Identification and Characterization of Quantitative Trait Loci (QTL) for Resistance to the Cyst Nematode (*Globodera pallida*) in the Potato (*Solanum tuberosum*)

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Für meine Eltern und meinen Bruder

Table of Contents

Abbreviations

ASO	Allele specific oligonucleotide
AUDPC	Area under the disease progress curve
BAC	Bacterial Artificial Chromosome
BGRC	Braunschweig Genetic Resources Center
BNA	Böhm-Nordkartoffel-Agrarproduktion
BSA	Bovine serum albumin
CAPS	Cleaved Amplified Polymorphic Sequence
CCD	charge-coupled device
CGN	Centre for Genetic Resources
cM	centi Morgan
DAPI	4', 6-Diamidino-2-phenylindol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
Dt	Dezi tons
FISH	Fluorescent *In Situ* Hybridization
FITC	Fluorescein isothiocyanate
GLKS	Gross Luesewitz Potato Collections
HC	Haplotype C
IPK	Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung
LB	Luria-Bertani
LD	Linkage Disequilibrium
LG	Linkage Group
LRR	Leucine Rich Repeat
MAS	Marker Assisted Selection
MCR	Maturity Corrected Resistance
NBS	Nucleotide Binding Site
PAD4	Phytoalexin-Deficient 4
PBS	Phosphate buffer solution
PCR	Polymerase Chain Reaction
PCN	Potato Cyst Nematode
QRL	Quantitative Resistance Locus/ Loci
QTL	Quantitative Trait Locus/ Loci
rAUDPC	relative Area under the disease progress curve
RFLP	Restriction Fragment Length Polymorphism
RKN	Root Knot Nematode
SaRa	Saka-Ragis
SCAR	Sequence Characterized Amplified Region
SD	Single Dominant
SGT1	Suppressor of G-Two allele of SKP1
SNP	Single Nucleotide Polymorphism
SOC	Super Optimal Catabolite
SSR	Simple Sequence Repeat

Abstract

This work focused on the identification and characterization of the genetic basis of important agronomic traits in the potato with main interest centered on resistance to the potato cyst nematode, *Globodera pallida.* A high level of genetic resistance to nematodes is an important aspect in breeding new potato varieties. The first step towards that is the localization of genetic factors controlling resistance on the potato map. We made use of information obtained from *experimental* diploid mapping populations to evaluate tetraploid breeding material for the *applied* research. We explored the genetic resistances present in the crop, which show a continuous variation and are assumed to be controlled by several loci which act collectively on the expression of resistance. These loci are referred to as quantitative trait loci (QTL) (Meyer et al. 1998).

The number of QTL for resistance was determined through an association mapping approach. Two tetraploid populations were genotyped with several markers either within or physically linked to candidate genes. Phenotypic data was supplied by the two breeding companies, Saka-Ragis and Böhm-Nordkartoffel. Significant associations with quantitative resistance were found for all marker types. Single nucleotide polymorphisms (SNPs) were shown to be the most efficient marker type to detect significant associations between markers and the resistance phenotype. Two of them served as basis for developing a PCR-based marker highly diagnostic for potato varieties with high resistance to *Globodera pallida* (Sattarzadeh et al. 2006). Based on an InDel in the sequence we designed an allele-specific CAPS marker for the quantitative detection of maturity corrected resistance to the oomycete *Phytophthora infestans*. Using this marker we provide solutions for the selection of superior potato genotypes through the development of easy-to-use DNA based molecular markers.

Fine mapping of a QTL for resistance to the nematode located on chromosome V was done by a combination of association tests and physical mapping to gain more insight in the genomic organisation of a QTL. We were able to delimit the size to a genetic region of 2 cM. Fluorescent *in situ* hybridization (FISH) was applied to obtain valuable complementary information regarding size and chromosomal position of this QTL. The

region was estimated to span less than 1 Mb and is located in the euchromatic region of the long arm of chromosome V. These findings will assist future map based cloning efforts in this region.

The detection of numerous loci in tetraploid breeding material associated with important agronomic traits provides valuable data which can be used to design additional markers not just for resistance against *G. pallida* but also for other traits, such as resistance to *Phytophthora infestans*, maturity, yield, and starch content.

Zusammenfassung

Ziel dieser Doktorarbeit war die Identifikation und Charakterisierung der genetischen Grundlagen von wichtigen agronomischen Merkmalen in der Kartoffel. Wesentlich war hierbei die Resistenz gegen den Kartoffelnematoden *Globodera pallida* (Stone 1973). Die starke Resistenz gegen den Nematoden ist ein wichtiger Aspekt in der Kartoffelzüchtung. Der erste Schritt hin zu resistenten Sorten ist die Kartierung der mitwirkenden genetischen Faktoren auf den Kartoffelchromosomen. Zu diesem Zweck wurden Informationen aus Versuchen mit diploiden Kartierungspopulationen für unsere Studie in tetraploidem Züchtungsmaterial evaluiert. Die Idee war, die Erkenntnisse der Forschung auf die Praxis zu übertragen und wichtige Informationen über die Struktur und Funktion des Genomes des tetraploiden Züchtungsmaterials zu erhalten. Wir untersuchten den Resistenzphänotypen, der eine Normalverteilung zeigt. Es wird angenommen, dass diese Form der Resistenz von mehreren Genorten gemeinsam verliehen wird. Diese Genomabschnitte nennt man „quantitative trait loci“ (QTL).

Die Anzahl der QTL für Resistenz wurde mittels einer Assoziationsstudie ermittelt. Zwei tetraploide Populationen wurden mit mehreren Markern genotypisiert, die, wie aus vorhergenenden Studien bekannt, mit Resistenzloci im Kartoffelgenom gekoppelt sind. Signifikante Assoziationen mit Resistenz wurden mit allen Markertypen gefunden. Punktmutationen „Single nucleotide polymorphisms“ (SNPs) bewiesen sich als effizienteste Marker, um signifikante Assoziationen zwischen Markern und Phänotypen zu dedektieren. Zwei SNP-Marker bildeten die Basis für die Entwicklung eines PCR-Tests diagnostisch für Sorten mit hoher Resistenz gegen *G. pallida* (Sattarzadeh et al. 2006). Basierend auf einem InDels entstand ein weiterer PCR-Test für die Identifizierung von Genotypen mit reife-korrigierter Resistenz gegenüber dem Oomyzeten, *Phytophthora infestans*. Hiermit bieten wir erste technisch einfach anwendbare Marker für die Selektion von überlegenen Genotypen für die markergestützte Züchtung.

Ein weiterer Aspekt dieser Arbeit war die Fein-kartierung von dem QTL für Nematodenresistenz auf Chromosom V. Hierfür bedienten wir uns einer Kombination aus Assoziationstests und physikalischer Kartierung. Wir konnten das QTL auf die genetische

Größe von 2 cM reduzieren. Ein Fluorescent-*in-situ*-hybridization Experiment (FISH) diente dazu, die physikalische Größe des Genomabschnittes zu messen und seine genaue Position auf dem Chromosom zu bestimmen. Der Abschnitt liegt im Euchromatin und beträgt maximal 1 Mb. Diese Ergebnisse sind wichtige Infomationen für die „map-based cloning"- Strategie in der Zukunft.

Die Identifizierung von mehreren Genomabschnitten in tetraploiden Züchtungsmaterial, die mit wichtigen agronomischen Merkmalen assoziiert sind, bietet wertvolles Datenmaterial für die Entwicklung von weiteren PCR-basierenden Markern. Dies beschränkt sich nicht nur auf die Resistenz gegen *G. pallida*, sondern auch auf andere Merkmale. Diese Merkmale sind Resistenzen gegen *P. infestans*, Reife, Ertrag und Stärkegehalt.

Chapter 1

General Introduction

The Potato (*Solanum tuberosum*)

The potato (*Solanum tuberosum*) originated in the highlands of South America, where it has been cultivated for more than 8000 years. Spanish explorers introduced the plant into Europe in the 16th century as a botanical curiosity (Brücher 1975; Figure 1.1). By the 19th century it was cultivated throughout the continent, providing an inexpensive food source for the workers of the Industrial Revolution. Today the potato is the fourth most important food crop in the world following wheat, maize and rice (www.cipotato.org). Disease control is a prerequisite for improving and maintaining yield and quality of the potato crop and since the potato became widely grown serious outbreaks of disease and crop failures and consequent social and economic effects have repeatedly provided incentive for improvement (Hide and Lapwood 1978). The worst famine, by far, was the Potato Famine of the 1840's. Starting in late 1845, the famine took an estimated one million lives, and drove another two million to travel to the Continent or to the United States (http://www.usna.edu/EnglishDept/ilv/famine.htm). The potato is prone to more than a hundred diseases caused by bacteria, fungi, viruses or mycoplasmas but fortunately relatively few reach serious proportions in any one growing areas. Late blight, caused by the oomycete *Phytophthora infestans*, is generally the most important disease wherever potatoes are grown (Hide and Lapwood 1978). In addition the potato cyst nematode, *Globodera pallida*, is the most destructive pest in potato cultivation in temperate regions.

