male flower trusses (Fig. 2).

QTL analyses were carried out with the software package MapQTL® 5.0 (VanOoijen, 2004) for the presence/absence of staminate flower trusses. Only RILs with at least three plants in each evaluation were considered, 73 in 2012 and 96 in 2013. Interval Mapping model (IM) was used first to detect consistent QTLs. Afterwards, and by using automatic cofactor selector tool (ACS), Multiple QTL model (MQM) was performed. LOD scores, phenotypic variation and QTL position were then estimated. A permutation test (10.000 cycles) was used to determine the LOD threshold with a confidence interval of 95%.

In order to search a candidate gene involved in the presence or absence of male trusses, tomato and *Arabidopsis* genome databases were scanned for CET genes described as associated to proteins involved in similar plant phenotypes. For instance, the melon protein sequences with high homology to that of SFT gene protein in tomato were found by BLAST in MELONOMICS genome sequence database (https://melonomics.net).

Fig. 2. Male flower trusses in one line of the melon RIL population TGR-1551 x 'Bola de Oro'.

Results and Discussion

Twenty-seven out of 73 RILs in 2012 and 32 out of 96 RILs in 2013 showed male flower trusses. Inflorescences displayed different internode lengths but all of them were indeterminate.

Only one significant QTL was detected by IM for presence/absence of male trusses in both evaluations 2012 and 2013 (Fig. 3) that was located on LG VIb (subgroup of LG VI). High LOD scores were calculated for markers ECM81, ECM133 and GCM303. For MQM analysis, marker ECM81 was automatically selected as cofactor. The QTL peak was located among the markers ECM81 and ECM133 (LOD 9.0 for 2012 and 11.8 for 2013). The percentage of phenotypic variation explained was high (46.1% for 2012 and 45% for 2013). Genetic distance between the markers ECM81 and ECM133 was 7.7cM in the RIL map.

The sequence of the protein encoded by tomato SFT gene was obtained from the tomato genome sequence database (http://solgenomics.net) and it was scanned for homology in the melon genome by BLAST. Six putative proteins were then identified, two of them showing a high homology: MELO3C010015P1, which is located in LG XII and, as expected, shows a high homology to the protein encoded by SFT gene in tomato. Another highly homologous protein was MELO3C006888P1, located in LG VI, the same linkage group where the QTL herein was detected. This protein shows a high homology to CEN-like protein 1 in tomato, and it is also related to CET1 in *Nicotiana* and Terminal Flower 1 (TFL1) in *Arabidopsis*. There are other similar proteins in melon but they showed lower homology than these two above: MELO3C024540P1 (CM3.5_scaffold68) and MELO3C021426P1 (CM3.5_scaffold47), both placed in non-anchored fragments and with similarities to CEN-like protein 2 (CET2); The protein MELO3C009465P1, located in LG

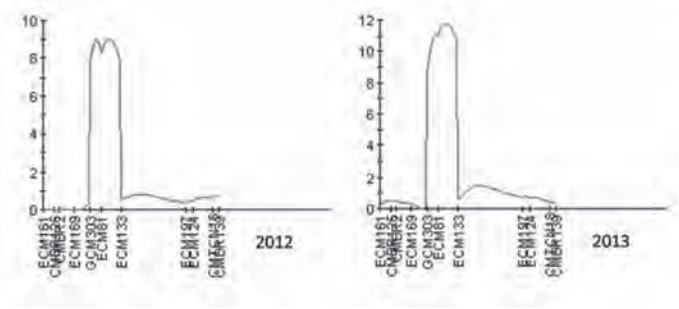

Fig. 3. Quantitative trait loci detected in linkage group VIb of melon genome by Multiple QTL mapping model analyses (95% LOD threshold) for presence/absence of male trusses in two evaluations (2012 and 2013).

XI and homologue to SP (SELF PRUNING) in tomato, and MELO3C009627P1, also placed in LG XI and with homology to Mother of FT and TFL1 proteins in *Arabidopsis*.

According to this first approach, the melon protein MELO3C006888P1, located between the genome positions 6,993,449 to 6,994,296 of scaffold00006, could be a putative candidate gene involved in the regulation of presence/absence of male inflorescence, since the DNA sequences associated are located on LG VI, the same linkage group where the QTL for male inflorescence was detected in our linkage map. The most tightly linked markers, ECM81 and ECM133, are located in that same scaffold between the positions 7,549,735 and 7,746,005. In order to finely map the QTL and to validate tightly linked or in-gene molecular markers, new and specific SNP markers are being currently designed to be evaluated in the RIL population and to be used in backcrossing generation of near-isogenic lines in the 'Bola de Oro' cultivar background. Also, a new evaluation of the RIL population will be carried out to evaluate, not only the presence/absence of male inflorescences, but also the number of male flowers in each one.

In melons, male flowers usually appear lonely or in groups of one, two or even five flowers per node and secondary stems. This is the first time in our knowledge that male flower distribution in inflorescences is described. This pattern might suppose an advantage for melons since multi-staminate flowering pattern could provide more pollen and during extended periods; therefore, it can provide chances of pollination of female flowers and improved melon yields may be expected in situations in which pollination could be compromised. Further studies of fine mapping of the candidate gene for this trait and generation of near-isogenic lines in and from the TGR-1551 x 'Bola de Oro' genetic map will help understanding of genetic basis for the trait.

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Effects of Low Night Temperature on Growth, Dry Matter Accumulation, and Distribution During Fruit Expansion in Melon

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Additional index words. *Cucumis melo* L., growth, dry matter accumulation, fruit development

Abstract: The effects of low night temperatures (9 °C and 12 °C) on dry matter accumulation and distribution of plant growth were analyzed with the material of "Yumeiren" melon (*Cucumis melo* L.) growing under normal and low night temperature circumstance simulated by phytotron. The results showed that 9 °C at night significantly reduced the height and stem diameter, and the extent was about 4.89% and 2.87 % respectively. The dry matter accumulations of roots, stems, leaves and fruits were limited by low night temperatures of 9 °C. Compared with control, the dry matter accumulation amounts of roots, stems, leaves and fruits decreased by 9.57 %, 7.71 %, 6.98 %, 25.44 % respectively after the treatment of 9 °C at night for 18 d. Furthermore, the total dry matter accumulation reduced by 16.22 %, and the dry matter accumulations were transferred from fruits to stems, leaves and other vegetative organs. However, in the whole treatment process, the treatment of 12 °C at night was appeared no significant effect on melon development and dry matter accumulation and distribution. These above results indicated that 12 °C at night might be suboptimal temperature for melon growth, while 9 °C at night obviously restricted the melon growth during fruit expansion.

Melon (*Cucumis melo* L.) is the rmophilic plant. Low temperature adversity stress will not only result in its fertility problems, but still can cause the death of the plant if more serious. At present, gardening plants on low temperature stress researches mainly involves the seedling growth of metabolic changes, but the researches on the influence of low temperature stress to fruit period are less. Researches aiming at melon low temperature adversity physiological major refer to the germination rate of seeds (Wolff et al., 1997; He, 2008), seeding growth (Edelstein et al., 2009), the influence of night temperature on the melon matter content, physiology and biochemistry and plant cold resistance during the period of flower bud differentiation (Edelstein and Nerson, 1994; Mavi and Demir, 2007) aspect on increasing plant cold-resistant of chemical protective (Wolff et al., 1997; He, 2008) and the influence of cryopreservation on the accumulation of sugar in fruit (Manzano et al., 1996; Lester et al., 2007). However, in the process of melon facility cultivation, plant is vulnerable to the negative impacts of the low temperature during fruit enlargement period, especially the low temperature at night. It directs impact on plant growth and fruit development and leads to serious affection of the quality and yield (Li, 2009). However, research in this regard is rarely reported. In the melon fruit enlargement period, the effect of low temperature at night on its growth and development is still not clear.

Therefore, in this research, melon was as experiment material. We analyzed the effect of low temperature at night on its growth and development during the fruit enlargement period to explore the low night temperature stress related factors. On this basis, we can prevent melon reproductive barriers technical measures in low temperature. This will be of great importance to improve melon production and quality in the low temperature seasons.

Materials and Methods

Plant materials and growth conditions. Seeds of the cultivar "Yumeiren" (*Cucumis melo L.*), a popular melon variety from Northeast China, were sown in sand/soil/peat (1:1:1 v/v) mixture in July in Shenyang Agricultural University. Plants were cultivated under glass greenhouse conditions (12h light, 300~1300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 26 ± 3 °C in day and 15 ± 3 °C in night, 40~60% relative humidity) for 30d.

Low night temperature stress treatment. Seven days after pollination, plants were moved into an environmental chamber to receive night (18:00 ~ 6:00 next day in 12h dark) temperature at

Acknowledgements. This work was financially supported by the Young Scientists Fund of the National Natural Science Foundation of China (Grant No. 31201641), the Science and Technology Committee of Beijing (Z131105000413), the National Basic Research Program of China (973 Program, Grant No. 2012CB126306), the Earmarked Fund for China Agriculture Research System (Grant No. Nycytx-35-gw23), and the Major Scientific Research Projects of Liaoning Province (Grant No. 2011215003), the Education Commission of Beijing (PXM2013_014207_0000_73, PXM2014_014207_ 000028) , the 2014 Education Teaching-Research Training for Undergraduate Students from the Education Commission of Beijing.

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15 °C, 12 °C and 9 °C treatments for 18 days. Light intensity (in μmol·m^{-2}·s^{-1}) during the day was 475 ± 50 from 6:00 to 10:00, 1045 ± 50 from 10:00 to 16:00, and 570 ± 50 from 16:00 to 18:00. Temperature and relative humidity in the growth chamber was maintained at 26 °C and at 50 %, respectively.

Dry matter measurements. Plant height (above cotyledons) and stem diameter (1 cm above the cotyledon) was measured in 0, 3, 6, 9, 12, 15, and 18 days after low night temperature treatment. The dry matter distribution ratio for each organ was calculated as following:

Dry matter allocation ratio = (dry matter weight/dry matter weight of whole plant) ×Plant dry matter

Data were analyzed by Excel 2003 and DPS, and mean separation was accomplished by the least significant difference (LSD) test at $P \leq 0.05$ or $P \leq 0.01$.

Results

Effect of low night temperature on plant height and stem diameter. As can be seen from Fig.1a and Fig.1b, during the fruit enlargement period, the plant height and stem diameter of melon plant increased gradually, especially in the early stage of the fruit enlargement increase obviously. The 9 °C treatment had an obvious inhibitory effect on the growth of plants. The height

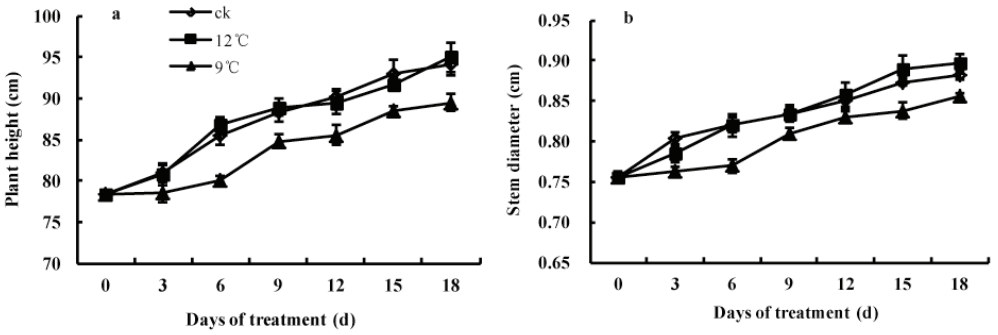

Fig. 1. Effect of different night low temperature on the height and stem diameter of melon plant.

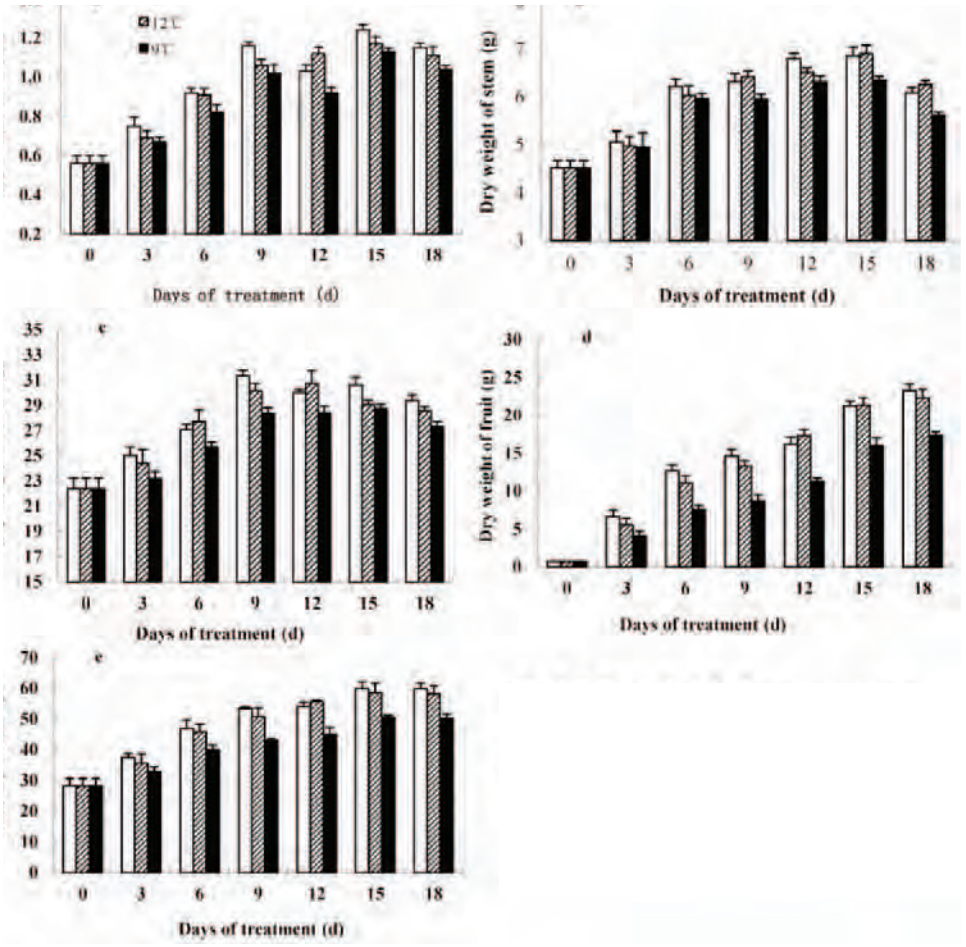

Fig. 2. Effect of night low temperature on dry matter accumulation of melon organs.

and stem diameter treated by night 9 °C was significantly lower than that of the control group. At 18 days after pollination, the plant height and stem diameter were decreased by 4.9 % and 2.9 %, however, there was no significant change when treated with the night 12 °C.

Effect of low night temperature on dry matter accumulation. Fig. 2 showed that dry matter accumulation was greatly limited at night 9 °C. Compared with the control, low night temperature 9 °C for 18 days resulted in decrease of dry weight in the root, stem, leaf and fruit by 9.6 %, 7.7 %, 6.9 % and 25.4 %, respectively. Overall, the dry weight accumulation was reduced by 16.2 %. However, at night 12 °C, no significant effect was observed on dry matter accumulation in any organs.

Effect of night low temperature on the distribution of dry matter. The distribution of dry matter in different organs is shown in Fig. 3. As compared with the control group, the dry matter partitioning ratios of root, stem, leaf were increased significantly at night 9 °C, while it was significantly decreased in the fruit. However, effects of nigh 12 °C on the distribution of dry matter accumulation were not significantly different. The results suggested that the night 9 °C resulted in dry matter transfer from the fruit to the stems, leaves and other vegetative organs.

Discussion

Effects of low night temperatures on the growth of melon plant during fruit enlargement. Melon plant growth is limited by low temperature, and the lower temperature is the higher extent of inhibition. The effects of low temperature on the growth of melon plant involve roots, stems, leaves and so on. Meanwhile, the low temperature time duration makes a intensifying inhibition for the biological production of melon plant. Extreme low temperature has bigger obstacle on melon seedling growth, since moving from 0 °C to 4 °C condition of treatment in the first day, the growth of plant is restrained completely. The growth recovery rate of melon plant on treatment of day and night low temperature is more slowly than its night low temperature. The results show that low night temperature also restrains the growth of plant during melon fruit enlargement period. This means that low temperature 9 °C at night significantly reduces the growth of plant height and stem diameter. Compared with control, the plant height and stem diameter was reduced and the extent is about 4.89 % and 2.87 % respectively processing for 18d. However, there was appeared no significant effect on plant height and stem diameter after 12 °C at night processing. These above results indicate that 12 °C at night is suitable temperature for melon growth, while 9 °C at night obviously restricted the melon growth during fruit enlargement period. We should take some protective or cultivation measures to reduce the damages on the plant growth caused by low temperature in facilities.

Effect of low temperature at night on melon dry matter accumulation and distribution during fruit enlargement period. Dry matter accumulation is the initial product that produced through photosynthesis including glucose and amino acids. They then are used to form both the plant assimilation substance, part of it used for the growth and development of various organs, and the other part accumulate as a temporary storage of substances in plant. Plant dry matter accumulation and distribution has a great influence on its own growth and development (Wei, 2002). On the basis of this, there are many researches about dry matter ac-

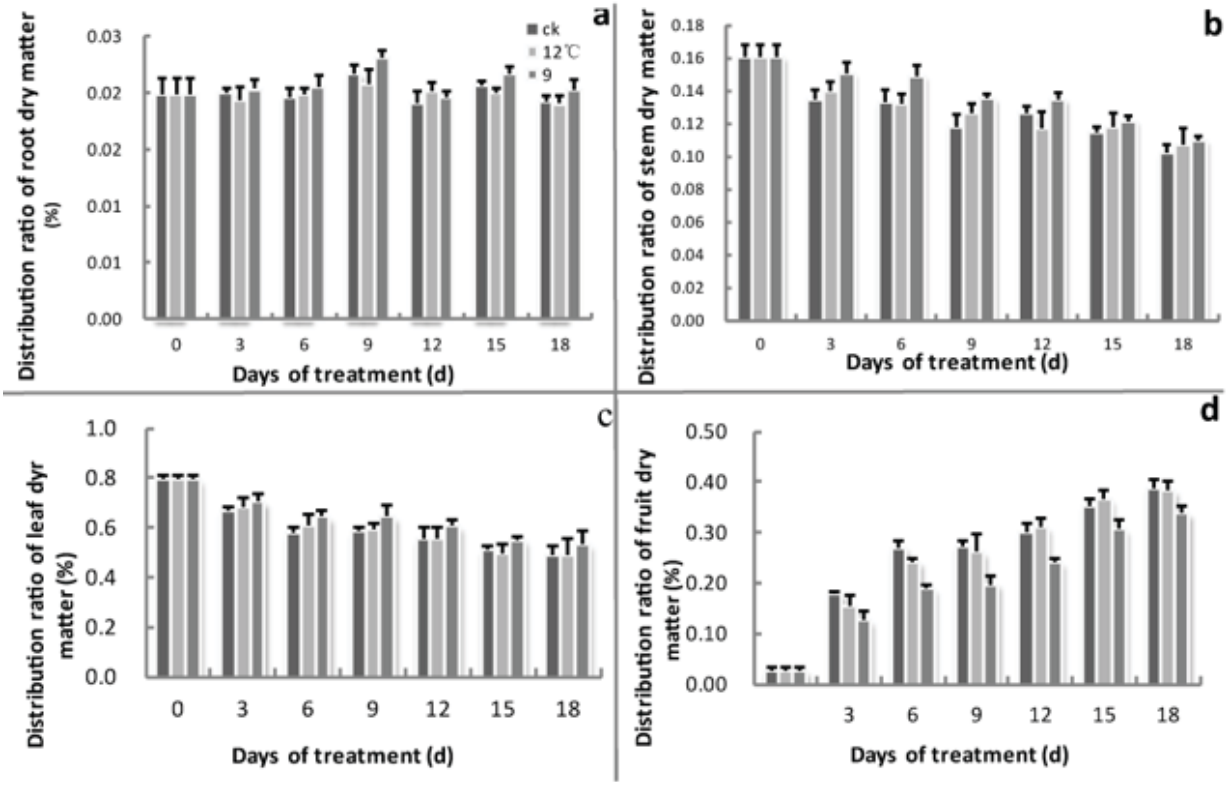

Fig. 3. Effect of night low temperature on the distribution of photosynthate of melon.

cumulation and distribution. Some of researches have improved that low temperature is an important environmental factors about substance accumulation and delivery (Walker et al., 1990).

Due to a long period of low temperature environment, seedling root activity reduced, the over ground part aged, it seriously affected the growth of seedlings. De ling Sun found that leaf area growth, dry matter accumulation, relative growth rate (RGR) and net photosynthetic rate (NAR) and chlorophyll content were significantly lower at cryogenic treatment than that at medium temperature and high temperature treatment processing (Wang and Wang, 2000). The results of analyzing the dry matter distribution rate of the seedling under different temperatures clearly showed that the dry matter distribution rate at roots under the low temperature was the highest while at the high temperature was the lowest, and the dry matter distribution rate of leaf under the intermediate temperature was the highest. Starch and sucrose are the main end products of photosynthesis. The transportation and distribution of photosynthetic products of the various organs was directly related to the growth of plants. The product of the photosynthesis is detained in the leaf and can't be transported if the sucrose library of plant is discordant. The results will cut down the leaf photosynthesis rate, the assimilation product and the dry matter accumulation. The conveyor direction of the sucrose synthetized in the cytoplasm is mainly decided by the carbohydrate consumption of the organs or the organizations demand for the sucrose. When the demand is larger, the sucrose formed in the cytoplasm transport to the organs or the organizations preferentially. The product of the photosynthesis is detained in the leaf and can't be transported if the sucrose library of plant is discordant. The results will cut down the leaf photosynthesis rate, the assimilation product and the dry matter accumulation. In the period of the fruit enlargement, the fruit is growing quickly, which is the mighty substance metabolic pool and at the same time, the vegetative organ is also at the same time.

The study showed that the dry matter accumulations of the various organs were limited by the low-temperature treatment of 9 °C at night and it changes the dry matter distribution rate. This leads to the dry matter accumulations transfer from fruits to stems, leaves and other vegetative organs and makes the photosynthesis products accumulate in the source, which indicates that the degree of the melon fruit sink strength decline may heavier than the vegetative organ and also influences the photosynthesis product transportation to the fruit.

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Effects of Exogenous Salicylic Acid on Seedling Growth, Photosynthetic and Chlorophyll Fluorescent Parameters in Melon Seedlings under Cadmium Stress

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Additional index words. melon, salicylic acid, cadmium stress, photosynthetic, chlorophyll fluorescent parameters

Abstract. We investigated the effects of 10~200 μmol·L^{-1} exogenous salicylic acid (SA) on 'Hamilü' melon seedlings growth, chlorophyll content, gas exchange parameters and chlorophyll fluorescence under cadmium (100 mg·L^{-1} Cd^{2+}) stress by substrates culture. The results showed that: there were significant effects of cadmium stress on the growth and photosynthesis function under. Treatment of 100 μmol·L^{-1} SA had the best alleviation effect on Cd^{2+} stress damage. At this concentration, the seedlings growth, net photosynthetic rate (P_n), stomatal conductance (G_s), transpiration rate (T_r), photosystem II (F_v/F_m), excitation capture efficiency of open centers (F_v'/F_m'), actual photochemical efficiency (Φ_{PSII}) and photochemical quenching coefficient (q_P) in leaves were significantly increased, while minimal fluorescence (F_0) and non-photochemical quenching coefficient (NPQ) were significantly decreased. The above results indicated that dosage effect of SA existed on the alleviation of Cd^{2+} stress in melon seedlings, and the best alleviating effect on Cd^{2+} stress damage was 100 μmol·L^{-1} SA, which was favorable for the seedlings to maintain photosynthesis, improve photochemical electron transport efficiency, capture and converse solar energy thus improving melon growth.

Cadmium (Cd) is a non-essential element for plant growth, and toxic at low concentration (Bouzid and Youcef, 2009). With the development of the mining, smelting industry, as well as Cd containing fertilizer application agricultural production, Cd containing irrigation with sewage, sludge etc., the soil Cd pollution is seriously increasing, possessing a threat to animal and human health since Cd is easily absorbed and accumulated by plants. Moreover, Cd stress is toxic to the plant itself; it can destroy the cell membrane structure and function of plant, inhibit photosynthesis and respiration processes, as well as their growth (Milone et al., 2003; Zhang et al., 2005).

Salicylic acid (SA) is a small phenolic molecule widely found in plant where it serves as an intracellular signaling molecule. SA regulates plant growth and induces tplant resistance to damage caused by adverse factors (Yang et al., 2013). Exogenous SA, to some extent, can relieve the damage of heavy metals to plants. For example, appropriate concentration of exogenous SA can significantly promote cabbage seed germination index, vigor index and seedling growth under Cr^{6+} toxic conditions (Zhao et al., 2008) and alleviate Lead stress on inhibition of cabbage seedling growth (Xia et al., 2012); 10 μmol·L^{-1} SA pretreatment can ease Cd^{2+} stress on inhibition of lettuce seedling growth at different degrees, promote chlorophyll content and photosynthesis function (Ren and He, 2010). However, there are few studies on effects of exogenous SA on seedling growth, photosynthetic and chlorophyll fluorescent parameters in melon seedlings under Cd^{2+} stress. Accordingly, our study focuses on the effects ofconcentration and treatment time of exogenous SA on seedling growth, photosynthetic and chlorophyll fluorescent parameters and other related indicators in melon seedlings under Cd^{2+} stress. We explore the feasibility of controlling Cd^{2+} stress with exogenous SA from the prospects of photosynthesis restriction, PSII quantum distribution and provide a theoretical basis to Cd^{2+} stress control with SA.

Materials and Methods

Plant culture and treatments. Seeds of the melon variety 'Hamilü' were provided by the Melon Research Group, Horticultural Research Institute, Shanghai Academy of Agricultural Sciences. SA was manufactured from Shanghai Sinopharm Group. The SA was first dissolved in 95% ethanol and then prepared with distilled water into 1000 μmol·L^{-1} mother liquor, stored at 4 °C and diluted as required test concentration. Cd^{2+} donor is $CdCl_2 \cdot 2.5H_2O$, AR.

The test was conducted in Horticultural Research Institute, Shanghai Academy of Agricultural Sciences. The seeds were rinsed thoroughly with distilled water and germinated on moist filter paper in an incubator at 30 °C. The germinated seeds were sown in plastic plates mixed with an garden soil, perlite, and peat (2:1:1) in a growth chamber at average of at 30 °C/20 °C day/night temperatures (12h/12h), photosynthetic photon flux density (PPFD) of 800–1000 μmol·m^{-2}·s^{-1} . Plants were irrigated daily with half-strength Hoagland nutrient solution.

Healthy seedlings at 3-4 leaf stage were selected for treatment. According to preliminary test results, the best effect on the alleviation of Cd^{2+} stress damage was observed in the treatment of 100 μmol·L^{-1} SA. Therefore, SA concentration were 0 (S_0), 10 (S_1), 50 (S_2), 100 (S_3) and 200 μmol·L^{-1} (S_4). We regarded the seedlings under 0- concentration SA and without Cd^{2+} stress treatment as our control (CK). Chlorophyll content, photosynthetic parameter and chlorophyll fluorescent parameter were measured at the second fully expanded leaf respectively under CD^{2+} stress 5 d and 10 d; the growth parameter was measured for 3 times after 10 d and 10 seedlings per treatment.

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Seedling growth. The shoot height (cotyledon node to the growing point) was measured with a ruler; caliper for stem diameter measurement (the cotyledon diameter along the expanded direction of cotyledon section); the leaf area was determined by a formula leaf length × width × 0.66, in which, leaf length and width were referred to the maximum value (Wang et al., 2009); thereafter, the seedlings were rinsed with distilled water and dried for shoot and root fresh weight.

Photosynthetic parameter. Net photosynthetic rate (*P*N), stomatal conductance (g_s), transpiration rate (*E*), and intercellular CO_2 concentration (C_i) were measured on the third fully expanded leaf using an infrared gas analyzer portable photosynthesis system (*LI-6400*, *LICOR Inc.,* Lincoln, NE, USA). During the measurements, PPFD was set to 800 μmol $m^{-2}\cdot s^{-1}$, the air relative humidity was about 85%, the leaf temperature was maintained at 25 °C and the ambient CO_2 concentration was about 400 $\mu L\cdot L^{-1}$. Measurements of photosynthesis were made once for each leaf and for six different leaves per treatment.

Chlorophyll fluorescent parameter. To determine the state of PSII, Chl fluorescence was measured using a portable pulse modulated fluorometer (PAM-2100, Walz, Effeltrich,Germany). Before each measurement, leaves were dark-adapted for at least 30 minutes. The minimal fluorescence of dark-adapted state (F_o) was determined by a weak modulated light, which was low enough not to induce any significant variable fluorescence. A 0.8-s pulse of the saturating light of 8000 $\mu mol\cdot m^{-2}\cdot s^{-1}$ was used on dark-adapted leaves to determine the maximal fluorescence of dark-adapted state (F_m). Then the leaf was illuminated with the actinic light (red led light) of 600 μmol $m^{-2}\cdot s^{-1}$. When the leaf reached steady-state photosynthesis, the steady-state fluorescence (F_s) was recorded and a second 0.8 s saturating light of 8,000 $\mu mol\cdot m^{-2}\cdot s^{-1}$ was applied to determine the maximal fluorescence of light-adapted state (F_m'). The actinic light was turned off, the minimal fluorescence of light-adapted state (F_o') was determined by the illumination with 3-s far-red light, photochemical quenching coefficient (q_P) was measured. The maximal quantum yield of PSII photochemistry (F_v/F_m), the effective quantum yield of PSII photochemistry (Φ_{PSII}), the efficiency of excitation capture of open PSII center(F_v'/F_m'), non-photochemical quenching (NPQ), and q_P were calculated as F_v/F_m, $(F_m' - F_s)/ F_m'$, F_v'/F_m', $F_m/F_m' - 1$, $(F_m' - F_s)/(F_m' - F_o')$, respectively (Chen et al., 2012).