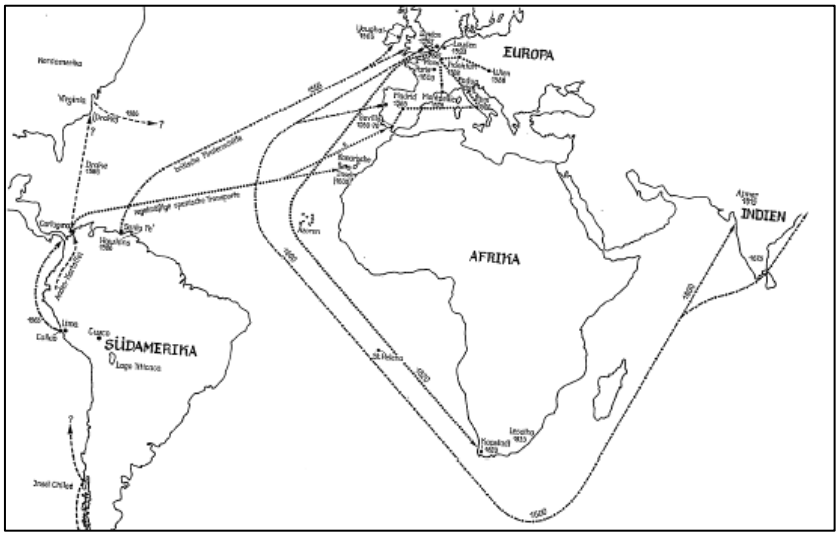

Figure 1.1 Migration of the potato (*Solanum tuberosum* L.) (Brücher 1975).

Conventional ways to control late blight or *G. pallida* on potato crops rely essentially on cultural and chemical methods but are costly and time consuming.
Increasing host plant resistance is a cost effective and environmentally friendly method of controlling pests and diseases. Breeding for resistance is therefore a major aim of companies involved in potato breeding.

The cultivated potato (*Solanum tuberosum* ssp. *tuberosum*) is a far from ideal species for genetic analysis: It is tetraploid (2n = 4x = 48) with tetrasomic inheritance and highly heterozygous owing to inbreeding depression after repeated selfing. One to four different alleles are present per locus, resulting in one homozygous and four heterozygous genotypes (Gebhardt and Valkonen 2001). Nevertheless the use of molecular markers enables the selection of favourable genotypes at an early time point in the potato breeding process. More than 60 mapped *R* genes and QTL for resistance to different pathogens of potato have been identified to date (reviewed in Gebhardt and Valkonen 2001).
The 'SOLanaceae Function Map for Pathogen Resistance' compiles the current knowledge on genomic positions of candidate genes having putative functions in pathogen recognition, defence signalling and defence responses (gabi.rzpd.de/projects/Pomamo/SolFunctionMap.html).

The parasitic root cyst nematode *Globodera pallida*

Globodera pallida (Figure 1.2) and *Globodera rostochiensis* originate from the Andes in South America (Evans and Stone 1977). From here they were most probably transported to Europe and distributed in many ways to different potato growing areas. The major import started approximately 150 years ago when potato tubers were brought to Europe for breeding purposes (Müller and Rumpenhorst 2000).
In Germany the first report on the appearance of *Globodera* was in 1913. In gardens near Rostock damage was observed on several potato plants. The cause for the damage was determined by Wollenweber to be the cyst nematode *Heterodera schachtii*. It was not until the 1930s that *H. schachtii* was acknowledged to be a new species and the name *Heterodera rostochiensis* was introduced. In 1972 a part of the population was recognized as a new species, which was named *Heterodera pallida*. Later both potato cyst nematodes

together with other species with round cysts were subsumed under the new genus *Globodera* (Müller and Rumpenhorst 2000).

Figure 1.2 *Globodera pallida* juvenile (J2) . Scale bar = 100 µm

Life cycle of the nematode: Second-stage juveniles hatch, under a stimulus from host root exudates, from eggs within cysts in the soil and invade the roots. Each individual nematode feeds on a group of cells in the pericycle, cortex, or endodermis, transforming them into a syncytium (feeding cell). The nematode remains here for the rest of its development, as it passes through two more juvenile stages to become either male or female. Females swell and break through the root surface but remain attached. They are fertilized by the vermiform, actively moving males. After copulation the males die and the females remain on the roots while eggs develop within them. Females are white when they protrude from the root surface and those of *G. pallida* remain so (Figure 1.3); those of *G. rostochiensis* pass through a golden yellow phase lasting 4-6 weeks. When the females are fully mature they die and their skin hardens and turns brown to become a protective cover (the cyst) around the eggs within. There are, on average, 500 eggs per cyst. At this point they generally drop from the surface of the root into the soil, where the eggs can either hatch immediately to attack the crop or remain dormant to act as a source of inoculum for future crops (www.eppo.org/QUARANTINE/quarantine.htm). Cysts can remain dormant for many years in the absence of solanaceous hosts (Jones & Jones 1974). The average size of soil and plant nematodes is about 1mm (Jones & Jones 1974).

Figure 1.3 *G. pallida* cyst on root of the susceptible potato variety 'Desiree'.
Scale bar = 100 µm.

Symptoms: The symptoms of attack by *Globodera* spp. are not specific. Patches of poor growth occur generally in the crop (Figure 1.4), sometimes with yellowing, wilting or death of the foliage. Even with minor symptoms on the foliage, the size of the tubers can be reduced (www.eppo.org/QUARANTINE/quarantine.htm).

Figure 1.4 Patches of stunted plants (NIVAP, the Netherlands Potato Consultative Institut, www.aardappelpagina.nl/explorer/)

Resistance to potato cyst nematodes

Cyst nematodes cause a variety of plant diseases, mostly in temperate regions of the world. Some cyst nematode species attack only a few plant species and are present over limited geographic areas, whereas others attack a large number of plant species and are widely distributed (Agrios 1997). About 60 parasitic nematode species feed on potato plants (Jensen et al. 1979). Species causing yield reduction are cyst nematodes of the genus *Globodera*, root lesion nematodes of the genus *Pratylenchus* and root knot nematodes of the genus *Meloidogyne* (Brodie 1999).

Globodera root cyst nematodes have as hosts *Solanum* species and among those important crops such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicon*), and eggplant (*Solanum melongena*) (Southey 1965). The most damage to *Solanaceae* is caused by *Globodera* species, followed by root knot nematode (RKN) species of the genus *Meloidogyne*.

An efficient way of protection against nematodes is the introgression of resistance genes. Introgression in the classical sense is the incorporation of genes from one individual or population into the gene complex or gene pool of another (Bradshaw and Mackay 1994). This breeding goal has been successfully achieved for resistance to *Globodera rostochiensis* by the introgression of the major *H1* gene for resistance from *S. tuberosum* ssp. *andigena* (Ellenby 1952) (Table 1.1). However the wide spread cultivation of varieties with resistance to *G. rostochiensis* favoured the multiplication of pathotypes of *G. pallida*, which are not affected by this resistance gene.

Several other *R* genes and QTL have been mapped, including genes for resistance to *G. rostochiensis*, *G. pallida* and *Meloidogyne chitwoodi* (reviewed in Gebhardt and Valkonen, 2001). Thirteen PCN resistance loci have been mapped on potato chromosomes III, IV, V, VII, IX, X, XI and XII (after Grube et al. 2000, Table 1.1). Eight resistance loci (*Gro1.4*, *Gpa4*, *Gpa*, *Gpa5*, *Gpr1*, *Gpa6*, *Gro1.2*, *Gro1.3*) confer partial resistance, while four of them (*H1*, *GroV1*, *Gpa2* and *Gro1-4*) confer absolute resistance to different *Globodera* species or pathotypes.

Table 1.1 Overview of mapped loci that confer resistance to nematodes in the potato.

LG	Gene/ Allele	SD/QTL	Species	Origin	Cloned	Reference
III	*Gro1.4*	QTL	*G. rostochiensis*	*S. spegazzinii*	No	Kreike et al. 1996
IV	*Gpa4*	QTL	*G. pallida*	*S. tuberosum* spp. *tuberosum*	No	Bradshaw et al. 1998
V	*Gpa*	QTL	*G. pallida*	*S. spegazzinii*	No	Kreike et al.1994
V	*Gpa5*	QTL	*G. pallida*	*Solanum spp.*	No	Rouppe van der Voort et al. 2000
V	*Grp1*	QTL	*G. pallida/ G. rostochiensis*	*Solanum spp.*	No	Rouppe van der Voort et al. 2000
V	*H1*	SD	*G. rostochiensis*	*S. tuberosum* spp. *andigena*	No	Gebhardt et al. 1993, Pineda et al.1993
V	*GroVI*	SD	*G. rostochiensis*	*S. vernei*	No	Jacobs et al. 1996
VII	*Gro1-4*	SD	*G. rostochiensis*	*S. spegazzinnii*	Yes	Paal et al. 2004
IX	*Gpa6*	QTL	*G. pallida*	*Solanum spp.*	No	Rouppe van der Voort et al. 2000
X	*Gro1.2*	QTL	*G. rostochiensis*	*S. spegazzinnii*	No	Kreike et al. 1993
XI	*Gro1.3*	QTL	*G. rostochiensis*	*S. spegazzinnii*	No	Kreike et al. 1993
XII	*Gpa2*	SD	*G. pallida*	*S. tuberosum* spp. *andigena*	Yes	Rouppe van der Voort et al. 1997, Van der Vossen et al. 2000

LG: Linkage group; SD: Single dominant locus; QTL: Quantitative trait locus (after Grube et al. 2000).

To date two nematode resistance genes have been cloned in the potato. The *Gro1-4* gene conferring resistance to pathotype Ro1 of the root cyst nematodes *G. rostochiensis* was cloned based on the candidate gene approach (Paal et al. 2004). *Gro1-4* encodes a protein of 1136 amino acids that contains a *Toll*-interleukin 1 receptor (TIR), nucleotide binding (NBS); leucin rich repeat (LRR) homology domains and a C-terminal domain with unknown function. The deduced *Gro1-4* protein differed by 29 amino acid changes from susceptible members of the *Gro1* gene family. *Gro1-4* is expressed, among other members of the family including putative pseudogenes, in non-infected roots of nematode resistant

plants. It was also demonstrated that the members of the *Gro1* gene family are expressed in most potato tissues.