Statistical analysis. All data were subjected to analysis of variance and expressed as the mean (minimum of three replications) ± standard deviation (SD). The significance of difference between the control and treatments was set at $P = 0.05$ by Duncan's *t*-test.

Results

Seedling growth. Melon seedlings were significantly inhibited under Cd^{2+} stress (S_0); shoot height, stem diameter, leaf area, shoot fresh weight, and root fresh were significantly decreased by 26.74 %, 24.39 %, 34.84 %, 30.66 %, 37.11 %, the root fresh weight of which suffered the largest decline, that was the maximum root growth inhibition (Table 1). All exogenous SA treatments promoted the seedling growth under Cd^{2+} stress, but different concentration SA had different effects on melon seedlings growth. 10 $\mu mol\cdot L^{-1}$ (S_1) and 50 $\mu mol\cdot L^{-1}$ (S_2) SA had no significant effect compared with S_0; the best effect on the alleviation of Cd^{2+} stress damage was observed in the treatment of 100 $\mu mol\cdot L^{-1}$ SA (S_3), compared with S_0 , shoot height, stem diameter, leaf area, shoot fresh weight, and root fresh weight increased by 36.29%, 21.61 %, 37.70 %, 34.68 %, and 41.46 %, respectively, but all significantly decreased compared with CK (except shoot height). Over 100 $\mu mol\cdot L^{-1}$ SA, seedlings growth decreased.

Photosynthetic parameters. Net photosynthetic rate (P_n), stomatal conductance (G_s),transpiration rate (T_r) of S_0 were significantly decreased by 58.37 %, 38.33 %, 68.18 % at 5 d; and 75.44 %, 59.11 %, and 73.37 % at 10 d, respectively, under CD^{2+} stress (Fig. 1). Treating with SA improved photosynthetic parameters, while a greater increase was reported at 10d than that of 5d. Compared with S_0, S_2, S_3 and S_4 improved P_n, G_s and T_r (except T_r at 5d) under Cd^{2+} stress significantly; and S_3 was the best concentration, P_n, G_s and T_r increased by 106.28 %, 42.26 %, 163.72 %, and; 191.22 %, 80.28 %, and159.15%, respectively, at 5d and 10d; there were no significant difference between S_1 and S_0. As indicated on Fig. 1C, intercellular CO_2 concentration (C_i) change was more complex: that of S_0 was significantly lower than that of CK at 5 d, but significantly higher than CK at 10 d; compared to S_0, for different concentrations of SA treatments, C_i was increased significantly at 5 d, but decreased at 10 d. Based on the above indexes analysis, it showed that exogenous SA can effectively alleviate Cd^{2+} stress on melon seedlings photosynthesis inhibition, so as to maintain a high photosynthetic rate.

Chlorophyll fluorescent parameters. Photosynthetic parameters (F_o) of S_0 were significantly increased by 25.57 % and 77.30 % respectively, greater increase was reported at 10d than that of 5d (Fig. 2A); compared with S_0, S_2, S_3 and S_4 decreased of F_o of melon seedlings under Cd^{2+} stress significantly, and S_3 had the best effect with decrease of 16.18% and 39.71 % at 5 d and 10 d, respectively; there were no significant difference between S_1 and S_0. The F_v/F_m represented PSII initial photochemical efficiency as a key indicator of photo inhibition. As shown as Fig. 2B, S_0 decreased of F_v/F_m by 19.41 % and 24.02 % at 5d and10d respectively compared with CK, greater decrease was reported at 10 d than that of 5 d; different concentration SA increased F_0 of melon seedlings under Cd^{2+} stress; S_3, was the best, which were increased by 21.71 % and 28.26 %, respectively, at 5d and 10d than these of S_0. Fig.2 C–E showed that the indicators, excitation capture efficiency of open centers (F_v'/F_m'), actual photochemical efficiency (Φ_{PSII}), and photochemical quenching coefficient (q_p) enjoyed consistent change with F_v/F_m. Compared with CK, F_v'/F_m', Φ_{PSII}, q_p of S_0 were significantly decreased by 28.70 %, 67.06 %, 33.30 % and 46.73 %, 82.75 %, 48.09 % respectively at 5d and 10d. Compared with S_0, S_2, S_3 and S_4 increased of F_v'/F_m', Φ_{PSII}, q_p under Cd^{2+} stress significantly, and S_3 had the best effect with increase of 26.28 %, 142.89 %, 44.73 % and 45.33 %, 225.21 %, 41.64 %, respectively, at 5d and 10d. NPQ reflects whether PSII antenna pigment-absorbed energy can be used for photosynthetic electron transport in the form of heat dissipation. As shown in Fig. 2F, compared with CK, NPQ of S_0 were significantly increased by 100.37 % and 228.24 %, respectively, greater increase was reported at 10d than that of 5d; compared with S_0, different concentration SA decreased NPQ of melon seedlings under Cd^{2+} stress (except S_1 group at 5d); S_3 was the best, which decreased 34.18% and 33.28% respectively at 5d and 10d.

Discussion

Previous studies have shown that at a certain concentration of Cd^{2+}, plant growth and biomass accumulation are significantly inhibited (Yu et al., 2008; Zhou et al., 2008). In agricultural production, it has been found that the application of exogenous SA can promote plant growth under salt damage, high temperature and heavy metal stress and other stress conditions (Yang et al.,

Table 1. Effects of exogenous SA on the growth of melon seedlings under Cd^{2+} stress.

Treatment	Shoot height/cm	Stem diameter/mm	Leaf area/cm²	Shoot fresh weight/g	Root fresh weight/g
CK	14.33 ± 0.91a	4.10 ± 0.36a	38.38 ± 3.48a	5.37 ± 0.28a	0.65 ± 0.04a
S_0	10.50 ± 0.90c	3.10 ± 0.26c	25.01 ± 1.44d	3.72 ± 0.33c	0.41 ± 0.03c
S_1	11.87 ± 0.65bc	3.13 ± 0.20c	25.20 ± 1.96d	3.79 ± 0.35c	0.47 ± 0.03c
S_2	12.17 ± 0.81bc	3.33 ± 0.15bc	28.41 ± 2.13cd	4.33 ± 0.31bc	0.50 ± 0.05bc
S_3	14.30 ± 1.25a	3.77 ± 0.36b	34.44 ± 2.59b	5.01 ± 0.40b	0.58 ± 0.05b
S_4	12.53 ± 1.21ab	3.43 ± 0.32bc	32.46 ± 3.18bc	4.99 ± 0.37b	0.52 ± 0.05bc

Note: Different letters in the same columns indicate significant differences among treatments at 0.05 level.

2013; Xia et al., 2012; Wang et al., 2012). Our study showed melon seedling biomass accumulation was significantly inhibited under CD^{2+} stress, especially the root fresh weight, while there were significant effects of exogenous SA on the growth and photosynthesis function under Cd^{2+} stress. The best effect on the alleviation of Cd^{2+} stress damage was observed in the treatment of 100 μmol·L^{-1} SA.

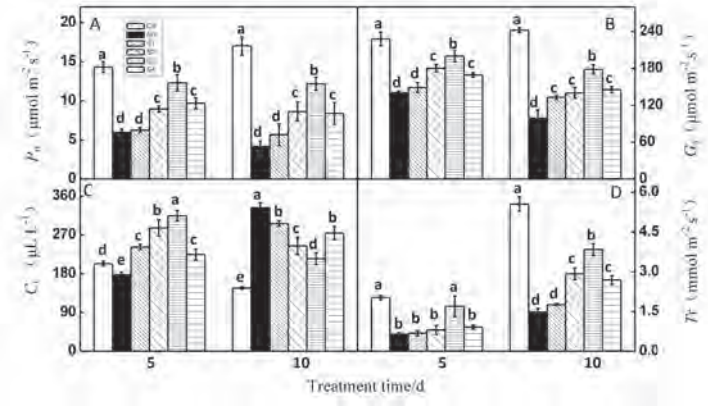

Fig 1. Effects of exogenous SA on photosynthetic parameters of melon seedlings under Cd^{2+} stress.

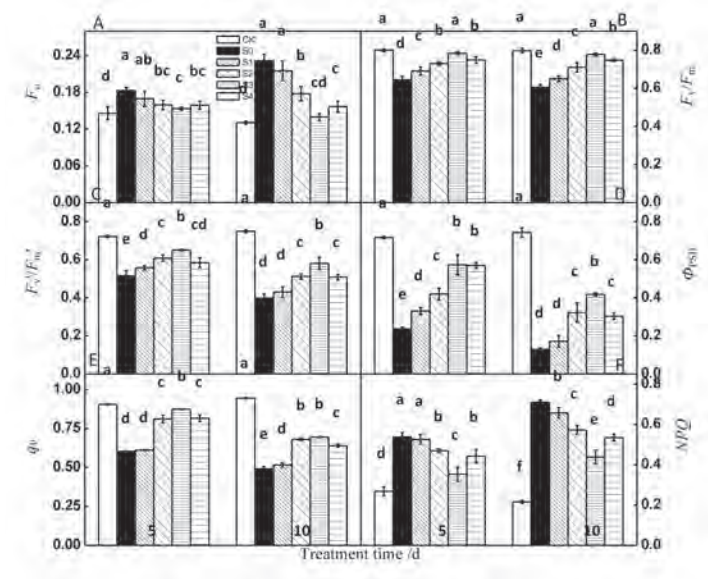

Fig 2. Effects of exogenous SA on chlorophyll fluorescent parameters of melon seedlings under Cd^{2+} stress.

Photosynthesis is a process that includes absorption, transfer and utilization of light energy. Under stress conditions, the major factors causing photosynthetic rate Pn decreased included stomatal limitation due to partially closed stomatas, and non-stomatal limitation caused by decreased mesophyll cell photosynthetic activity limitations (Berry and Downton, 1982). Exogenous SA treatment alleviated P_n, G_s and T_r decline under Cd^{2+} stress; for 10d treatment, increase rate of C_i was significantly lower, indicating that exogenous SA treatment can alleviate Cd^{2+} stress's non-stomatal limitation on melon seedlings photosynthetic function, limit melon mesophyll cells' photosynthetic activity decrease, and maintain a high photosynthetic rate. This was consistent with test results of Shi et al (2009).

Chlorophyll fluorescence is an important approach of detection and analysis of plant photosynthesis, providing a wealth of information for light system study and electron transfer process; is also an ideal probe of plant physiology and photosynthesis study, as well as relationship between plants and environmental stress. In the study, F_o and F_v / F_m decreased, indicating that Cd^{2+} stress caused melon seedlings photoinhibition phenomenon, and inhibited PSII activity(Zhu et al., 2010). In addition , decreased F_v'/F_m', $\Phi_{PS\ II}$, and q_p under Cd^{2+} stress made QA from the PSII acceptor to QB electron transfer suppressed, photosynthetic electron transfer branch reaction as molecular oxygen as the receptor enhanced, excitation capture efficiency of PSII reaction center declined (Zhao et al., 2013). NPQ rise showed increased heat dissipation trend under Cd^{2+} stress so as to avoid photoinhibition under stress, which is consistent with Chin et al's findings on chlorophyll fluorescence characteristics of salix leucopithecia under Cd^{2+} stress(Qian et al., 2011). Meanwhile, among different concentrations of exogenous SA treatment , 100 μmol·L^{-1} SA treated melon seedlings saw significant increase of F_v / F_m, F_v ' / F_m ', $\Phi_{PS\ II}$, and q_p than single Cd^{2+} stress treatment, accompanied by significant F_0 and NPQ decrease, indicating that exogenous SA could reduce the light inhibition caused by Cd^{2+} stress by starting xanthophyll cycle's heat dissipation mechanism, enhance the photochemical energy formation, thereby effectively alleviating the Cd^{2+} stress damage on melon seedlings photosynthesis mechanism, and maintaining the normal function of PSII.

In summary, exogenous SA can relieve Cd^{2+} stress in melon seedlings with dose effect; and 100 μmol·L^{-1} SA showed the best effect of increasing its net photosynthetic rate, thereby enhancing the ability of plants to adapt to Cd^{2+} stress environment, increasing seedlings biomass, as well as effectively alleviating Cd^{2+} stress damage.

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Single Nucleotide Polymorphism in Genes Accounting for Ethylene Biosynthesis and Perception in Melon Varieties, and Its Functional Validation

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Additional index words. *Cucumis melo*, shelf life, ACC oxidase, ethylene receptor.

Abstract. Ethylene is a key phytohormone responsible for fruit ripening and the ripening phenotypes mainly depend on the ability of ethylene production and perception. We previously performed EcoTILLING for the ethylene biosynthesis *CmACO2* and the receptor *CmERS1* genes among 46 melon cultivars, and identified several single nucleotide polymorphisms (SNPs) among these cultivars. In this study, we aimed to validate whether the SNPs affected fruit ripening characters. Two missense mutations were identified for the *CmACO2* among cultivars with long shelf life. To examine the effect of these SNPs on enzyme activity, in vitro enzyme activity assay was performed. Wild type *CmACO2* and each missense mutations were expressed in *E. coli*, and the recombinant proteins were used for assay for the ACC oxidase activity. The ACO activity carrying each missense mutation was higher than that of control, suggesting that these mutations had significant impact on the ACO activity. On the other hand, three missense mutations were identified for the *CmERS1* among cultivars with long shelf life. We then generated transgenic tomato lines in which each mutant gene was overexpressed under a constitutive CaMV 35S promoter. Preliminary characterization of transgenic lines showed altered fruit development, suggesting a possible role of the SNPs in fruit development.

Ethylene is a key phytohormone inducing fruit ripening and the ripening phenotypes are generally the result of the degree of ethylene production and perception. Melon (*Cucumis melo*) is an attractive model for studying fruit ripening due to the wealth of genetic resources exhibiting two types of fruit ripening phenotype, climacteric and non-climacteric types (Ezura and Owino, 2008). We previously performed EcoTILLING of genes involved in ethylene biosynthesis (*CmACS2, CmACO1, 2, 3*) and ethylene perception (*CmETR1, 2, CmERS1*) among 46 melon cultivars including five subspecies, *cantalupensis*, *reticulatus*, *chinensis*, *inodorus* and *conomon*, and identified several SNPs among these cultivars (Shibata et al., 2012). We found two missense mutations (V81M and P104S) for the *CmACO2* and three missense mutations (V86M, T377N and D508E) for the *CmERS1*, among cultivars showing long shelf life of fruits.

This study aims to validate whether these SNPs influence gene function and fruit ripening characters. We first examined the effect of two missense mutations found in *CmACO2* on the enzyme activity to produce ethylene in vitro. Next, we examined the effect of missense mutations found in *CmERS1* on ethylene sensitivity and fruit ripening by overexpressing each of these mutations in the model tomato cultivar Micro-Tom.