The *Gpa2* gene, which confers resistance to a small set of populations of the potato cyst nematode *G. pallida*, has been mapped to the same 6 cM interval on chromosome 12 of the potato as the virus resistance gene *Rx*. From the sequence similarity between *Gpa2* and *Rx1*, it is clear that there is an evolutionary relationship between the two genes. Sequence diversity is concentrated in the LRR region and in the C-terminus (Van der Vossen et al. 2000). *Gpa2* is a member of the leucine zipper, nucleotide binding, leucine rich repeat family of plant genes (Rouppe van der Voort et al. 1997). Molecular analysis of the locus resulted in the identification of cluster harbouring four highly homologous genes in a region of approximately 115 kb. Although the precise mechanism of *Gpa2*-mediated resistance is unknown, preliminary results suggest that it is not associated with a localized necrosis response (A. Goverse, personal comment). This is in contrast to the response mediated by the nematode-resistance genes *Mi-1* from the tomato (Milligan et al. 1998), and *H1* from the potato (Rice et al. 1985).

Resistance mechanisms

R genes are monogenetic. Plants possess genes for resistance (*R* genes) directed against avirulence genes of pathogens or pests. The discovery of matched specificity between single host *R* genes and single pathogen *AvR* genes was made by Harold Flor in the 1940s (Flor 1971) and is the source of the term gene-for-gene interaction. *R* gene mediated resistance is able to activate defence mechanisms more rapidly and effectively than basal defence pathways, such as synthesis of phytoalexins or pathogenesis-related (PR) proteins, which inhibit the spread of the pathogen after a successful infection. *R* gene mediated resistance is often associated with a hypersensitivity response during which the cells in the vicinity of the infection site undergo programmed cell death. In the cell the earliest signs of *R* gene dependent resistance mechanisms are calcium influx, alkalization of the extracellular space, protein kinase activation, production of reactive oxygen species and nitric oxide, and transcriptional reprogramming (Bent 1996).

R genes can be divided into five structural classes (Takken et al. 2000; Figure 1.5). Most *R* genes characterized so far encode leucine-rich repeats (LRRs) shown to be involved in protein-protein interactions (Hwang et al. 2000).

LRR-containing *R* proteins can be divided into two classes: those in which the predicted gene product contains an extracellular LRR and a membrane anchor; and those in which the *R* gene product is predicted to be cytoplasmic. Cytoplasmically located *R* gene products are characterized by the presence of a conserved region containing a nucleotide binding site (NBS) and a C-terminal LRR region (Hwang et al. 2000).

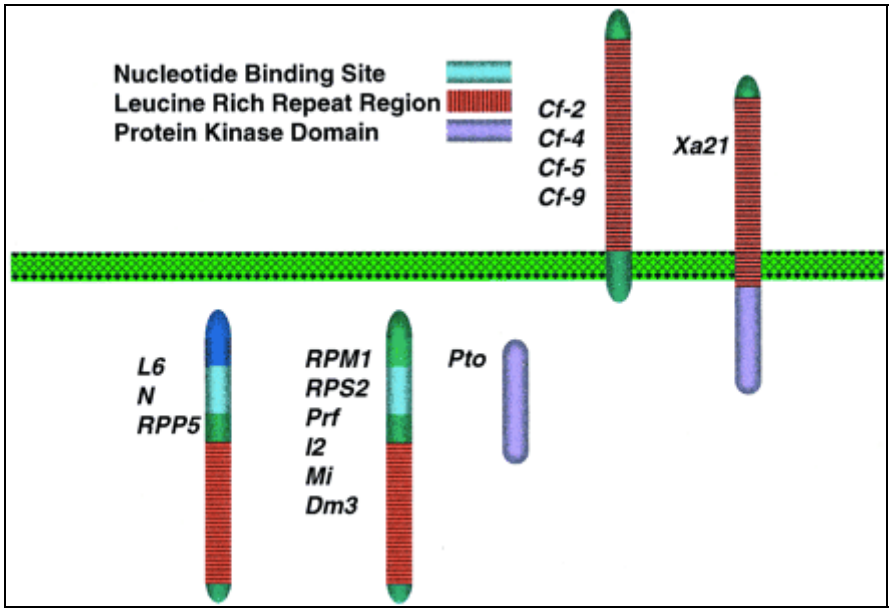

Figure 1.5 Major protein motifs shared between the deduced products of cloned resistance genes (Michelmore and Meyers 1998).

However in nature most genetic variation in readily observable traits is polygenic (Tanksley 1993), meaning that phenotypic variations result from the segregation of alleles at multiple quantitative trait loci (QTL) with effects that are sensitive to the genetic, and external environments.

Major challenges for biology are to map the molecular polymorphisms responsible for variation in agriculturally important complex traits (Mackay 2001). A number of factors for quantitative resistance to root cyst nematodes have been identified and mapped (Table 1.1 and Gebhardt and Valkonen 2001 for review). Causative candidates for the expression of a quantitative trait are genes with a possible structural or regulatory function. This

makes the identification of the causative gene a difficult task. To date three QTL were cloned in the tomato, the *Brix9-2-5* for fruit sugar content (Fridman et al. 2000), *Ovate* for fruit shape (Liu et al. 2002), and *fw2.2* for fruit weight (Frary et al. 2000). No QTL have been cloned so far in the potato.

Both potato cyst nematodes species display different pathotypes or virulence groups. Three pathotypes Pa1, Pa2 and Pa3 of *G. pallida* have been distinguished based on differentials (Table 1.2, Kort et al. 1977). *G. pallida* field populations are however not uniform with respect to pathotype composition. Populations containing mixtures of pathotypes Pa2 and Pa3 (Pa2/3) are currently the most common in potato cultivation in middle Europe. The detection and description of these pathotypes parallels the history of breeding for resistance.

Initially only two genes, the *H1* gene from the *Solanum tuberosum* ssp. *andigena* (CPC 1673) (Ellenby 1952) and the *H2* gene from *Solanum multidissectum* (Dunnett et al. 1961), were known. Both are dominant genes, which account for almost 100% of resistance against avirulent pathotypes. When populations were discovered which could overcome the resistance, new sources for resistance were introgressed from wild *Solanum* species and became part of the breeding programs. Wild species, such as *S. spegazzinii* (Caromel et al. 2003) became part of the breeding programs for resistance.

Table 1.2 International pathotype scheme for *G. rostochiensis* and *G. pallida* (Kort et al. 1977).

Differentials	Pathotypes							
	Ro1	Ro2	Ro3	Ro4	Ro5	Pa1	Pa2	Pa3
Solanum tuberosum ssp. tuberosum	+	+	+	+	+	+	+	+
Solanum tuberosum ssp. andigenum CPC 1673	-	+	+	-	+	+	+	+
Solanum kurtzianum- hybr. KTT 60-21-19	-	-	+	+	+	+	+	+
S. vernei hybr. 58.1642/4	-	-	-	+	+	+	+	+
Solanum vernei- hybr. (VTN2) 62.33.3	-	-	-	-	+	-	-	+
Solanum vernei- hybr. 65.346/19	-	-	-	-	-	+	+	+
Solanum multidissectum P55/7	+	+	+	+	+	-	+	+

Interestingly, it was determined that only Ro1 and Pa1 are considered to be true pathotypes. The remaining ones (Ro2, Ro3, Ro4, Ro5, Pa2, Pa3) should be understood as

virulence groups, which consist of varying complexes of different virulence (European and Mediterranean Plant Protection Organization 1985).

In Germany the varieties considered resistant are described in the 'Kartoffelschutzverordnung'. A tested variety is considered resistant when resistance ranges between 25 and 50% depending on environmental conditions. This value is calculated as Pf//Pi value (Pi: initial population density, Pf: final population density after 12-14 weeks).

Association mapping

For the past two decades, the dominant study design for investigation of the genetic basis of inherited disease has been linkage analysis in families (Carlson et al. 2004). Linkage analysis searchs for regions of the genome with a higher-than-expected number of shared alleles among affected individuals within a family. This indicates that somewhere within this linked region is a disease-predisposing allele. Closely related individuals tend to share large regions of the genome inherited from the same recent ancestor (Carlson et al. 2004). Association studies are carried out with unrelated individuals making use of the higher frequency of recombination events which may have occurred in the past. Consequently molecular makers associated with an interesting allele are expected to be closer together (Cardon and Bell 2001 for review). Further advantages are a higher allelic diversity present in the population, and the random collection of individuals without the need to generate mapping populations. First association studies in potato showed the variation in tetraploid breeding material regarding the invertase locus (Li et al. 2005), resistance to late blight (Gebhardt et al. 2004), resistance to *Verticillium albo-atrum* (Simko et al. 2004a), and resistance to *Verticillium dahliae* (Simko et al. 2004b).

Objective of this thesis

Objective of this study was the identification and characterization of important agronomic traits in tetraploid potato breeding material with focus on the resistance to *Globodera pallida*. At this main interest was to characterize a region on the upper arm of chromosome V in more detail since it is known to harbour a wealth of resistance factors to different pests and pathogens including *G. pallida* (Chapter 4). The project aims can be summarized as follows (a) identification of QTL for different agronomic traits using association mapping in the main discovering associations with resistance to *G. pallida;* (b) development of PCR marker assays to facilitate the development of superior varieties in commercial potato breeding programs; (c) fine mapping and characterization of the major QTL conferring resistance to the potato cyst nematode *G. pallida* pathotypes Pa2/Pa3 on chromosome V.

Figure 1.6 shows the aspects dealt with in each of the following chapters.