Materials and Methods

Full-length cDNA of *CmACO2* was isolated from a cDNA library of *Cucumis melo* L. cv. Vedrantais by a PCR-based method, and ligated into PCR-II-Blunt-TOPO vector (Invitrogen). Missense mutations were introduced by PCR-based methods. Wild-type (no mutation), mutants carrying a single mutation (mutation D or E in Fig. 1A), and the mutant carrying both mutations (D+E) were ligated into an expression vector, pET-15b (Novagen). The constructed vectors were introduced into *E. coli* strain BL21 Star (DE3), which was then used for mutated protein expression. After confirmation of protein expression and purification of expressed protein, the purified protein was subjected to a ACC oxidase activity assay as described in Nakatsuka et al. (1997).

Full-length cDNA of *CmERS1* was also isolated from the same cDNA library by a PCR-based method, and ligated into PCR-II-Blunt-TOPO vector (Invitrogen). Missense mutations were introduced by PCR-based methods, and mutated genes (mutation A, E, and G in Fig. 1A) were ligated into a plant transformation vector, pBI121. The constructed vectors were introduced into *Agrobacterium tumefaciens* strain GV2260, which was used for tomato transformation. A model tomato cultivar, Micro-Tom (*Solanum lycopersicum* L.) was used for tomato transformation by the method of Sun et al. (2006). Diploid plants among regenerated plants were screened by flow cytometry, and subjected for genomic Southern blotting analysis. Transgenic plants with a single copy of the transgene were selected and subjected for further characterization of fruit development. Regenerated plants with non-mutated and mutated transgenes were grown in a growth room at 25°C in 16 hrs light/8hrs dark period.

Results and Discussion

CmACO2 recombinant proteins with or without missense mutations were expressed and purified from the soluble fraction

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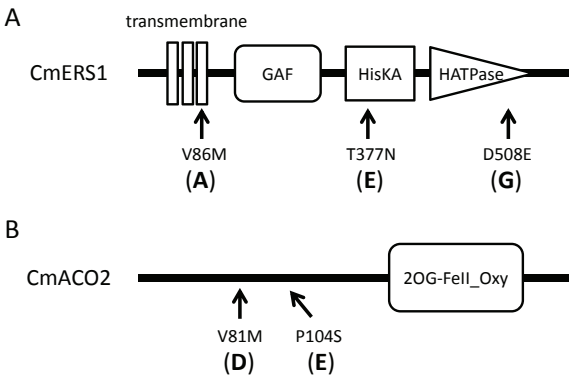

Fig. 1. Missense mutations in *CmACO2* and *CmERS1* detected among melon cultivars showing long shelf life of fruits. Arrows show missense mutations. Alphabets and numbers under arrows indicate amino acid of reference melon, the position and changed amino acid by missense mutation in a direction from left to right. Domains were searched by SMART analysis. Transmembrane: transmembrane domain; GAF: GAF domain; HisKA: His Kinase A domain; HATPase: Histidin Kinase-like ATPases domain; 2-OG-FeII_Oxy: Fe^{2+} and 2-oxoglutarate-dependent dioxygenase domain (Pfam domain).

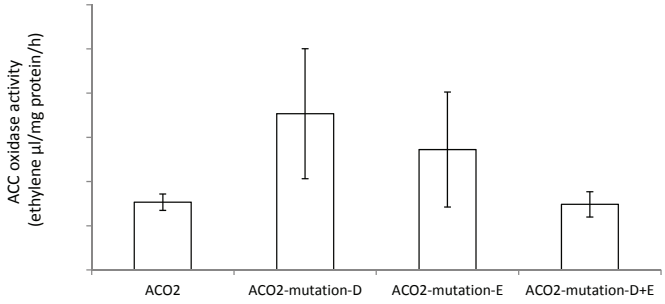

Fig. 2. Enzyme activity of recombinant *CmACO2* protein. Recombinant ACO2 protein with missense mutations D, E and D+E was expressed in *E. coli*. Purified recombinant protein by His-Trap column was subjected for enzyme activity assay. Values are the means ± SE (n = 2 or 3).

of *E. coli* cultures (data not shown). The recombinant proteins were purified by the His-Tag method, and subjected to the enzyme activity assay of ACO to monitor the ethylene production. All recombinant proteins confirmed the ACO enzyme activity (Fig. 2), but the enzyme activity of CmACO2 with mutations D and E was 2.3 and 1.8 times higher than that of wild-type CmACO2, suggesting that these missense mutations enhanced the enzyme activity. This was unexpected because E mutation was found some cultivars belong to *conomon* subspecies (ssp) with long shelf life, whereas higher ethylene production may accelerate fruit ripening herein decreasing shelf life. Although melon cultivar with only mutation D was not detected, mutation D+E was found some cultivars belong to *inodorus* ssp with long shelf life. However the enzyme activity was about the same as wild-type CmACO2. Thus, these mutations found in *CmACO2* are unlikely associated with long shelf life in melon. It has been reported that CmACO1 is a major factor contributing to the ACO activity during fruit ripening in melon cv. Vedrantais (Lassere et al., 1997). Nevertheless, although it is possible that CmACO2 activity occupies a minor portion among total ACO activity during melon fruit ripening, further biochemical studies of CmACO1 and CmACO2 enzyme activity in melon fruits with long shelf life are required.

The mutated *CmERS1* gene with each of missense mutations A, E, G or E+G was transformed into a model tomato cultivar, Micro-Tom. About 200 cotyledon explants were subjected for *Agrobacterium* inoculation, and we obtained 48, 16, 31 and 50 rooted plants for the *CmERS1* with missense mutations A, E, G and E+G, respectively. Diploid plants confirmed by flow cytometer analysis were 30, 11, 19 and 21 for the *CmERS1* with missense mutations A, E, G and E+G, respectively. Genomic Southern blotting analysis revealed the presence of 8, 4, 7 and 7 plants that carried a single copy of *CmERS1* gene with missense mutations A, E, G and E+G, respectively. Next, fruit development of the transgenic plants (T_0 generation) was examined. After planting, 7, 3, 4 and 6 lines for the *CmERS1* with missense mutations A, E, G and E+G, respectively, set fruits and the duration to breaker and red stages through fruit development was examined (Fig. 3). Prolonged fruit development to breaker stage was observed in lines A#4, A#28, E#3, G#6 and G#12 in which each mutated

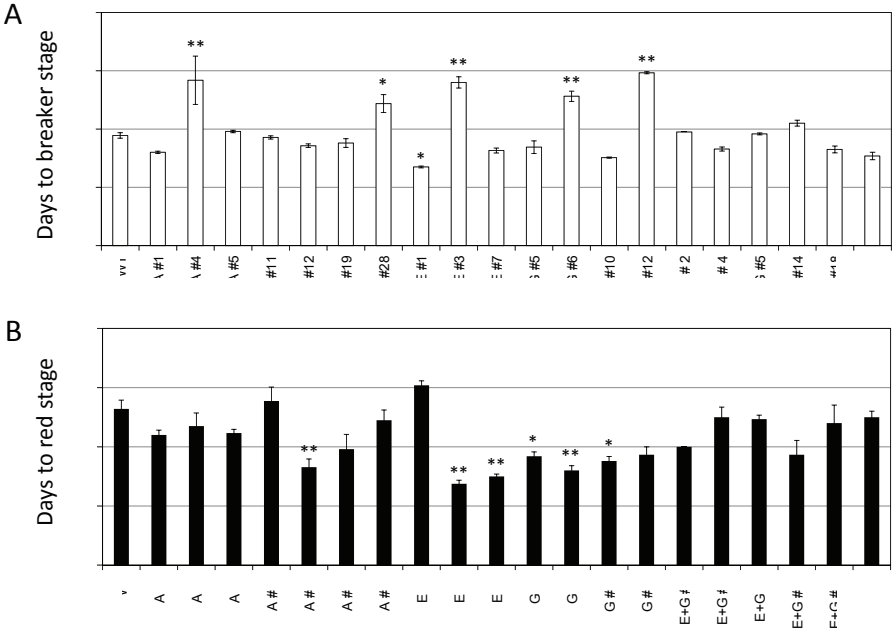

Fig. 3 (left). Characterization of fruit development in transgenic Micro-Tom plants (T_0) overexpressing CmERS1 with missense mutations A, E, G or E+G and the wild type (WT). **(A)** Days to breaker stage after pollination. **(B)** Days to red stage after breaker stage. The values indicate the mean and standard errors (n = 3-5). The level of significance compared with the WT was determined using Tukey-Kramer test (*P < 0.05, **P < 0.01).

CmERS1 is overexpressed. Earlier fruit development to breaker stage was observed in line E#1. However, among lines with missense mutation E+G, no lines showed altered fruit development (Fig. 3A). Transgenic lines A#12, E#3, E#7, G#5, G#6, and G#10 required shorter time to reach ripe red stage, although no transgenic lines showed prolonged fruit ripening. Among lines with missense mutation E+G, no transgenic lines showed altered fruit development (Fig. 3B). Although alterations in fruit development were observed among T_0 plants, whether the phenotypic alteration was due to the effect of transgenes was unclear in this preliminary characterization. Precise characterization must be carried out using homozygous lines for each transgene.

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Involvement of Ethylene in the Sexual Expression and Sex Determination of Watermelon (*Citrullus lanatus*)

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Additional index words. aminoethoxyvinylglycine, ethephon, hormonal treatments, rootstocks.

Abstract. Cultivars of watermelon are mainly monoecious. In this paper we demonstrate that although ethylene has a feminizing role in sex determination of female flower buds, arresting stamen development in female flowers in cucumber, melon and squash, it has a masculinizing effect in sexual expression in watermelon. In contrast to what occurs in other cucurbit species, ethylene delays the transition to female flowering and reduces the number of female flowers per plant in watermelon. The monoecy of watermelon is unstable under high temperature conditions, and many female flowers are converted into bisexual ones when greenhouse temperature exceeds 30 ºC. This monoecy instability is produced by a reduction of ethylene in floral buds before sex determination. In fact, high temperature reduces ethylene production in apical shoots, and bisexual flowers produce significantly less ethylene than female ones. Moreover, the conversion of female into bisexual flowers can also be achieved by external treatments with the ethylene inhibitor AVG, which indicates that watermelon female floral buds require a minimal level of ethylene to complete their normal development and maturation. External treatments with ethylene and AVG clearly indicate that ethylene delays the induction and the production of pistillate flowers in watermelon. This finding was also confirmed by the results from grafting experiments using two *Cucurbita pepo* genotypes that contrast for both ethylene production and sensitivity, *Bolognese* (*Bog*) and *Vegetable Spaghetti* (*Veg*), as rootstocks.

Ethylene is the main regulator of sex expression in the different cultivated species of the *Cucurbitaceae* family. The level of ethylene in the floral bud at the earliest stages of development is essential to determine its sex phenotype. In cucumber, melon and squash, sex determination and proper development of female flowers require much more ethylene than male flowers (Boualem et al., 2008, 2009; Li et al., 2009; Martínez et al., 2014). Ethylene in these three species promote the transition from male to female flowering, as well as the ratio of female to male flowers (Manzano et al., 2011, 2013). These data contrast with the masculinizing effect of ethylene in watermelon, since it was reported that treatments with ethylene-releasing agents result mostly in the suppression of ovary development (Rudich and Zamski, 1985), while inhibitors of its biosynthesis and perception hastened the appearance of the first female flowers and increased the female to male flower ratio in watermelon (Rudich and Zamski, 1985; Sugiyama et al., 1998).

By using different approaches in this paper we found that ethylene regulates differentially two sex-related developmental processes in watermelon: the sexual expression on one hand, and the sex determination and floral development of individual floral buds on the other hand. As in other cucurbits, the determination and development of watermelon female flowers requires ethylene; nevertheless, the regulation of watermelon sexual expression by ethylene seems to be completely opposite to that occurring in the other cultivated species of the family.

Material and Methods

All the experiments were carried out in the commercial cultivars *Premium* and *Fashion* (Nunhems). The plants were grown in the spring-summer season under standard greenhouses conditions in the area of Almería. Some of the experiments were carried out in a climate controlled chamber. For grafting experiments, *Premium* was grafted on different *Cucurbita* rootstocks: the commercial hybrid *Shintosa Camelforce* F1 (Nunhems), and two inbred lines of *Cucurbita pepo* subspecies *pepo* of the Vegetable Marrow Group, *Vegetable Spaghetti* (*Veg*) and *Bolognese* (*Bog*), that contrast for ethylene production and sensitivity (Manzano et al., 2010).

The treatments with aminoethoxyvinylglycine (AVG) and ethephon were performed in the apical shoots of plants of watermelon having 3 true leaves. The production of ethylene was measured in the main shoot apices of watermelon plants at the male and female phases of plant development, as well as in the flower buds throughout five different stages of floral development. The plant organs were excised from the plant and incubated at room temperature for 6 h in hermetic glass containers in the dark. Ethylene production was determined by gas chromatography. All these methods were already reported in Manzano et al. (2010, 2011, 2013).

Results and Discussion

The sexual expression of the triploid seedless cultivar *Fashion*, and the diploid *Premium* was assessed by studying the

Acknowledgments. The authors thank the collaboration of the company Nunhems Spain. This work was supported by grant P12-AGR-1423, funded by Junta de Andalucía, Spain. Z.M. acknowledges FPU program scholarships from MEC, Spain. S.M. is funded by grant PTA2011-479-I from the Spanish Ministry of Science and Innovation.

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sexual phenotype of each flower in the first 30 nodes of at least 10 plants. When plants were growing below 30 ºC, flowers of both cultivars were either male or female. However, when the greenhouse temperature raised above 30°C (36°C and 47°C), both cultivars developed bisexual flowers (data not shown). The higher cultivation temperatures and the occurrence of bisexual flowers were also associated with a decrease in the production of ethylene in the plant apex (data not shown). This monoecy instability evidenced under high temperature conditions, has been also recently detected in different cultivars of *Cucurbita pepo*, and was found to be associated with an allelic variant of the ethylene biosynthesis gene *CpACS27A* (Martínez et al., 2014), the ortholog to *CmACS7* and *CsACS2*, which are responsible for stamen arrest in the female flowers of monoecious cultivars of melon and cucumber, respectively (Boualem et al., 2008, 2009; Li et al., 2009).

To examine the production of ethylene in male, female and bisexual flowers, buds at different stages of floral development were collected from *Premium* plants. The developmental stages were separated on the basis of their corolla length, ranging from 4 ± 1 mm in F1 to 22 ± 2 mm in F5 (Fig. 1A). The level of ethylene produced by female flowers was significantly higher than that produced by male flowers in all developmental stages analyzed (Fig. 1B). Moreover, in both male and female flowers, the production of ethylene tends to increase upon maturation of the flower (Fig. 1B). Throughout the development of bisexual flowers, ethylene production was similar to that of male flowers (Fig. 1B). These data indicates that the female flowers of watermelon have the same requirements of ethylene as those of melon, cucumber and zucchini for their correct development, and that the occurrence of bisexual flowers could be the consequence of a reduction of ethylene in the floral bud as has been reported in zucchini and cucumber (Manzano et al., 2011; Yamasaki et al., 2003).