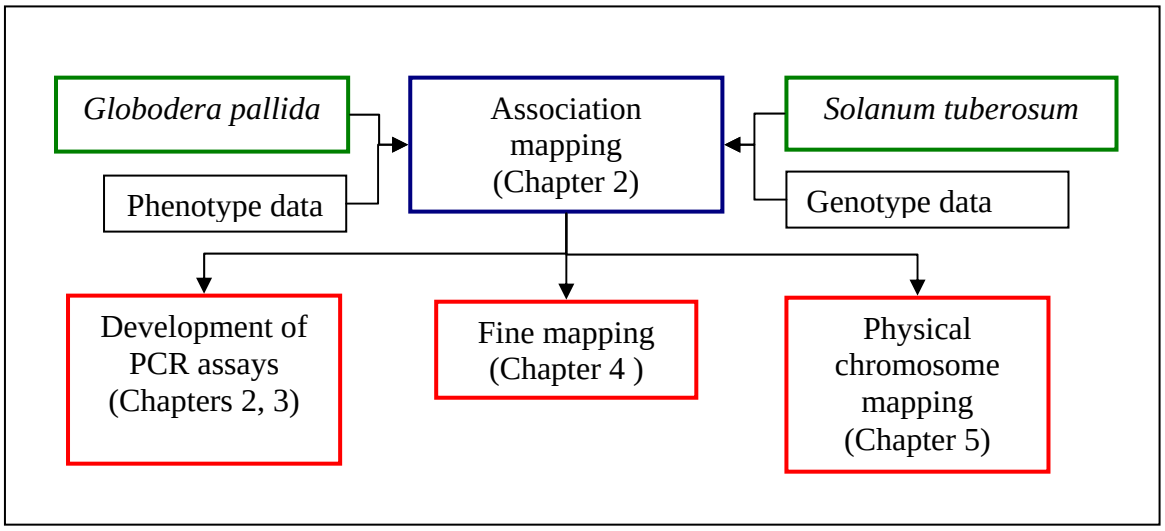

Figure 1.6 Strategy of the PhD work. The black boxes contain aspects for which information was available. The blue box frames basic work for which genotypic and phenotype data was assembled. Following the association mapping study, three additional projects (red boxes) were carried out to obtain a more accurate picture of the region on the upper arm of chromosome V.

Chapter 2

Detection of associations between candidate gene loci and quantitative traits

Abstract

Two populations of 96 tetraploid varieties and breeding clones each, which are related by descent, were evaluated for resistance to *Globodera pallida*, *Phytophthora infestans*, maturity, maturity corrected resistance (MCR), starch content, and yield. The same populations were genotyped for SNPs, SSR, CAPS, SCAR and ASO markers either within or physically linked to candidate genes. Associations with quantitative resistance were found for all marker types. One allele specific marker developed upon sequence information was shown to be associated with MCR and can be used in breeding programs for marker assisted selection (MAS).

Introduction

For the past two decades, the prevalent study design for investigating the genetic basis of inherited diseases has been linkage analysis in families (Carlson et al. 2004). Traditionally, linkage mapping relies on the linkage disequilibrium (LD) between markers and trait values that occur within mapping populations or families. However, LD occurs also in unrelated (associated) populations. Association studies are carried out with unrelated individuals making use of the higher frequency of recombination events which may have occurred in the past. Association mapping of QTL is based on marker-trait LD in such populations, identifying events that created association in the relatively distant past (Jannink and Walsh 2002). Assuming that many generations, and therefore meioses, have elapsed recombination will have removed association between a QTL and any marker not tightly linked to it. Thus association mapping allows for much finer mapping than standard bi-parental cross approaches. It has become the method of choice for identifying the inheritance of complex traits with SNPs as the main marker used for genotyping in humans (Jorde 2000; Hinds et al. 2006), animals (Palsson and Gibson 2004) as well as plant genomes, such as potato (Simko et al. 2004a, 2004b; Gebhardt et al. 2004; Li et al. 2005). The idea is to identify markers with allele-frequency differences between groups of individuals displaying a significant difference in the phenotype of interest compared to control individuals. When enough segregating markers are scattered throughout the entire genome, it is theoretically possible to detect and characterize all genes affecting a quantitative trait except in areas on the chromosome where the recombination frequencies are low and the detection of polymorphisms is limited. Today, molecular linkage maps covering the entire genome are available for quantitative trait studies in many organisms including the potato (Tanksley 1993).

In contrast to quantitative resistance to *Phytophthora infestans*, which is controlled by factors on every potato chromosome, quantitative resistance to *Globodera pallida* seems to be controlled mainly by few QTL with large effects (reviewed in Gebhardt and Valkonen 2002). Genetic control of *P. infestans* in the foliage can be race-specific, controlled by the dominant alleles of resistance genes (*R* genes), but can also be effected through the action of general resistance which is considered to be more durable and based on polygenes (Umaerus and Umaerus 1994; Wastie 1991). In potato, 11 *R* genes (*R*1-*R*11) have been

identified originating from *S. demissum* (Malcomson and Black 1966; Shaw 1991). However, the immunity reaction of the resistant plants can be easily overcome by the appearance of new virulent factors of the pathogen (Micheletto et al. 2000). Breeding efforts are therefore directed towards the increase of quantitative resistance which is longer lasting than race-specific resistance. Caveat is the correlation between quantitative resistance to *Phytophthora infestans* and late maturity in temperate climates, which is an undesirable characteristic (Collins et al. 1999; Oberhagemann et al. 1999; Visker et al. 2003; Bormann et al. 2004). To break the correlation between resistance and maturity, resistance factors that do not affect maturity or are separated by recombination from the genes controlling maturity must be identified (Bormann et al. 2004).

Marker-assisted selection (MAS) in early generations in the greenhouse could accelerate breeding for resistance. Special disease and quality tests are often required to find potential new cultivars; a diagnostic marker can improve the efficiency of selection.

The objectives of this study were (a) increasing the mapping resolution of known QTL conferring resistance to the potato cyst nematode *G. pallida*; (b) evaluation of the genotypic data additionally for resistance to *P. infestans*, maturity, maturity corrected resistance (MCR), starch content, and yield; (c) detection of novel QTL of interest for the improvement of the characteristics of the potato; (d) development of molecular markers tagging QTL for the selection of superior genotypes in potato breeding.

Materials and Methods

Plant material

Plant material of two populations of tetraploid potato breeding clones and varieties were provided by two breeding companies. Saka-Ragis Pflanzenzucht GbR (SaRa) supplied 120 individuals and Böhm-Nordkartoffel-Agrarproduktion (BNA) supplied 109 individuals. For genotyping 96 individuals for each population were used.

Plant genomic DNA extraction: Young, healthy potato leaves were harvested, freeze dried (Eps1-15, Typ 1815, Christ Gefriertrocknung GmbH, Osterode, Germany) and stored in

air-tight containers at -20°C. Total genomic DNA was extracted from 0.3g – 0.4 g freeze dried leave material according to Bormann et al. (2004).

Concentration measurement of DNA: The quality and quantity of DNA was measured by comparison of band-intensity on ethidium bromide stained agarose gels with a DNA molecular weight standard or alternatively by the absorbance at 260 nm using a photometer (Smart Spec™ 3000, BioRad, München, Germany).

Genotyping

Standard PCR reaction: Amplicons were generated from 50 ng genomic DNA template in 20 µl total volume containing buffer (20 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl), including 0.25 µM of each primer (Table 2.2), 0.2 mM of dNTP and 1.0 unit *Taq* DNA polymerase (Invitrogen Life Technologies, Freiburg, Germany). Standard cycling conditions were: 3 min initial denaturation at 94°C, followed by 39 cycles of 1 min denaturation at 94°C, 1 min annealing at the appropriate T_m and 1 min extension at 72°C. Reactions were finished by 8 min incubation at 72°C. PCR products were examined for quality on ethidium bromide stained agarose gels.

Purification of PCR products for sequencing: ExoSAP-IT® (USB Corporation, Cleveland, USA) was used for simplified PCR clean-up. 2 µl of ExoSap were added to 5 µl of PCR product and the mixture was incubated for 15 min at 37°C and inactivated at 80°C for 15 min.

Sequencing of PCR amplicons: DNA sequences were run at the MPIZ DNA core facility using Applied Biosystems Abi Prism 3730 sequencer (Weiterstadt, Germany) and BigDye-terminator v3.1 chemistry. Premixed reagents were from Applied Biosystems. Oligonucleotides were purchased from Invitrogen, Life Technologies, Freiburg, Germany or QIAGEN GmbH, Hilden, Germany.

Detection of single nucleotide polymorphisms (SNPs): Sequencing data of all genotypes were aligned using the multalin interface page (Corpet 1988) to detect polymorphisms. For further detection and quantification tracefiles (Figure 2.1) were subjected to the Data Acquisition and Analysis Software (DAx) (van Mierlo 2006).

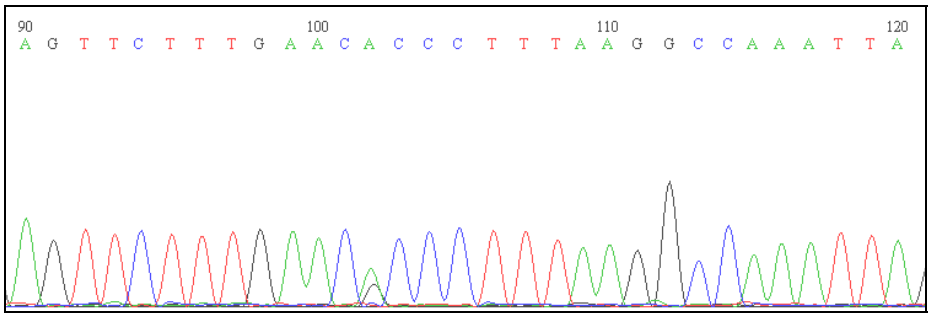

Figure 2.1 Section of a sequence (tracefile) with a SNP at position 102.