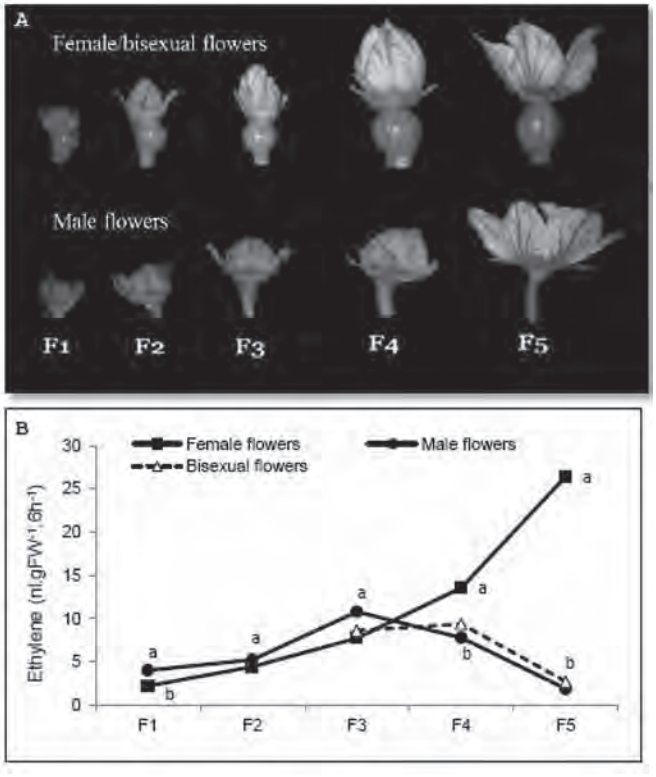

Fig. 1. Ethylene production in flowers of watermelon cv. *Premium*. **(A)** The different developmental stages of male, female and bisexual flowers were separated on the basis of the corolla length. **(B)** Evolution of ethylene production throughout flower development. Each point is the mean ± SE of at least five replicates. Different letters indicate statistical differences between the three studied floral phenotypes at each specific stage of development ($P \leq 0.05$; n = 5).

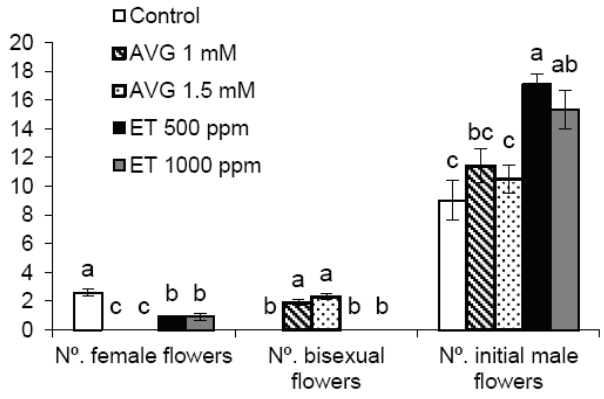

Fig. 2. Effect of treatments with ethephon (ET) and AVG on the sexual expression of watermelon plants cv *Premium*. Bars represent standard errors. Columns with different lowercase letters are statistically different by Tukey's test at $p \leq 0.05$.

External treatments with ethephon and AVG confirmed the involvement of ethylene in the abort of stamen during female flower development, but also altered significantly the sexual expression and sex determination of watermelon cv. *Premium* (Fig. 2). Ethephon delayed flowering in such a way that no flower buds were developed until nodes 5 or 6, delayed female flowering transition to node 12-14, and reduced the number of female flowers per plant (Fig. 2). No bisexual flowers were observed in plants treated with ethephon. AVG treatments, however, did not significantly alter the sexual expression of watermelon plants, although promoted a conversion of female into bisexual flowers (Fig. 2).

The effect of rootstocks on watermelon sexual expression was studied by grafting *Premium* plants on three rootstocks genotypes, the *Shintosa Camelforce* F1 rootstock, a commercial hybrid, commonly used in this area; as well as two genotypes of *C. pepo*, *Bog* and *Veg*, that differ in both ethylene production and ethylene sensitivity (Manzano et al., 2010). The results are in concordance with those of external treatments with ethephon and AVG. Although no alteration was found in the female flowering transition, rootstocks were able to alter the number of male and female flowers per plant (Fig. 3). When grafted on *Bog* rootstock, which have higher ethylene production and sensitivity than *Veg*, *Premium* produced lower number of female flowers per plant than when it was grafted on *Veg* (Fig. 3).

Results regarding sexual expression are contrary to what happens in cucumber, melon and zucchini, where ethylene treatments reduce the initial male phase of development and increase the female to male flower ratio, while treatments with ethylene inhibitors AVG or silver thiosulphate (STS) delay female flowering and reduce the female to male flower ratio (Byers et al., 1972; Manzano et al., 2011; Perl-Treves, 1999; Rudich, 1990). In watermelon our results confirmed those of Rudich and Zamski

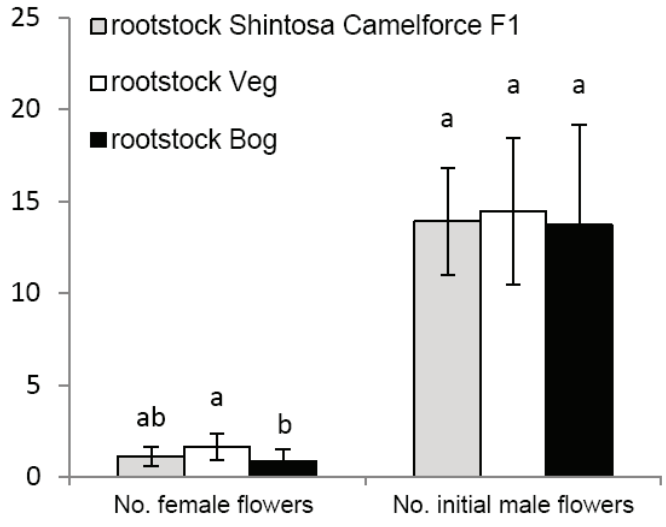

Fig. 3. Effect of different *Cucurbita* rootstocks on the sexual expression of the watermelon cv. *Premium.* Bars represent standard errors. Columns with different lowercase letters are statistically different by Tukey's test at $P \leq 0.05$. Plants of the watermelon cv. *Premiun* were grafted onto three rootstocks: '*Shintosa Camelforce* F1' *Cucurbita* hybrids (Nunhems), and onto *Veg* and *Bog C. pepo* cultivars.

(1985) and Sugiyama et al. (1998) indicating that ethylene has a masculinizing effect, delaying female flowering transition, and reducing the number of female flowers per plant. Moreover, these data indicate that the control of sex expression, i.e. the female flowering transition and the female to male flower ratio, is completely separated from the control of sex determination and development of individual floral buds. The two processes are regulated by ethylene, but the mechanisms controlling them should be separated in place and/or time. As stated previously, sex determination and proper development of individual floral buds depends on ethylene production in the floral meristem during very early stages of development (Boualem et al., 2008, 2009; Li et al., 2009; Martínez et al., 2014). The transition to female flowering and the female to male flower ratio are processes that should be regulated in the apical meristem of the plant. This is likely to be so in other cucurbit species, but given that in melon, cucumber and zucchini, the two processes were regulated in the same way, they had not been separated yet.

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Yield and Quality Response of Spaghetti Squash (*Cucurbita pepo*) Cultivars Grown in the Southeastern United States

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Abstract. New opportunities due to the local food movement have created increased interest in "locally grown" specialty crops like spaghetti squash in the southeastern US. The goal of this study was to evaluate several cultivars of spaghetti squash to determine their adaptation to the growing environment in the southeastern United States, especially yield and fruit quality among cultivars. The test was conducted in Laurel Springs, in western North Carolina. The study was arranged as a randomized complete block design with four replications and eight cultivars. Cultivars were direct seeded on 6 June 2013 on black plastic mulch with drip irrigation, followed by three harvests at 83, 90, and 104 days after planting. Plants were spaced 0.61 m in-row with 3.04 m between row centers. Growing conditions were challenging since there was over 100 cm rainfall during the growing season. The cultivar that yielded the most tonnage (35.8 Mg·ha^{-1}) and fruit number (39,540 fruit·ha^{-1}) was 'Unique'. The next highest yielding cultivars were Stripetti and Trifetti, while the lowest yielding cultivars were 'Small Wonder' and 'Tivoli'. 'Unique' had a small fruit size that averaged 0.9 kg and had an orange yellow flesh, while the largest fruit size of 2.2 kg was produced from 'Trifetti' which had the traditional pale yellow or off white flesh. Average flesh thickness was greatest for 'Trifetti' (32.3 mm) while the thinnest flesh was obtained with 'Unique' (26.2 mm). 'Unique' had the highest lutein (0.2 mg/100 g fwt) content, and pro vitamin a compounds alpha carotene (0.1 mg/100 g fwt), and beta carotene (1.1 mg/100 g fwt) when compared with the other cultivars.

Squash is divided into summer and winter types (Paris, 1994). Summer squash is harvested and consumed as immature fruit, and consist of yellow straightneck, yellow crookneck, scallop, marrow, and zucchini (Robinson and Decker-Walters, 1997). Winter squash is harvested and consumed as mature fruit and consist of acorn, buttercup, butternut, delicata, hubbard, kabocha, turban and spaghetti. Winter squash can be stored for several months after harvest and consumed during the winter (Paris, 1994; Brecht, 2004). USDA does not report production area or yield among the various types of vegetable squashes (i.e., zucchini, hubbard, spaghetti squash, etc.) nor between summer and winter squash. Rather, they report all squash production area planted and harvested, yield, price and value by combining both fresh market and processing squash (USDA, 2014). In 2013, in the United States, about 16,500 ha of squash was planted, 17.1 MT·ha^{-1} was the average yield, with total production valued at approximately $235 million.

Spaghetti squash is commonly grown and sold across the United States. However, it has received relatively limited study on yield and nutritional value. Most spaghetti squash cultivars are cylindrical and have a solid rind (Beany and Stofella, 1997) and a pale yellow flesh (Robinson and Decker-Walters, 1997). Interest was piqued in spaghetti squash in subsequent years that followed with the release of the orange-flesh, bush type cultivar 'Orangetti' in 1986 (Paris, 1993) and another orange-flesh cultivar 'Hasta La Pasta' (Beany and Stofella, 1997; Beany et al., 1998). The 'Hasta La Pasta' cultivar was reported to be preferred to the traditional 'Vegetable Spaghetti' cultivar in terms of yield, nutritional value and consumer preference.

Several spaghetti squash cultivars have been released in the past 10 years. Growers are interested in diversifying their business operation, and consumers are health conscious today and are interested in the nutritive value of what they eat. Most winter squash types contain almost twice the calories per serving of spaghetti squash, which has only 42 calories per cup, according to the USDA National Nutrient Database (http://www.livestrong.com/article/86012-spaghetti-squash-nutrition-value/). Spaghetti squash is low in fat, with less than 0.5 grams of fat per cup, which makes spaghetti squash a good choice for weight-loss or weight-management plans. The objectives of this study were to survey the spaghetti cultivars for yield, size, shape, internal flesh thickness, and internal nutritional composition, to make cultivar recommendations for the southeastern United States.

Materials and Methods

Eight spaghetti squash cultivars (Goldetti, Seeds by Design; Pinnacle, Hollar Seed; Primavera, Sakata Seed; Small Wonder, Hollar Seed; Stripetti, Hollar Seed; Tivoli, Sakata Seed; Trifetti, Seeds by Design; Unique, DP Seeds) were evaluated for yield (weight and number per ha, fruit size, and flesh thickness) and

Acknowledgement. Funding in part for this research by the North Carolina Vegetable Growers Association, and Outgrow Hunger.

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for internal nutrition/phytochemical evaluations (% soluble solids content, pH, lutein, alpha and beta carotene, retinol, and vitamin A) for fruit grown at the Upper Mountain Research Station, Laurel Springs, North Carolina.

Black plastic mulch and drip irrigation was used in this study. Cultivars were direct seeded on 6 June 2013 and row centers were 3.04 m with an in-row spacing of 0.61 m. Plots were 6.1 m long and contained 10 plants. The study was arranged as a randomized complete block design with four replications, with a total maximum number of plants per cultivar being 40.

There were three harvests; 28 August 83 days after planting (DAP), 4 September (90 DAP), and 18 September (104 DAP). Each ripe fruit was harvested and weighed; from that, the weight and number per ha, and average fruit weight were determined. Five fruit were selected from each plot and the length and width of fruit were measured. Three of the five fruit were cut from stem to blossom end and the flesh thickness to the nearest mm was measured at the top (stem end), on both middle sides and the bottom (blossom end) of the fruit. Flesh thickness was determined by the average value from the four measured positions of the fruit.

After harvest, three to five fruit per cultivar were transported from the research station to the Plants for Human Health Institute in Kannapolis, North Carolina. Spaghetti squash were cut longitudinally (stem to blossom end) through the ground spot into quarters and seeds removed. One quarter was steamed until tender (about 10 min on full power using 600 watt microwave). Internal flesh was scooped out and pureed with a homogenizer (Polytron Brinkmann, ThermoScientific). Percentage soluble solids content was determined by placing 0.5 ml puree onto a digital refractometer (Atago Model P100). Flesh pH was determined by insertion of a stainless steel microelectrode probe into the puree. Carotenoid profile was determined on extracts by high performance liquid chromatography (Hitachi Elite La Chrom, Tokyo, Japan) equipped with autosampler and binary pumps. Extracts were obtained by mixing 0.5 g of puree with hexane:ethanol:acetone (Fish et al., 2002). The upper layer was filtered through a 0.25 uM PTFE filter into brown HPLC vials. Vials were placed in an autosampler and a 20 µl injection made onto C_{30} YMC 4.6 x 250 mm S-3 um column (Waters), with oven temperature of 35°C. Peaks were determined by photodiode array at 470 nm. A gradient system consisting of 3 solvents and a flow rate of 1 ml/min was used. Solvent A was 50 mM ammonium acetate in methanol, B was isopropyl alcohol and C was tetrahydrofuran containing 250 ppm (BHT), with 0.05% triethylamine (TEA) in all solvents. The flow program used was 90 % A and 10% B linear gradient, then 54% A, 35% B and 11% C for 24 min, then linear gradient to 30 % A, 35% B and 35% C for 19 min and returned to initial conditions (90% A and 10% B) over 10 min. Data were collected and processed using Hitachi D-2000 Elite HPLC system manager software.

Results and Discussion

The growing season was unusually wet, with over 50 cm of precipitation in a two week period in July, and over 100 cm over the growing season. Nonetheless, good yields were obtained, beginning 83 DAP, with an average of yield over all three harvests of 22.6 Mg·ha^{-1} across all eight spaghetti squash cultivars (Table 1). The majority of the spaghetti squash were harvested by the second harvest or 90 DAP (data not shown). The harvest times are similar to those reported by Beany et al. (1998) who harvested the fruit in their studies as early as 83 DAP to as late as 100 DAP.

Table 1. Yield response and average fruit size of spaghetti squash cultivars.

Cultivar	Seed Co.	Mg·ha^{-1}	Number fruit/ha	kg/fruit
Goldetti	Seeds by Design	17.4	8,877	1.9
Pinnacle	Hollar Seed	23.7	16,677	1.4
Primavera	Sakata Seed	22.3	13,852	1.6
Small Wonder	Hollar Seed	11.2	10,759	1.1
Stripetti	Hollar Seed	28.9	15,870	1.8
Tivoli	Sakata Seed	12.8	7,667	1.7
Trifetti	Seeds by Design	27.5	12,641	2.2
Unique	Seeds by Design	37.2	39,540	0.9
Mean		22.6	15,735	1.6
LSD (0.5)		12.3	7,809	0.2

Planting date: 7 June 2013; Harvest Dates: 29 August, 4 September, 18 September 2013.

Table 2. Spaghetti squash cultivar fruit size, L/D ratio, and flesh thickness[z].

Cultivar	Length (cm)	Width (cm)	Length/ Width	Flesh Thickness
Goldetti	26.4	13.0	2.0	27.5
Pinnacle	19.3	13.2	1.5	28.5
Primavera	19.6	13.5	1.4	31.7
Small Wonder	15.5	12.7	1.2	26.8
Stripetti	23.4	13.2	1.8	30.9
Tivoli	19.8	13.0	1.5	31.4
Trifetti	33.8	12.7	2.7	32.3
Unique	18.5	11.2	1.6	26.2
Mean	22.1	13.0	1.7	29.4
LSD (0.5)	2.0	0.8	0.2	2.4

[z]Five fruit were measured from each plot for a total of 20 fruit per cultivar for length and width, while three fruit were measured from each plot for a total of 12 fruit per cultivar for flesh thickness.

Yields in our study were better than the 17.1 Mg·ha^{-1} average yield reported across all squash types (USDA, 2014). Total cultivar yields differed substantially with 'Unique' producing the most tonnage (Table 1). Other cultivars yielding 4.9 Mg·ha^{-1} or more than the average yield were 'Stripetti' and 'Trifetti'. The lowest yielding cultivars were 'Small Wonder' and 'Tivoli'.

'Unique' produced two to five times more fruit than the other cultivars (Table 1). This was in part because 'Unique' was the only cultivar with an average fruit weight less than 1 kg. 'Small Wonder' produced the next smallest fruit size of 1.1 kg, and produced yields in terms of fruit number that were more comparable to the other cultivars. Cultivars producing second and third highest number of fruit per ha were 'Pinnacle' and 'Stripetti', respectively. 'Pinnacle' fruit have similar characteristics and size comparable to the standard 'Vegetable' spaghetti cultivar (Beaney and al.,1998), while cultivars 'Goldetti' and 'Trifetti' produced much larger average size fruits nearing or exceeding 2.0 kg (Table 1).

The higher fruit weights of 'Goldetti' and 'Trifetti' was also manifested in fruit size as these cultivars produced the longest fruit of 26 and 34 cm, respectively (Table 2). In contrast, the shortest length fruits (less than 20 cm) were 'Small Wonder', 'Tivoli', and 'Unique'. The acceptance of large size spaghetti squash fruits by the consumer needs to be determined, much like Beany et al. (1998) reported with the newly introduced orange-flesh cultivar 'Hasta La Pasta'. Fruit width was similar across nearly all cultivars evaluated; the exception was 'Unique', which had an average fruit

Table 3. Flesh composition of spaghetti squash, Laurel Springs, 2013.[z]

Cultivar	SSC (%)[x]	pH	Lutein	---Carotene--- Alpha	Beta	Retinol	Pro Retinol per serving 120 g	IU Vit A
			--------ug/100g fresh weight--------					
Goldetti	4.6	5.7	35.7	24.2	467.5	40.0	48.0	79.9
Pinnacle	5.4	5.6	38.0	6.4	98.3	8.0	10.2	16.9
Primavera	5.3	5.5	16.2	28.8	144.8	13.3	15.9	26.5
Sm Wonder	4.9	5.6	18.9	22.2	381.6	32.7	39.3	65.5
Stripetti	5.7	5.4	7.6	11.5	184.1	15.8	19.0	31.6
Tivoli	3.9	5.7	27.6	21.3	141.9	12.7	15.3	25.4
Trifetti	5.4	5.4	43.0	13.7	227.6	19.5	23.4	39.1
Unique	3.7	5.7	183.8	95.9	1127.0	97.9	117.5	195.8
Mean	4.9	5.6	46.4	28.0	346.6	30.0	36.1	60.1

[z]Values determined from 3–5 fruit samples per cultivar.
[x]SSC = Soluble solids content.

width of 11.2 cm, nearly 2 cm less than the 13 cm average among the other 7 cultivars (Table 2). The length width ratio provides perspective of fruit shape. The more traditional ratio ranges from 1.4 to 1.8 and includes 'Pinnacle, 'Primavera', 'Stripetti', 'Tivoli', and 'Unique'. 'Small Wonder' produced slightly longer than round fruit (L/W=1.2), while 'Goldetti' produced elongated (L/W=2.0) and Trifetti extremely elongated (L/W=2.7) fruit (Table 2). The average flesh thickness differed among cultivars and ranged from 26.8 to 32.3 cm, with 'Unique' having the thinnest flesh and 'Trifetti' having the thickest flesh.

Sweetness for 'Unique' and 'Tivoli' was relatively low, 3.7 and 3.9, respectively, compared with the other cultivars (Table 3). The slightly higher pH for 'Unique' and 'Tivoli' than the other cultivars may be indicative that the fruits were overripe. However, the pH was very similar among cultivars and ranged from 5.4 to 5.7. The percentage soluble solids content ranged from 3.7 to 5.7, which is not very sweet. Because of this, spaghetti squash can be prepared in both sweet and savory dishes (http://www.livestrong.com/article/86012-spaghetti-squash-nutrition-value/). The spaghetti squash are different than most of winter squashes which are much sweeter (Robinson and Decker-Walters, 1997). The lutein content (0.2 mg/100 g fwt) was highest in the dark gold flesh of 'Unique', exceeding other cultivars 4 to 8 fold. This increased lutein content may provide benefits in keeping the eyes safe from free radicals and the high-energy photons of blue light (Johnson et al, 2000). Similarly, 'Unique' had the highest provitamin A relatives, alpha carotene (0.1 mg/100 g fwt), and beta carotene (1.1 mg/100 g fwt) compared with the other cultivars. Beta carotene was the dominant pigment identified in spaghetti squash flesh. There was no beta crytoxanthin detected in the fresh tissue. Spaghetti squash as a source of vitamin A was low, the best being 2%.

Summary

Spaghetti squash offer an option for commercial growers to diversify their crops. Selection of cultivars is a key consideration when producing any crop. Several of the vegetable spaghetti cultivars that were high yielding were 'Unique', 'Trifetti', 'Pinnacle', and 'Stripetti'. Consumer preference and acceptance of the cultivars in this study for size or taste are not known. Consumers are more health conscious so may be interested in 'Unique' for its phytochemical compounds.

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Identification of the *Cucumis melo* 'Canary Yellow' Gene Using a Genetic Algorithm

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Additional index words. *Cucumis melo*, RNA-seq, bulk segregant analysis, flavonoids.

Abstract. A common method to identify genes is the candidate gene approach that is based on predictions based on sequence similarity and transcriptional co-regulation to known relevant genes. A major limitation of utilizing this approach occurs when no candidate gene is available or predictable or when there are too many of them. An algorithm is a solution to a problem in a finite number of steps. We have developed an algorithm for gene discovery based on high magnitude genotyping, preferably by RNA-seq, of defined bulks of phenotypes from segregating populations. We demonstrate here the utilization of this algorithm to identify a gene affecting rind pigmentation in melon by exploring the genetic mechanism underlying 'Canary Yellow' fruit rind. RNA-seq analysis of small bulks of developing fruit harvested from F_3 families, derived from a cross between a 'Canary Yellow' and non-yellow melons, was used to identify the gene responsible for this phenotype and the biochemical changes that are associated with allelic variation in this gene. An F-Box Kelch-protein like gene, *CmFBK*, controls the metabolic flux towards flavonoids in the rinds of 'Canary Yellow' melon fruit but downstream phenylpropanoids products in non-yellow rind melons. The function of this gene was validated by virus induced gene expression and by stable transformation of tomato plants. Additional genes controlling fruit set and quality in melon and other species are now being analyzed and discovered using this algorithm.

Complete understanding of the genetic mechanisms that regulate plant and fruit traits requires gene cloning and associating DNA polymorphism (genome and epigenome) with trait variation. A common methodology for gene discovery is the candidate gene approach. This approach is based on attempts to associate polymorphism in homologs in the genome of the plant we are studying, to a gene that had been shown to be associated with the investigated trait in another plant species or in another organism. When no candidate gene is available or when there are too many candidate genes, we can use traditional map based cloning which is tedious, time consuming and requires large, preferably advanced, segregating populations.

Flavonoids are secondary phenylpropanoid-derived metabolites ubiquitous to plants. Flavonoids are synthesized from the amino acid phenylalanine by a well-established pathway ubiquitous to all terrestrial plants. In recent years, there has been increasing interest in flavonoids as health benefit agents in human diet. Flavonoids are antioxidants which have been thought to possess anticancer, neuroprotective, cardioprotective and anti-inflammatory activities (reviewed by Romano et al., 2013).

Our general understanding is that flavonoid accumulation is largely regulated at the transcriptional level, by transcription factors (TFs) that specifically control the expression of structural genes of the flavonoid biosynthetic pathway (Spelt et al., 2000; Matus et al., 2010; Nesi et al., 2001; Ramsay et al., 2005; Hartmann et al., 2005). Expression of these TFs is regulated by a combination of endogenous factors and exogenous stimuli (Loreti et al., 2008; Harmer et al., 2000; Lillo et al., 2008).

Data regarding flavonoid accumulation in the Cucurbitaeae family is scarce (Krauze-Baranowska and Cisowski, 2001; Imperato, 1980; Jaramillo et al., 2011). This is surprising because of the vast distribution of these pigments in the plant kingdom. Recently we showed that the yellow flavonoid pigment naringenin chalcone (NarCh) is accumulated as the main flavonoid in the fruit rind of some varieties of melon, including 'canary yellow' type varieties such as 'Noy Amid' ('NA'). Segregation analysis in an F_2 population originated from a cross between 'NA' and 'Tendral Verde Tardio' ('TVT'; a non-NarCh accumulating line) indicated that fruit rind accumulation of NarCh in this population is governed by a single gene, independently from the accumulation of chlorophylls and carotenoids pigments. An attempt to associate this regulating gene with structural genes of the phenylpropanoids pathway was not successful leaving us with the hypothesis of the involvement of a TF in fruit rind NarCh accumulation (Tadmor et al., 2010). Numerous TFs, belonging to different TF families that have been associated with flavonoid biosynthesis regulation, are present in the melon genome, making the candidate gene approach problematic for the identification of the melon gene that regulates fruit rind NarCh accumulation.

We have developed an algorithm for gene discovery, based on deep sequencing of bulks of tails from segregating populations. The preferred sequencing method is RNA-Seq which provides both single nucleotide polymorphism (SNP) and differentially expressed genes (DEG) that distinguish between the bulks. The between bulks SNP data enables mapping of the trait. Phenotypic characterization of the bulks combined with DEG data enables elucidating the 'global' effect of the candidate gene and the genetic mechanism that leads to the trait under investigation. The function of the gene can now be validated based on changes discovered during the phenotyping process (Fig. 1). We have successfully

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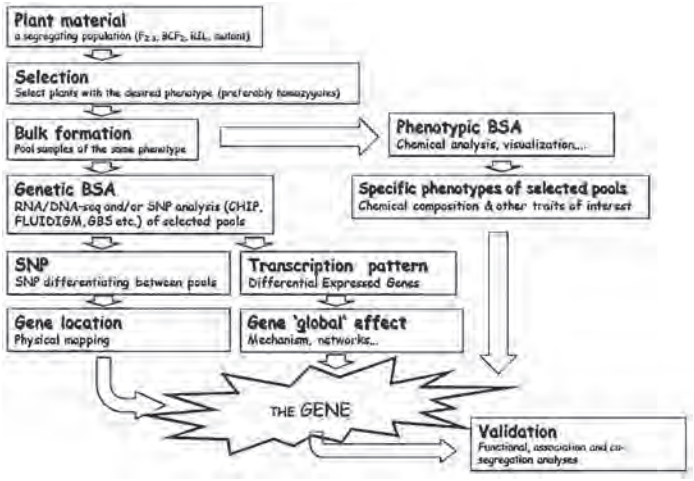

Fig. 1. A schematic representation of our 'genetic algorithm' for gene discovery.

used this genetic algorithm for the discovery of several genes governing melon fruit quality and plant performance traits. We have utilized our genetic algorithm to discover the genetic factor that regulates NarCh accumulation and to uncover biochemical changes related to NarCh accumulation in melon fruit rinds.

Materials and Methods

Plant material. A set of 16 melon varieties were selected to represent rind color variation in *C. melo*. They included representatives of five major taxonomic groups of melon with both climacteric and non-climacteric fruit ripening (Table 1). Plants were grown under conventional conditions in the field and in the greenhouse at the Newe Ya'ar Research Center.

An $F_{2:3}$ segregating population was established by crossing 'NA' - a NarCh accumulating melon inbred line, and 'TVT' – a non NarCh accumulating inbred line (Tadmor et al., 2010). Fruit rind color visualization of the progenies identified 12 F_3 families that are all yellow and thus are homozygous to NarCh accumulation and 7 F_3 families that are all white and thus do not accumulate visible amounts of NarCh. Thirty plants of each of these 19 families were grown and open pollinated by bees in the field of Newe Ya'ar. Female flowers were tagged at the day of anthesis and three biological replications of fruit rind from each F_3 family were sampled during three fruit developmental stages: 10DAA (days after anthesis), 20DAA, and ripe fruit.

Nucleic acid extraction, RNA-Seq and data analyses. DNA and RNA extractions and preparation of cDNA libraries were performed using routinely utilized protocols in our laboratory. Preparation of strand-specific RNA-Seq libraries and sequencing of the libraries on Illumina HiSeq2000 were performed in the 'Bio-Tech Center' at the University of Illinois at Urbana-Champaign, following the manufacturer's instructions (Illumina, USA). Each of these libraries yielded 20 million of 100bp reads. RNA-Seq reads were aligned to the melon reference genome. Based on the alignments, SNPs between yellow and white fruit bulks were identified, and differentially expressed genes (DEG) between bulks at each fruit developmental stage and within bulks at each fruit developmental stages were also identified.