Detection of marker alleles: Marker alleles (SCAR, CAPS, ASO, and SSR) were scored as absent or present of the PCR fragment.

Microsatellite Analysis

PCR was performed in a total volume of 20 µl containing 10x standard reaction buffer (Amplicon), 0.2 mM dNTPs, 3.0 mM $MgCl_2$, 0.25 µM of each primer, 25 ng template DNA and 1unit *Taq* polymerase.

PCR conditions: Initial denaturation for 3 min at 94°C, 2 min at the respective annealing temperature (Table 2.1), 90 sec at 72°C, 30 cycles of 1 min at 94°C, 1 min at T_a and 45 sec at 72°C, and one cycle at final extension for 5 min at 72°C.

Electrophoresis was run in 30 mM TAE buffer on SPREADEX gels (Elchrom Scientific, Cham, Switzerland) using the SEA 2000 Electrophoresis System (Elchrom Scientific). Gels were run at 50°C at 120 volts according to manufacturer's instructions.

Gels were staining after electrophoresis (10% SYBR™ Gold in 30mM TAE buffer) for 45 min and destained for 30 min in a mixture containing: 20 ml of 30 mM TAE, 30 ml H_20 and 0.5 ml 100x Destaining Solution (Elchrom Scientific).

SSR alleles were numbered and scored according to their presence on the gel starting with the smallest size band.

Table 2.1 SSR markers scored in the BNA and SARA population to assess population structure.

SSR marker	Primer sequence 5'-3'	Size range (bp)	TA°C	LG
StI 004	f-gct gct aaa cac tca agc aga a r-caa cta caa gat tcc atc cac ag	78 - 112	60 – 54*	VI
StI 007	f-tat gtt cca cgc cat ttc ag r-acg gaa act cat cgt gca tt	115 - 146	60 – 54*	XII
StI 009	f-gcg aaa acc ttg aag caa ct r-ctg ctg ttg ctg ttg atg gt	262 - 322	60 – 54*	I
StI 013	f-cca ctt cct cca ctt cca aa r-cca tgg ttg cac caa cta ga	240 -340	60 – 54*	III
StI 022	f-tct cca att act tga tgg acc c r-caa tgc cat aca cgt ggc ta	114 - 145	63	VIII
StI 023	f-gcg aat gac agg aca aga gg r-tgc cac tgc tac cat aac ca	160 - 280	60 – 54*	X
StI 024	f-cgc cat tct ctc aga tca ctc r-gct gca gca gtt gtt gtt gt	149 - 186	60 – 54*	II
StI 028	f-ata ccc tcc aat ggg tcc tt r-ctt gga gat ttg caa gaa gaa	170 - 217	60	XI
StI 031	f-agg cgc act tta act tcc ac r-cgg aac aaa ttg ctc tga tg	123 - 141	60 – 54*	I
StI 047	f-act gct gtg gtt ggc gtc r-acg gca tag att tgg aag cat c	128 - 170	60 – 54*	VIII
StI 058	f-caa gca cgt tac aac aag caa r-ttg aag cat cac ata cac aaa ca	77 - 103	60 – 54*	V
STM 0001	f-agt att caa ccc att gac ttg ga r-tag aca agc caa gct gga gaa	113 - 174	60	VI
STM 0030	f-aga gat cga tgt aaa aca cgt r-gtg gca ttt tga tgg att	122 - 191	53	XII
STM 0037	f-aat tta act tag aag att agt ctc r-att tgg ttg ggt atg ata	75 - 90	48	XI
STM 0038	f-aac tct agc agt att tgc ttc a r-tta ttt agc gtc aaa tgc ata	108	54	II
STM 1043	f-att tga att gaa gaa ctt aat aga a r-cac aaa caa aat act gtt aac tca	226	53	VII
STM 1052	f-caa ttt cgt ttt ttc atg tga cac r-atg gcg taa ttt gat tta ata cgt aa	200 - 268	59	?
STM 1097	f-tga ttt agt tgc ttg ttt g r-gct ttc gat cct aat aca cc	90 - 160	54	VII
STM 1106	f-tcc agc tga ttg gtt agg ttg r-atg cga atc tac tcg tca tgg	150 - 200	60	X

SSR marker	Primer sequence 5'-3'	Size range (bp)	TA°C	LG
STM 2012	f-gcg gcc gct tct cag cca a r-tct cgt tca atc cac cag atc	247	64	X
STM 3012	f-caa ctc aaa cca gaa ggc aaa r-gag aaa tgg gca caa aaa aca	168 - 213	57	IX
STM 3023b	f-aag ctg tta ctt gat tgc tgc a r-gtt ctg gca ttt cca tct aga ga	183 - 193	50	IV

*Touch down PCR, StI markers: Feingold et al. 2005; STM markers: Milbourne et al. 1998

Cloning of PCR products for the development of the CAPS marker

PCR conditions: In a final volume of 20 µl PCR buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl); 2.0 mM $MgCl_2$; 0.2 mM of each dNTPs; 0.4 µM of each primer and 1 unit of *Taq* polymerase (Invitrogen, Life Technologies, Karlsruhe, Germany) were mixed together with 50 ng template DNA. PCR reaction conditions were 93°C for 2 min followed by 40 cycles of 92°C for 45 sec, 55°C for 45 sec and 72°C for 60 sec with a final elongation time of 10 min at 72°C.

Cloning of PCR products: PCR products were cloned using the pGEM®-T Vector System according to the manufacturer's instructions (Promega Corporation, Madison, USA). For each transformation, 25 µl competent cells of *E. coli* strain DH10B (Elektromax, Invitrogen, Life Technologies, Karlsruhe, Germany) were mixed with 1 µl ligation mixture and transferred to an electroporation cuvette. Electroporation was done at 1.8 volts on a MicroPulser Electroporator (BioRad Laboratories, UK).

The cells were transferred to a 1.5 ml eppendorf tube containing 600 µl SOC medium and incubated at 37°C for 90 min. 20 µl bacteria suspension was plated on the white and blue selective Luria-Bertani-media (LB) containing ampicillin (100 µg/ml), IPTG (8 µl/ml) and X-Gal (40 µl/ml). Petri-dishes were incubated at 37°C for 14-20 h.

Screening: Single white colonies were picked with a sterile tip and dipped in a PCR master mix to amplify the target insert of the plasmid using the specific primers. In parallel, the single colony was streaked on a selective LB medium containing ampicillin (100 µg/ml), IPTG (8 µl/ml) and X-Gal (40 µl/ml). Once the correct PCR product was obtained, the corresponding colony was propagated for plasmid DNA isolation and sequencing with the T7 Promoter Primer 5′-TAATACGACTCACTATAGGG-3′. Plasmid DNA isolation was performed using the QIAGEN Plasmid Midi Kit according to manufacturer's instructions (QIAGEN GmbH, Hilden, Germany).

Detection of polymorphisms between alleles and development of the CAPS marker: Sequencing data of the clones were aligned using the multalin interface page (Corpet, 1988) to detect polymorphisms. Polymorphism discriminating the alleles associated with either resistance or susceptibility could be identified at position 211 bp and used to design a CAPS marker. The web-based program, dCAPS Finder 2.0 (Neff et al. 1998) was used to developed the CAPS marker. Primers sequences used 5'-3': f- TGA GGA ATT GTA TCT CAT TGT TTG AAC TTA C and r- GAG AAC ATG TAC ATC CAT GAC. The forward primer ends with two mismatches at positions 209 bp and 210 bp in the sequence to allow the restriction enzyme cleavage of the primer of the allele associated with higher resistance. 13 µl of the PCR product was digested with 2 units of the restriction enzyme BseN1 (Bsr1) for 3 h at 65°C with 10 x reaction buffer in a 20 µl reaction volume (Fermentas, Life Sciences). The PCR products were separated on a 3% agarose gel (MetaPhor®, Cambrex Bio Science Rockland, USA) for 3 h at 80 volts.

Table 2.2 Polymorphic markers scored by polymerase chain reaction (PCR) and amplicon sequencing in the BNA and SaRa populations. ***** Primers were designed upon sequence information

LG	Marker name	Primer sequence (5'- 3')	Amlicon size (bp)	TA (C°)	Assay	Reference
II	*StPAD4-1*	f-GAA TTT TAT GCA ATT TGA ATT TTC	330	60	Sequence	Pajerowska et al. 2005
		r-CGG CAT GGA CCA TTG CCG GA TC				
III	*StSGT1-1*	f-GCC GTT GAC CTC TAC ACT C	370	60	Sequence	Pajerowska et al. 2005
		r-ACC AAT TAA CAG AAA CAC AGG*				
		fn-ACA CTC AAG CCA TAA CGA TG				
IV	*54I8L*	f-GGT GTC TTG AGT ATT GTC G	350/480/500	58	CAPS/*TaqI*	Park et al. 2005
		r-CCA CTT TTT CCT TTG CCT G				
IV	*STM5140*	f-GCT ATT GTT GCA GAT AAT ACG	188/192	55	SSR	Bryan et al. 2004
		r-GCC ATG CAC TAA TCT TTG A				
V	*BA213c14t7*	f-CAA TTG ATT CAT TTT ATG TAG CGA G	650/710	56	Sequence, SCAR	Rickert 2002
		r-TCT TGA CGC AAA CCT CTG CGA G				
V	*239E4left*	f-GGC CCC ACA AAC AAG AAA AC	340	56	Sequence	Bakker et al. 2004
		r-AGG TAC CTC CAT CTC CAT TTT GTA AG				
V	*Jen1*	f-CAA CGT TAC TGA GAA CTA C*	620/950	55	ASO	unpublished
		r-ATA TCG AAT ACT GAG TGA AC*				
V	*Haplotype C*	f-ACA CCA CCT GTT TGA TAA AAA ACT	276	60.5	ASO	Sattarzadeh et al. 2006
		r-GCC TTA CTT CCC TGC TGA AG				
V	*Haplotype A*	f-ATT CTC ACC AGC AGT CTT A	121	52	ASO	unpublished
		r-ATC AAT ATT TAT AAT AGC TGG T				

LG	Marker name	Primer sequence (5'- 3')	Amlicon size (bp)	TA (C°)	Assay	Reference
V	*ScarU14*	f-GGG CTT GTA TAA GAC CTC CGA GAG G	260	56	SCAR	Jacobs et al. 1996
		r-CCC TTC CTT GGG TAG TTT GAG CG				
V	*GP179*	f-GGT TTT AGT GAT TGT GCT GC	570	58	SCAR	Meksem et al. 1995
		r-AAT TTC AGA CGA GTA GGC ACT				
V	*BA47f2*	f-TAA CCA ACA TTA TCT TCT TTG CC	650	55	ASO	Gebhardt et al. 2004
		r-GAA TTT GGA GAG GGG TTT GCT G				
V	*R1*	f-CAC TCG TGA CAT ATC CTC ACT A	1400	55	ASO	Ballvora et al. 2002
		r-CAA CCC TGG CAT GCC ACG				
V	*BA151m8AB*	f-GAC ACA GAT CCG AAG CCT ATC ACA*	380	56	ASO	Gene bank AY 730335
		r-AAC GTA CTA TTC GTA TTT CGA AGA*				
V	*239E4left*	f-TGA GGA ATT GTA TCT CAT TGT TTG AAC TTA C*	560	56	CAPS	Bakker et al. 2004
		r-GAG AAC ATG TAC ATC CAT GAC*				
V	*CosA*	f-CTC ATT CAA AAT CAG TTT TGA TC	210	55	SCAR	Gebhardt et al.2004
		r-GAA TGT TGA ATC TTT TTG TGA AGG				
VIII	*LOX**	unpublished	800/1500	56	CAPS/*EcoRV*	Trognitz et al. 2002
IX	*STH (PR10) **	unpublished	710	49	CAPS/*TaqI*	Trognitz et al. 2002
XI	*STM5130*	f-AAA GTA CAG CGA AGA TGA CGA C	295/300	56	SSR	Bryan et al. 2004
		r-TTA CCT TTG CAA CCT TGC C				
X	*BA81l15t3*	f-CTG TTG GGT CTT CCT ATA AGT TGG*	240	58	Sequence	Gene bank CG783071
		r-TGA AAC CAC TAA ACA TGA CAT TTT G*				
XII	*Gpa2*	f-GCA GAT ATA ACC ACA CTA GCT C*	380	58	ASO	van der Voort et al. 1997
		r-ATG CTC CAT TTC GAC TTC CC*				

Figure 2.2 Banding patterns of PCR markers used in this study. Seventeen markers were scored for polymorphic bands indicated with arrows. The left lane shows a 100 bp ladder (Fermentas), exceptions are marker *239E4left* with the marker shown on the right; marker *Haplotype C* with a 1 kbp ladder (Invitrogen *) and the the two SSR markers *STM5130* and *STM5140* with size ladder M1 (Elchrom Scientific^{+}).

Phenotypic data

Phenotypic data were obtained for seven traits in the BNA population and five traits in the SaRa population.

BNA population: Maturity, late blight resistance (AUDPC), maturity corrected resistance (MCR), resistance to *G. pallida* Pa2, tuber starch content (TSC), tuber yield (TY) and tuber starch yield (TSY).

SARA Population: Maturity, late blight resistance (AUDPC), maturity corrected resistance (MCR), resistance to *G. pallida* Pa2/Pa3, tuber starch content (TSC).

Statistical analysis: Statistical analysis was based on the mean values over the two test years 2004/2005. Exception was the assessment of resistance against *Globodera pallida* in the BNA population, which was only analyzed for the year 2004. Phenotypic data was generated for 109 individuals in the BNA population and 120 individuals in the SaRa population. The distribution of the data was obtained using the One-Sample-Kolmogorov-Smirnov-Test. The data were found to be normal distributed when the Asymptotic Significance (2-tailed) was > 0.2.

SNP analysis was performed using the free software R (http://www.r-project.org). For phenotypic data showing normal distribution the Anova test was used and the Kruskal Wallis tests was selected for data not normally distributed.

Marker alleles were analyzed for significant associations ($p < 0.01$) using the T-test for normally distributed data and not normally distributed data were analysed using the Mann-Whitney test. All tests were computed using the software SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Figure 2.4 shows histograms of the traits under investigation.

Assessment of resistance to G. pallida: Both tests were carried out according to Kort et al. (1977) (Chapter 1). For this assessment BNA used the population 'Kalle' with resistance to pathotype Pa2, individuals known to be highly susceptible were analyzed with a default score of 200 cysts. The SaRa population was evaluated for pathotypes Pa2/Pa3, population 'Chavornay'. Individuals of the Saka-Ragis population were tested for resistance to *G. pallida* at the 'Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern' (Rostock, Germany). Individuals of the BNA population were tested at the 'Landwirtschaftskammer Niedersachsen' (Hannover, Germany).

Field assessments were located at Windeby (Schleswig-Holstein) in northern Germany for the SaRa population and in Ebstorf (Niedersachsen) for the BNA population (Figure 2.3).

Figure 2.3 Map of Northern Germany with the locations of the breeding companies indicated with red circles.

Assessment of resistance to late blight: The Area under the disease progress curve (AUDPC) is a resistance parameter calculated from the percentages of leaf area affected, which are estimated at different time points during the epidemic. When data have been collected sequentially through the season, the AUDPC is calculated in order to compare cultivars or treatments. The AUDPC was calculated according to Campbell and Madden (1990).

*Relative AUDPC (rAUDPC)**:*** Field measurements were carried out over a different time frame each year (between 40 and 48 days). Therefore each AUDPC value was divided by the total area possible (= total number of evaluation days x 8 (BNA) or 9 (SaRa) resulting in the relative AUDPC (rAUDPC). Thus the rAUDPC values may be compared over the different years. BNA scored on a scale from 1-9, SaRa scored on a scale from 0-9.

Maturity corrected resistance (MCR): A regression curve rAUDPC against maturity was computed. For each rAUDPC measurement the difference to the regression curve was calculated, which resulted in negative values for resistant plants and positive values for susceptible plants (= maturity corrected resistance) in the SaRa population, when maturity was scored on a scale from 1-9 (german system); the opposite is correct for BNA data, due to scores of maturity form 9-1 (dutch system). The phenotypic data were calculated in which the resistance was corrected for the maturity effect, resulting in a value for resistance independent from maturity.

Maturity: The maturity of uninfected plants grown for multiplication was evaluated based on the senescence of the plants using an 1 to 9 scale (1= very early maturing and 9= very late maturing in the SaRa population; 1= very late maturing and 9= very early maturing in the BNA population). The plants were compared with standard potato varieties with known maturity type according to the procedures of the 'Bundessortenamt' (BSA, Hannover, Germany).

Tuber starch content in percent (TSC): The starch content of fresh potatoes is correlated with potato density. A sample of 5050 g potato in a net basket is weighed above water and then again immersed in clean water of maximum 18°C (International Starch Institute Science Park Aarhus, Denmark).

Tuber yield (TY) and tuber starch yield (TSY): The tuber yield (TY) and tuber starch yield (TSY) were weighed in Dt/ha.