SNP and qRT-PCR analyses. SNP were detected with High Resolution Melt (HRM) analysis using an Eco Real-Time PCR system (Illumina) and analyzed in Eco version 4 software. qRT-PCR analysis utilized the same instrument and same software.

Flavonoids extraction and analysis. Flavonoids were extracted in methanol. Glycosylated flavonoids were visualized under UV light (320nm) after being fractionated by HPTLC (silica gel 60, Merck) followed by DPBA (2-Aminoethyl diphenylborinate) staining. DPBA incites fluorescence of glycosylated flavonoids under UV light. Mass spectral analyses were also conducted on the same methanolic extracts with and without enzymatic hydrolysis using LC-MS-TOF (LC: 1290 infinity system, MS-TOF: 6224-TOF-LC-MS, Agilent technologies).

Microscopy. DPBA stained tissue cuts were observed in Olympus BX61 microscope coupled with U-HGLGPS illumination system, Olympus 20x/0.50 objective, using U-MNBV2 filter (excitation 420-440nm, emission >475nm). Images were collected with a digital camera (DP73, Olympus), image processing was performed with cellSens Dimention software (Olympus).

Tomato seedling fluorescence was observed on Leica M205FA stereomicroscope, Leica 10472649 Planapo 0.63x objective, under GFP3 filter. Images were collected with a digital camera (DFC495, Leica), image processing was performed with Leica Application Suit V3.8 software.

Functional analyses of a candidate gene. Once a candidate gene was selected we confirmed its function by chemical analysis of Virus Induced Gene Expression (VIGE) melon leaves and of stable transgenic tomato fruit rinds (Gal-On et al., 1997).

Results and Discussion

After preparation of plant samples we performed both phenotypic Bulk Segregant Analysis (BSA) and genetic BSA (Fig. 1). The phenotypic BSA included microscopic and chemical analysis of the parental lines and the bulks while the genetic BSA included RNA-Seq of two replications in each developmental stage of each bulk, and SNP analysis of recombinants coming from heterozygous F_3 families identified during the search for homozygous yellow F_3 families.

Phenotypic BSA. Microscopic analyses of fruit rind under visible light and under UV light after staining with DPBA indicated that: 1. NarCh is not glycosylated and it is accumulated in the outer cuticle of the fruit rind; 2. There is additional yellow fluorescing compounds that are accumulated in the fruit rinds of NarCh accumulating fruit; and 3. Fruit rind that do not accumulate NarCh accumulate other compounds displaying blue fluorescence in their rinds. TLC of methanolic extracts of fruit rinds of 16 melon varieties (Table 1), fruit rinds of 12 F_3 families of the 'yellow' bulk, 7 F_3 families of the 'white' bulk and leaves of all of them indicated that: 1. yellow fluorescent compounds were always associated with the accumulation of NarCh and the presence of blue fluorescence compounds were always associated with the absence of NarCh in fruit rind; and 2. Melon leaves always displayed both NarCh and yellow fluorescent compounds independently of the flavonoid patterns displayed in fruit rinds. This indicated that the putative regulator of fruit rind flavonoids accumulation is not active in the leaves. Yellow and blue fluorescent bands of fruit rind methanolic extracts were taken out from the TLC plate. Utilizing LCMS, we fractionated these bands with and without enzymatic glycosylation. A set of authentic standards that had the same accurate mass as the LCMS peaks was used to identify the fluorescent bands by co-migration. These analyses identified five compounds that were specifically accumulated in the NarCh accumulating melon rinds in addition

Table 1. List of varieties analyzed in this study.

Variety	Taxonomic Group	Ripening mechanism	Color of ripe fruit rind	NarCh
Eshkolit	reticulatus	climacteric	yellow	+
Dulce	reticulatus	climacteric	green–orange	–
Arka Jeet	candalak	climacteric	orange	+
Aremenian Yard Long	flexuosus	non-climacteric	white	–
Noy Amid	inodorus	non-climacteric	yellow	+
Yellow Canary	inodorus	non-climacteric	yellow	+
Rochet	inodorus	non-climacteric	dark green	+
Tendral Verde Tardio	inodorus	non-climacteric	dark green	–
Tam–Dew	inodorus	non-climacteric	white–yellow	–
Branco	inodorus	non-climacteric	white	–
Branco Oval	inodorus	non-climacteric	white	–
Gold King	inodorus	non-climacteric	yellow	+
Noy Yizre'el	cantalupenesis	climacteric	Green–orange	–
Vedratais	cantalupenesis	climacteric	yellow–green	–
Ananas	cantalupenesis	climacteric	orange–green	+

to NarCh: naringenin, luteolin, dihydrokaempferol, kaempferol, and quercetin, all are flavonoids located downstream to NarCh in the flavonoid biosynthetic pathway. Three blue-fluorescing compounds were found to be specifically accumulated in the non-NarCh accumulating lines: caffeic acid, ferulic acid, and aesculetin, all belong to a side chain of the flavonoids biosynthetic pathway upstream to chalcone synthase (Fig. 2). This indicates that NarCh accumulation is a visible part of a larger metabolic flux change; when NarCh is accumulated the metabolic flux changes from following the general phenylpropanoid pathway, accumulating caffeic and ferulic acid derivatives to the flavonoid pathway accumulating different flavonoids. The genetic factor that regulates this metabolic flux does it in a tissue specific manner, only in the fruit rinds. Shifts of flux between these two pathways were previously reported due to mutation in structural genes of the pathway (Basseau et al., 2007), or in transcription factor (Fornalé et al., 2010). Understanding the biochemistry of NarCh accumulation set the background for a better understanding of the process and for the functional analysis to be performed once a candidate gene is selected using the genetic BSA.

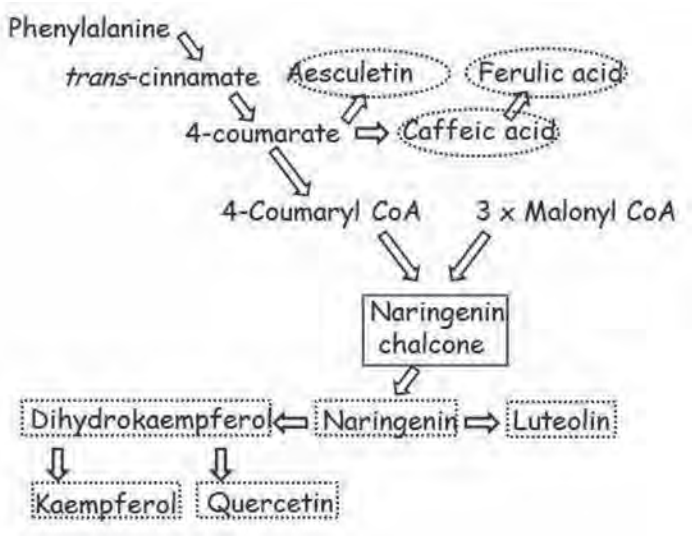

Fig 2. LCMS analysis assisted by comparison to authentic standards identified five compounds that were concomitantly accumulated in NarCh accumulating fruit rinds (framed with dotted rectangles) all are flavonoids located downstream to NarCh (framed with solid rectangle). Three compounds that specifically accumulated in the NarCh lacking lines (framed with dotted ellipses) all belong to the continuation of the general phenylpropanoid pathway, a branching of the flavonoid biosynthetic pathway.

Genetic BSA. The transcriptome-BSA strategy allows all SNPs that are unlinked to the trait of interest ('canary yellow' rind in this case) and expression differences that are not associated with the trait, to segregate statistically equally between the two bulks, thus presenting a specific differential enrichment of the genetic factors governing flavonoid accumulation. Two sets of data were derived from the RNA-Seq data: SNP and DEG. We decided that a SNP is considered to be 'real' when at least 10 reads are available for this region in each of the bulks and when the difference between the two bulks is larger than 90%. Under these conditions, we have identified a chromosomal region of ~100,000bp in which our gene resides. When searching for homozygous 'yellow' F_3 families we identified many heterozygous F_3 families that segregated for fruit rind color. HRM SNP analysis combined with visual phenotyping of ~800 segregants derived from 14 heterozygous F_3 families narrowed down the candidate locus to a smaller area that included less than 20 genes. One of these genes, coding for an F-Box Kelch repeat like protein (*CmFBK*), was selected to be our candidate gene. A DNA marker developed based on polymorphism in this gene completely segregates with NarCh accumulation in the large segregating population as well as in the germplasm collection.

We functionally proved *CmFBK* metabolic flux alteration activity in melon leaves infected with *CmFBK*-VIGE and in tomato fruit rind that grew on stable *CmFBK* transgenic plants. Tomato is a model species for the study of fleshy fruit ripening. In tomato, NarCh is the major flavonoid accumulated in the outer epidermis cell layer of mature fruit peel (Hunt and Baker, 1980). The accumulation of NarCh was found to be positively regulated by the MYB12 TF, since a mutation in this gene results in a colorless epidermis phenotype (Adato et al., 2009; Ballester et al., 2010). Interestingly, the melon *CmFBK* was functional in this system.

In summary, using our genetic algorithm, we identified a unique tissue specific regulation of the flavonoids metabolic flux. We managed to clone and functionally analyze *CmFBK*, an F-box Kelch protein that regulates fruit rind flavonoids accumulation. FBK proteins are mediators for ubuiquitination of

proteins, targeting them for proteolysis while the Kelch repeat is probably responsible for substrate specificity. The exact target of *CmFBK* is still unknown.

The same genetic algorithm is now being used to identify additional important genes in the cucurbit family.

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Grafting Extends Field Storage Life of Seedless Watermelon

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Additional index words. *Citrullus lanatus,* functional food, shelf life, lycopene, citrulline.

Abstract. Quality of watermelon fruit is a combination of firmness, full red color, sweetness, and perceived shelf life in field and market; fresh cut watermelon must also have low drip loss. Watermelon grafting is widely used outside of the US to avoid soil borne pathogens and abiotic stresses. Commonly used rootstocks and seedless watermelon scions were used to determine best fruit productivity and marketable yield in the southeastern US relative to non-grafted plants. In 2012, fruit identified as ripe were left in the field on the vine 0 to 2 weeks to test the relative holding life, then used for fresh cut studies (held an additional 14 days at 3 °C) to determine firmness, drip loss, and composition. Depending on scion and rootstock cultivar, lycopene content increased slightly (up to 10%) with grafting while citrulline content decreased 10 to 30%. Holding grafted fruit in the field for 1 week increased citrulline content by 20%. Firmness was increased 10 to 40% by grafting. Percentage drip loss of fresh cut product was reduced to one third or one half using grafted watermelons regardless of fruit holding time in field. Grafted watermelons harvested at 0 or 1 week field holding resulted in acceptable fresh cut product after 14 days cold storage. Our results indicate that grafted watermelon can improve firmness and reduce drip loss in fresh cut watermelon without loss of sugars or lycopene, and that grafting extends field shelf life an extra week compared to non-grafted watermelons.

Watermelon fruit have a field shelf life harvest window of about 7–10 days from time of full flesh color. In the United States, where seedless watermelons make up 95 % of the retail market, soluble solids content (SSC) is expected to be at least 10%. Other components of quality are flesh color (light pink to full red, but not orange tinted or dark red) and flesh firmness of about 1.6 kg or 4 N (as measured by a flat probe of 0.8 cm diameter in heart and locule areas). Although usually not done in the field or packhouse, fruit pH can be a helpful marker of ripeness. Fruit that are just turning fully ripe have a pH of 4.5 to 5; those fully ripe of 5 to 6; and those overripe a pH over 6.5. Knowledge of relative pH is especially helpful in fresh cut products, as a higher pH can lead to more rapid microbial breakdown.

Additional indicators of quality in watermelon include total lycopene and citrulline content. These compounds have been found to help with human health issues, such as increased arterial flexibility and ability to scavenge free radicals (Figueroa et al., 2012).

Grafting of watermelon has been done for centuries in some parts of the world, but is a relative newcomer to the U.S (Hassell et al., 2008). In the United States, soil-borne diseases that can no longer be controlled by fumigation have driven research into grafted watermelon. The present study was done using scions of seedless watermelon cultivars and several types of rootstocks in order to determine best outcomes for eastern U.S. watermelon production.

Materials and Methods

Seeds of the rootstock genotype interspecific hybrid squash cv. Carnivor (Syngenta Seeds, Boise, ID) were sown in 72-cell plug trays (5.715 × 2.54 × 3.962 cm cell depth, bottom diameter, top diameter, respectively (TLC Polyform, Inc., Minneapolis, MN) filled with a fertilizer-free, soilless mix (76 % sphagnum peat, 25 % perlite) (Sun Gro Horticulture, Agawam, MA). No additional fertilizer was applied throughout the experiment. Seeds were planted following standard greenhouse production practices. Plants were grown in a double-layer polyethylene greenhouse covered with 6-mil Klerk's K50 Clear (Klerks Hyplast, Inc., Chester, SC).

When the cotyledons had unfolded, but not expanded (about 6–8 days for 'Carnivor' rootstock), seedlings were individually treated with 20 µL 6.25 % Fair 85 (Fair Products, Inc., Cary, NC) fatty alcohol applied to the meristem of the rootstock, as described by Daley and Hassell (2014). Rootstocks were held in the greenhouse for 14 days following treatment before grafting. The cultivars 'Tri-X 313', 'Fascination', 'Sugar Heart', 'Sugar Coat', and 'Sugar Red' were used as scion material, and seeds were sown following growing procedures outlined by Hassell and Schultheis (2002), on the same day as the 14-day rootstocks. Extra plants that were not used for grafting were grown as controls.

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Grafting was performed using the one-cotyledon method, as described by Hassell et al. (2008), with the hypocotyl extracted from the roots. After seven days in the healing chamber, grafted plants were removed and allowed to grow in the greenhouse for one week.

Plants were field transplanted in early May and harvested in late June, 2012. The experiment was a randomized complete-block design with 5 replications. Plots were 116 meters in length, 2.7 meter center spacing and 0.9 meter in-row spacing between plants. Fields were located at the Clemson University Coastal Research and Education Center in Charleston, South Carolina (GPS location: lat. 32°47'37'N, long. 80°03'54'W) (Google Earth, 2008). Soil type was Yonges loamy fine sand (Thermic Typic Endoaqualfs).

Fruit ripeness was determined following guidelines described by Maynard (2001). Sixty fruits from each cultivar were flagged and marked for harvest (30 from grafted and 30 from non-grafted plants). Twenty fruit from each cultivar were then removed from the field, dipped in a 10% chlorine bleach solution, and placed in a cooler (3 °C) for 24 h. These fruit were classified as week 0 after full ripeness. This same procedure was followed for weeks one and two after full ripeness, eventually removing all flagged fruit from the field. After 24 h, fruit were removed from the cooler and cut in half widthwise. A 3 cm slice was taken from the first half and four 3 cm squares were cut form the heart section and four 3 cm squares were cut from the locule section. They were then weighed and places on a mesh screen inside a TS 24 RS Safe-T-Fresh container (Shelton, CT) and placed back inside the 3 °C cooler for 14 additional days at which time they were removed, re-weighed and leakage collected and measured. An additional 3 cm slice was then taken from the first half of the fruit where pH, soluble solids, and firmness data were collected on each fruit. With the remaining half section, two scoops were taken from the center of the fruit using an ice cream scoop, placed in a liter bag and held at –80°C until shipment to Kannapolis, North Carolina for further analysis.