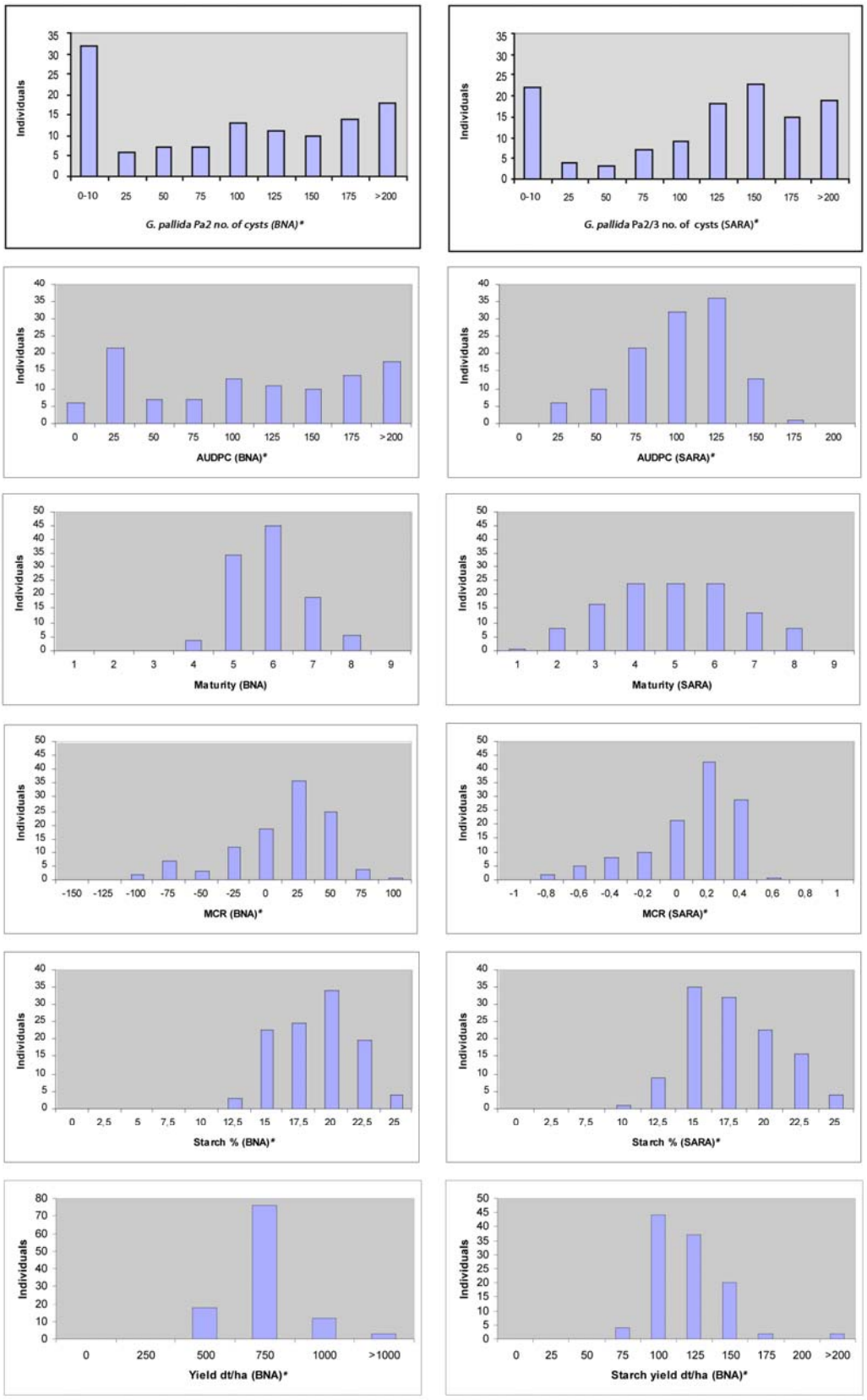

Figure 2.4 Histograms of the phenotypic data of the BNA population and SARA population.The histograms were created using Microsoft Excel.

* The values on the x-axes display categories including all individuals with values inbetween the previous category.

Results

Phenotypic analysis

Phenotypic data for resistance to *G. pallida, P. infestans*, MCR, starch content, yield and starch yield were analyzed using the Kolmogov-Smirnov test for distribution. Data regarding the starch content and the maturity were normally distributed in both populations, AUDPC was found to be normally distributed only in the SaRa population. All remaining data sets were not normally distributed (Figure 2.4). The T-test and the ANOVA were used for the data with normal distribution and the Mann Whitney and the Kruskal-Wallis test were applied for the other data sets. Maturity scores of the SaRa population ranged between 1 and 8. The variability of the genotypes chosen by BNA was between 4 and 8, excluding the very late genotypes of classes 1-3. The phenotypic data sets are diverse for both populations, because traits were evaluated according to different methods with the exception of the starch content. This trait was measured in percent and ranged in both populations between 12% and 25%. Both populations were evaluated separately.

Population structure

Nineteen SSR markers (Feingold et al. 2005; Milbourne et al. 1998) distributed on eleven chromosomes were scored for 2-7 different alleles and employed to analyze population structure. For this a ‘genetic distance analysis’ and a ‘principal component analysis’ was carried out and resulted in no significant subdivision, indicating a homogenous population structure (Dr. Heckenberger, personal comment).

Genotypic analysis

The two populations were genotyped for SNP and InDel markers. Further, the two populations were fingerprinted with different DNA marker systems, such as Simple Sequence Repeats (SSR), Sequence Characterized Amplified Regions (SCAR), Cleaved Amplified Polymorphism (CAPS), and Allele specific oligonucleotide (ASO) taking into consideration that, depending on the type of molecular marker used, it may not always be possible to distinguish between each of the heterozygous genotypes, or to distinguish from the homozygous genotypes. Most of the selected markers were previously found to be linked to QTL for resistance to either *P. infestans* or *Globodera* species (reviewed in Gebhardt and Valkonen 2002).

Sequence data were obtained from five different loci, namely *BA213c14t7*, *BA81l15t3*, *StPAD4-1*, *239E4left* and *StSGT1-1*. In total, 68 SNPs could be evaluated for association with the different traits. In addition 17 polymorphic PCR-based markers displaying a sum of 23 marker alleles were scored. Markers were chosen due to their position close to QTL or because they were previously identified as plant defense genes associated with quantitative resistance to potato late blight, such as lipoxygenase (*LOX*), *STH* (Manosalva et al. 2000) or Phytoalexin-Deficient 4 *PAD4* (Glazebrook et al. 1997). Nine PCR-based markers were located on the upper arm of chromosome V in a region known to harbour QTL for different agronomic traits (Chapter 4) and in addition the sequence of marker *BA213c14t7* on this segment was generated. Figure 2.5 shows the location of all markers used in this study on the 12 chromosomes of the potato function map.

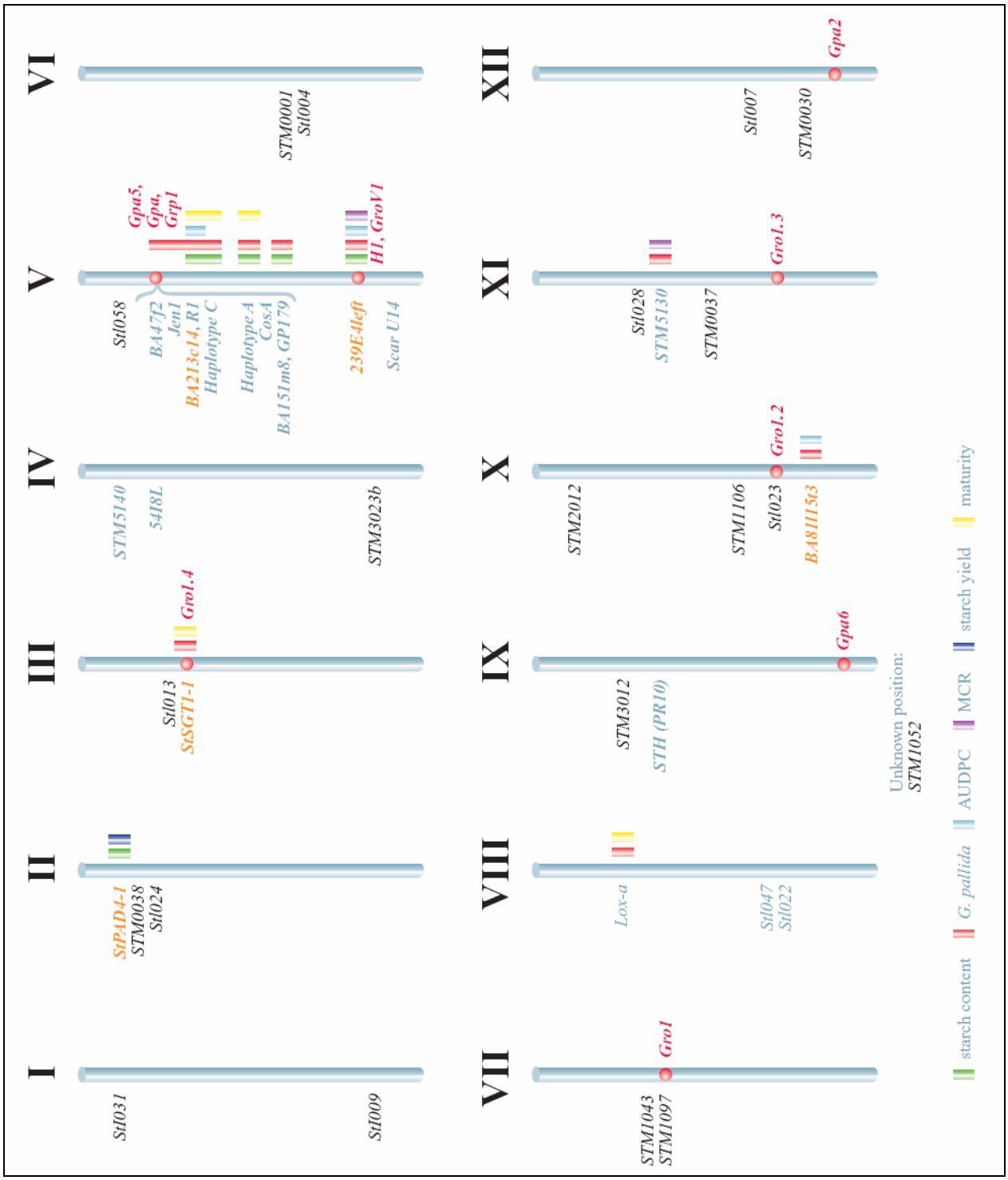

Figure 2.5 The potato function map. Twelve linkage groups (LG) corresponding to the 12 potato chromosomes are shown schematically. The loci for nematode resistance are written in bold italic red letters. Sequenced markers are in orange, SSRs used for assessment of population structure in black and PCR-based markers in bold blue letters. Different colours of the bars right to the LGs indicate associated regions on the chromosome with different agronomic traits. Red: *G. pallida*; light blue: AUDPC; dark blue: starch yield; green: starch; violet: MCR.

Association mapping in the BNA and SaRa populations

Five of the PCR-based markers on the upper arm of chromosome V, as well as SNPs deduced from the sequence information *BA213c14t7*, were found to be significantly associated ($p < 0.01$) with at least one of the traits previously detected in this region. In some cases SNPs were found to be associated with more than one trait in both populations, e.g. BA213c14t7_SNP218 was associated with *G. pallida*, maturity and starch content in the BNA population and *G. pallida* and AUDPC in the SaRa population (Table 2.5). Besides numerous SNPs from *BA213c14t7*, which were associated with *G. pallida* in the SaRa population, were associated with starch in the BNA population (Table 2.5).