Watermelon samples were thawed and homogenized (Polytron, Brinkmann). Aliquots were diluted with water, placed in cuvettes, and total lycopene determined by absorbance of transmitted light using a Hunter Ultra Plus colorimeter equipped with xenon lights following the method of Davis et al. (2003).

For the analysis of free citrulline and arginine amino acids, 0.1 g freeze dried flesh was weighed into an Eppendorf tube and extracted with 1 mL of 0.02 N H Cl (CEC, 1998) and diluted as needed with the extraction solvent. Samples were analyzed using a Hitachi Model L-8900 Analyzer (Hitachi High Technologies, Dallas, TX) fitted with an analytical column (#2622SC PF, 40 mm length, 6.0 mm i.d.) and guard column. Separation of the amino acids was done using a gradient of borate buffers (PF type, Hitachi High Technologies, Dallas, TX) and a temperature gradient of 30 °C. to 70 °C with additional changes provided by Hitachi personnel (Shinichi Otaka, private communication, 2013). Post column derivatization was performed by the instrument using ninhydrin. The instrument used visible detection at a wavelength of 570 nm. Standard curves (5 to 50 nM) were prepared by making serial dilutions of L-citrulline and L-arginine in 0.02 N HCl.

Results and Discussion

Grafted watermelons had firmer fruit for all weeks sampled than those not grafted (Fig 1). Grafted fruit harvested on week 0 or week 1 had a better fresh cut product than those harvested

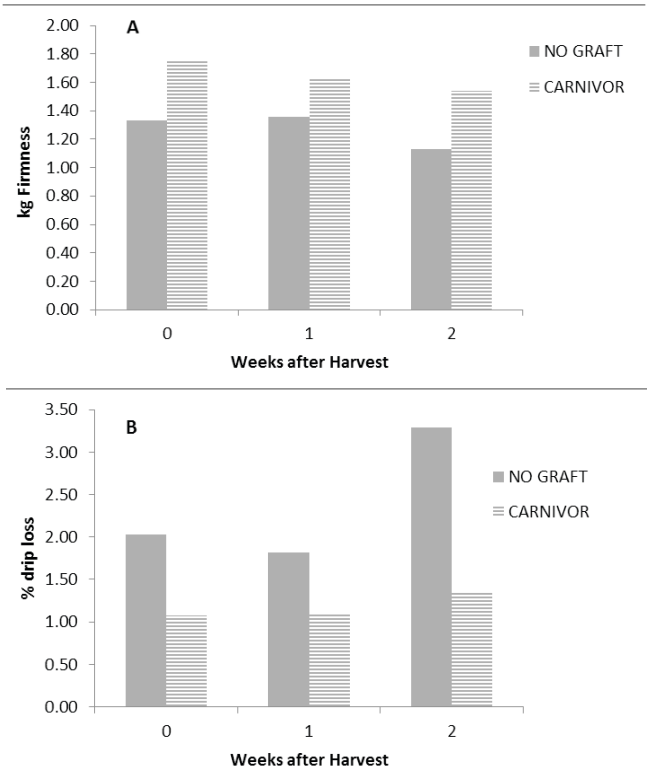

Fig. 1. Differences in firmness **(A)** and drip loss **(B)** in watermelon cubes after 14 days storage at 3 °C averaged over five scions. Cubes were cut from fruit of grafted ('Carnivor' rootstock) or not grafted seedless scions ('Fascination', 'Trix313', 'Sugar Heart', 'Sugar Coat', 'Sugar Red') and held at 0, 1, or 2 weeks in the field after full ripeness before cutting, 2012. Rootstock type significantly different within weeks after harvest for each variable.

week 2 while not grafted fruit were suitable for fresh cut only when harvested on week 0. The primary loss of quality was flesh firmness. Drip loss, the amount of juice that is lost from the cut cubes, increased in fruit harvested on week 3. The pH of the fresh cut product increased steadily as the watermelon fruit aged in the field and was at 7.2 for not grafted and 6.7 for grafted after 2 weeks field storage followed by 2 weeks fresh cut storage (data not shown).

Soluble solids and citrulline content changed less significantly than pH or total lycopene in field stored watermelons after week 2 (Table 1). Total lycopene content was consistently higher in watermelon fruit from scions grafted to the interspecific squash hybrid 'Carnivor' than fruit from not grafted plants. In contrast, citrulline content decreased in fruit from grafted plants, while arginine showed no consistent changes with scion, rootstock, or duration of field storage. Total lycopene may be an indicator of increased vine vigor and subsequent increased carbon for incorporation into the carotenoid pathway. Citrulline is a mostly water soluble, non-essential amino acid found in cucurbits, that acts as a storage mechanism to protect nitrogen in the plant in times of drought (Kawasaki et al., 2000). Differences in citrulline content between grafted and not grafted watermelon may result from overall reduced stress in fruit of grafted plants.

Table 1. Composition of watermelon fruit from Tri-X-313 or Sugar Red scions not grafted or grafted to Carnivor rootstock after 0, 1, or 2 weeks of field storage.

Rootstock	Week	SSC	pH	Lycopene (mg/kg)	Citrulline (g/kg)	Arginine	Citrulline + Arginine (g/kg)
				Tri-X-313			
No Graft	0	11.9a	5.87a	58.76a	2.53a	0.79a	3.32a
	1	12.1a	5.67b	54.38a	2.45a	0.77a	3.22ab
	2	12.1a	5.72b	48.55b	2.31b	0.71a	3.02b
Carnivor	0	11.4b	5.62b	60.67a	2.06b	0.66a	2.72b
	1	12.5a	5.55b	59.54a	2.35a	0.74a	3.09a
	2	11.7ab	5.76a	54.3b	2.32a	0.57a	2.89b
				Sugar Red			
No Graft	0	12.0b	5.42c	62.56a	3.54a	0.82a	4.36a
	1	12.7a	5.74b	62.49a	3.62a	0.78a	4.40a
	2	12.4a	5.84a	50.95b	3.26b	0.86a	4.12b
Carnivor	0	11.1b	5.26c	54.82b	2.02b	0.70a	2.72b
	1	11.9a	5.59b	66.77a	2.55ab	0.78a	3.33a
	2	11.7a	5.69a	60.48ab	2.81a	0.69a	3.50a

Means significantly different within column and weeks, Tukeys, $P < 0.05$.

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Identification of QTL Involved in Melon Domestication and Evolution

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ADDITIONAL INDEX WORDS. Introgression lines, pulp area, soluble solids content, fruit weight

ABSTRACT. Wild melons (*Cucumis melo* L.) usually produce very small-sized fruits lacking edible pulp. From an F_2 population between a wild accession of *C. melo* (Trigonus) and the cultivar 'Piel de Sapo' (PS) maintained *in vitro*, a genetic map with 107 SNPs covering 12 linkage groups (LGs) was constructed. The genetics of domestication traits were studied in this population: fruit shape (FS) and weight (FW), and edible pulp area (PA) and sugar content (SSC). After evaluating the population in three different assays, QTL with stable effects were identified in at least two trials: 12 for FS, 6 for FW, 1 for PA and 1 for SSC. The most robust QTL were *sscqt5.1* for SSC in the LG V, *paqt6.1* for PA in the LG VI and *fwqt8.1* for FW in the LG VIII, detected in all three trials. In all cases, the Trigonus alleles decrease the value of such characters. Therefore, these QTL are good candidates for searching gene regions involved in the processes of melon domestication and evolution. All of them have been validated in advanced backcross families (BC2S1 and BC2S2). The phenotypic differences observed in plants carrying the exotic allele were always highly significant when compared to those that carried the PS allele or to the parental 'PS' itself. Currently, introgression lines of these three QTL are being generated, and will be used for fine-mapping.

The origin of the melon cultivation is still controversial, but recent phylogenetic studies point to Asia as the primary center of origin (Sebastian et al., 2010). Regarding the fruit, some of the most important characters affected during the process of domestication are shape, weight, sugar content and the thickness of the edible pulp. The aim of this study was to identify QTL responsible for these characters with a crucial role in the process of melon domestication, as well as the validation of their effects in advanced backcross populations.

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Materials and Methods

The 192 F_2 plants of the mapping population were derived from a single cross between the wild accession Trigonus and the elite cultivar 'PS', both *C. melo* (Fig. 1). Trigonus produces a small, round and pulp lacking fruit, whereas fruits coming from 'PS' are big, oval-shaped and very sweet (Monforte et al. 2005). This population was asexually propagated *in vitro*, which allowed us to perform three different assays, in two locations (Valencia and Zaragoza) in 2011, and in Zaragoza in 2012. Fruit shape (FS) and edible pulp area (PA) were calculated from digital images with Tomato Analyzer 3.0 (Brewer et al 2006) and sugar content

(SSC) as ºBrix with a refractometer. All statistical analyses were performed using JMP v5.1.2 software for Windows (SAS Institute Inc., Cary, NC).

DNA was isolated from young leaves of the F_2 mapping population according to Doyle and Doyle (1990), with slight modifications and genotyped with a total of 107 SNP markers using Sequenom MassArray iPLEX and Illumina Veracode assays at Servicio de Investigaciones Biomédicas, Unidad Central de Investigación (University of Valencia, Valencia, Spain) and Centro Nacional de Genotipado (Madrid, Spain), respectively.

The linkage map was constructed using MAPMAKER 3.0 (Lander et al. 1987) and the distances were calculated with the Kosambi function (Kosambi 1944). QTL analysis was performed by composite interval mapping (CIM, Zeng 1993) using Windows QTL Cartographer 2.5 (Wang et al. 2007). The LOD score threshold $p<0.05$ was calculated by a permutation test with 1000 resamplings. The QTL were named according to the nomenclature system by Díaz et al. (2011).

The most robust QTL were validated in different families of advanced backcrosses (BC2S1 and BC2S2). The effects of the QTL identified for pulp area (*paqt6.1*) were verified in three different experiments. In 2012, two BC2S1 lines (12M124 and 12M125) carrying the PS and Trigonus alleles for the QTL, respectively, were assayed in Valencia. The same year, a BC2S1 family (12M59) with the markers linked to *paqt6.1* segregating and the control PS were evaluated in Paiporta (Valencia). In 2013, three BC2S2 families were tested in Zaragoza, two of them homozygous Trigonus (13M1 and 13M2) and the third one (13M3) homozygous PS, as well PS. Similarly, in 2012 BC2S2 families (12M60 and 12M61) with segregating markers linked to *sscqt5.1* and *fwqt8.1*, respectively, were evaluated together with the parents in Paiporta. The cosegregation of marker genotypes and the phenotypes was assessed by ANOVA.

Results and Discussion

Fruits from the cultivar PS and the exotic accession Trigonus exhibited very different phenotypes for the traits studied in this work (Table 1 and Fig. 1). Regarding the F_1 hybrid, it exhibited higher values only for FS (Table 1), suggesting the existence of best-parent heterosis. A continuous distribution of values for all traits was observed in the F_2, as expected in a quantitative trait.

QTL were found for all the characters studied. Twenty of them were identified in at least two trials: 12 for FS, 6 for FW, 1 for PA and 1 for SSC. Those with the most robust effects were: *sscqt5.1* (for SSC in LG V), *paqt6.1* (for PA in LG VI) and *fwqt8.1* (for FW in LG VIII), that were detected in all the three assays. All of them displayed a negative additive effect value, so the Trigonus allele causes a decrease in the values of those traits, producing smaller fruits with a reduced and less sweet pulp.

The QTL *paqt6.1* was validated in three different assays. In 2012, two BC2S1 families carrying the PS and Trigonus alleles for the marker linked to that QTL in homozygosis (12M124 and 12M125, respectively) were evaluated in Valencia. The exotic allele produced a 10% reduction in the PA value. In parallel, the BC2S1 segregating family for the markers in the QTL region (12M59) was tested in Paiporta (Valencia). The differences in the PA values were always highly significant when Trigonus and “PS” alleles were compared to each other. In 2013, the effects of the QTL were verified in Zaragoza by comparing three BC2S2 families, two carrying the Trigonus allele in homozygosis (13M1 and 13M2) and another one homozygous for the

Table 1. Means ± standard deviations for fruit weight (FW), fruit shape (FS), pulp area (PA), soluble solids content (SSC) for parents and F_1 and F_2 progenies across three locations.

Location	Year	Genotype	FW	FS	PA	SSC
Valencia	2011	PS	2485.0±356.4	1.3±0.0	0.7±0.0	10.7±2.8
		Trigonus	41.0±1.4	1.2±0.0	0.4±0.0	5.5±0.0
		F_1	294.5±12.0	1.8±0.2	0.6±0.0	6.5±0.7
		F_2	245.4±135.5	1.4±0.2	0.6±0.0	6.7±1.5
Zaragoza	2011	PS	1106.0±97.6	1.5±0.0	0.7±0.0	8.8±0.1
		Trigonus	17.0±1.40	1.2±0.0	0.3±0.1	4.2±0.2
		F_1	201.5±9.2	2.1±0.2	0.6±0.1	5.2±1.3
		F_2	152.7±100.1	1.5±0.3	0.5±0.1	5.2±1.3
Zaragoza	2012	PS	2139.9±470.0	1.4±0.1	0.7±0.1	9.7±2.8
		Trigonus	21.4±4.5	1.2±0.1	0.3±0.0	nd[z]
		F_1	342.5±113.3	1.8±0.2	0.5±0.0	7.9±1.1
		F_2	178.8±109.2	1.5±0.2	0.5±0.1	7.5±1.2

[z] nd: not detected.

PS allele (13M3). The families 13M1 and 13M2 produced fruits with significantly lower PA values.

In 2012, the QTL *sscqt5.1* and *fwqt8.1* were validated by studying families BC2S1 (12M60 and 12M61, respectively). In both cases, the homozygous Trigonus for the markers in the region of both QTL produced fruits with highly significant reduced values of SSC and FW, respectively.

Currently, introgression lines for each of the three QTL (*paqt6.1*, *sscqt5.1* and *fwqt8.1*) are being generated. These lines will be used in future works to map the QTL more precisely, what could ultimately lead to their cloning.

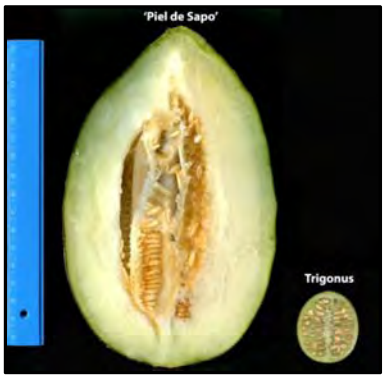

Fig. 1. Longitudinal sections of 'Piel de Sapo' and Trigonus fruits.

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