SNPs from *StPAD4-1* were associated with starch content and starch yield, SNPs from *StSGT1-1* with *G. pallida* and maturity, *BA81l15t3* with *G. pallida* and AUDPC, and *239E4left* with AUDPC, MCR, maturity, starch and *G. pallida* (Table 2.5). Marker locus *LOX* was associated with later maturity and higher resistance to *G. pallida* (Table 2.4) and alleles of SSR marker *STM5130* were found to be associated with higher resistance to *G. pallida* as well as MCR. MCR was also detected with SNP211 of sequence *239E4lef*t on the lower arm of chromosome V.

In total six SNPs were associated with maturity in the BNA population and none with AUDPC while ten SNPs were found to be associated with AUDPC in the SaRa population and none were found for the trait maturity (Table 2.5).

Regarding the PCR based marker alleles we detected one marker associated with maturity in the SaRa population but again no markers associated with late blight in the BNA population (Table 2.4).

A number of SNPs and PCR based marker alleles were found to be associated with one or more of the selected agronomic traits which are summarized in table 2.3. Exception was the trait yield without any detected associations. The SNP scoring shows that in most cases the same nucleotide allele is associated with the superior direction of effect of the traits (Table 2.5). For instance BA213c14t7_SNP139, nucleotide allele A is associated with higher resistance to *G. pallida* in both populations as well as higher starch content in the BNA population. For the trait maturity the result was not clear using the Boxplot function in the software SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

The allele frequency indicates the presence of the allele in the population. Similarly we calculated the frequency of nucleotides for SNP markers. These distributions are comparable in both populations.

Table 2.3 Summary of markers significantly associated with different traits.

Aspect	Population	PCR-based marker alleles	SNPs
Markers scored in each population	BNA	23	68
Significantly associated markers	Total	10	26
	BNA	7	20
	SaRa	7	20
Markers[1] found in both populations	Total	4	15
Markers[1] found in both populations for the same trait[3]	Total	2	0
Markers[1] for more than one trait	Total	4	17
	BNA	3	6
	SaRa	1	7
Markers[1] AUPDC	Total	2	10
	BNA	0	0
	SaRa	2	10
Markers[1] Maturity	Total	3	6
	BNA	3	6
	SaRa	1	0
Markers[1] MCR	Total	2	1
	BNA	0	0
	SaRa	2	1
Markers[1] *G. pallida* Pa2	BNA	6	6
Markers[1] *G. pallida* Pa2/3	SaRa	2	16
Markers[1] Starch	Total	3	17
	BNA	3	15
	SaRa	1	2
Markers[1] Starch yield[2]	BNA	0	1
Markers[1] Yield[2]	BNA	0	0

nt= not tested; [1]significantly associated at $p < 0.01$; [2]traits only assessed in the BNA population; [3]*G. pallida* excluded due to the different pathotypes used in the assessment.

Table 2.4 Significantly associated marker alleles analyzed using the T-test or the Mann-Whitney Test. The level for significance was $p < 0.01$. +/- displays the direction of effect indicating whether the presence of the marker band is significantly associated with positive or negative characteristics of the trait. The allele frequency indicates the presence of the allele in the population.

Marker	Population	Allele frequency	*G. pallida*	AUDPC	MCR	Maturity	Starch	Yield
GP179 570 bp	BNA	0.39					0.002-	
	SaRa	0.32	0.001-				0.000-	
Haplotype A 121 bp	BNA	0.30	0.000+			0.000-	0.000+	
	SaRa	0.19	0.000+			0.002-		
Haplotype C 276 bp	BNA	0.16	0.000+			0.000-	0.000+	
	SaRa	0.16	0.000+				0.007+	
Jen1 620 bp	BNA	0.11						
	SaRa	0.15		0.001+				
Jen1 950 bp	BNA	0.16	0.007-					0.003+
	SaRa	0.16						
BA47f2 650 bp	BNA	0.10						
	SaRa	0.19		0.005+				
LOX, *EcoRV* 1500 bp	BNA		0.001+			0.000-		
	SaRa							
239E4left, *BsrI* 560 bp	BNA	0.56						
	SaRa	0.61		0.000-	0.005+	0.007+		
STM5130 295 bp	BNA	0.64	0.001+					
	SaRa	0.50			0.006-			
STM5130 300 bp	BNA	0.54	0.000+					
	SaRa	0.42						

Table 2.5 Significantly associated marker alleles analyzed using the Anova or the Kruskal-Wallis Test. The level for significance was $p < 0.01$.

Marker	Population	*G. pallida*	AUDPC	MCR	Maturity	Starch	StarchYield*
BA213c14-t7_SNP78	BNA					0.009 (T/ 0.48)$^{+}$	
	SaRa	0.007 (T/0.30)$^{+}$					
BA213c14-t7_SNP96	BNA					0.009 (C/0.46)$^{+}$	
	SaRa	0.006 (C/0.33)$^{+}$					
BA213c14-t7_SNP139	BNA	0.000 (A/ 0.17)$^{+}$			0.000 (?)	0.000 (A/0.17)$^{+}$	
	SaRa	0.001 (A/0.32)$^{+}$					
BA213c14-t7_SNP214	BNA					0.006 (T/0.14)$^{+}$	
	SaRa	0.019 (C/0.84)$^{+}$	0.005 (C/0.84)$^{+}$				
BA213c14t7_SNP218	BNA	0.005 (A/0.42)$^{+}$			0.008 (?)	0.000 (C/0.58)$^{+}$	
	SaRa	0.008 (C/0.68)$^{+}$	0.006 (C/0.68)$^{+}$				
BA213c14t7_SNP244	BNA	0.004 (C/0.46)$^{+}$				0.001 (T/0.54)$^{+}$	
	SaRa	0.001 (C/0.38)$^{+}$					
BA213c14t7_SNP253	BNA					0.000 (?)	
	SaRa						
BA213c14t7_SNP273	BNA					0.000 (G/0.57)$^{+}$	
	SaRa	0.004 (G/0.66)$^{+}$	0.005 (G/0.66)$^{+}$				
BA213c14t7_SNP274	BNA	0.000 (T/0.08)$^{+}$			0.000 (T/0.08)$^{+}$	0.002 (T/0.08)$^{+}$	
	SaRa	0.000 (T/0.05)$^{+}$					
BA213c14t7_SNP284	BNA					0.000 (G/0.58)$^{+}$	
	SaRa	0.001 (G/0.66)$^{+}$	0.004 (G/0.66)$^{+}$				
BA213c14t7_SNP289	BNA					0.006 (A/0.45)$^{+}$	
	SaRa	0.001 (A/0.34)$^{+}$					
BA213c14t7_SNP305	BNA					0.000 (C/0.57)$^{+}$	
	SaRa	0.001 (C/0.66)$^{+}$					
BA213c14t7_SNP345	BNA					0.000 (T/058^{+})	
	SaRa	0.002 (T/0.66)$^{+}$	0.004 (T/0.66)$^{+}$				
BA213c14t7_SNP347	BNA	0.001 (?)				0.002 (T/0.48)$^{+}$	
	SaRa	0.002 (T/0.38)$^{+}$					

Marker	Population	G. pallida	AUDPC	MCR	Maturity	Starch	StarchYield*
BA213c14t7_SNP381	BNA						
	SaRa	0.006 (A/0.69) [+]	0.006 (A/0.69) [+]				
BA81l15t3_SNP218	BNA						
	SaRa		0.000 (G/0.13) [+]				
BA81l15t3_SNP219	BNA	0.008 (T/0.84) [+]					
	SaRa						
StSGT1-1_SNP_179	BNA	0.005 (C/0.72) [+]					
	SaRa						
StSGT1-1_SNP_183	BNA	0.004 (G/0.17) [+]			0.008 (A/0.83) [+]		
	SaRa	0.016 (G/0.03) [+]					
StSGT1-1_SNP_249	BNA				0.001 (C/0.44) [+]		
	SaRa	0.000 (G/0.49) [+]					
StPAD4-1_SNP95	BNA						0.008 (T/0.28) [+]
	SaRa						
StPAD4-1_SNP130	BNA					0.004 (T/0.26) [+]	
	SaRa						
239E4left_InDel84	BNA						
	SaRa					0.000 (T/nt) [+]	
239E4left_SNP189	BNA				0.002 (?)		
	SaRa	0.004 (G/0.18) [+]	0.000 (G/0.18) [+]			0.002 (G/0.18) [+]	
239E4left_SNP207	BNA						
	SaRa		0.007 (C/0.11) [+]				
239E4left_InDel211	BNA						
	SaRa		0.000 (T/nt) [+]	0.001 (T/nt) [+]			

[+] Letters in brackets show the nucleotide allele associated with the superior nucleotide allele and the frequency it occurred in the population; (?) indicates that a nucleotide could not be assigned clearly to the direction of effect.

Development of a PCR assay detecting an allele associated with MCR

An InDel at position 211 bp in the sequence of marker *239E4left* was found to be associated with MCR in the SaRa population. The amount of phenotypic variation in MCR explained by this allele was calculated to be about 8%. The use of sequencing data for marker-assisted selection in breeding programmes is not feasible due to time constraints and the high costs of the sequencing procedure. To counteract these problems, a CAPS

marker for the detection of this associated polymorphism was developed, which separates the alleles upon presence and absence of the InDel.

For this, the nucleotide sequence of individual alleles was determined via cloning of genotypes S3 and S19 (Figure 2.7). A BLAST was performed against the Unigene Sequence database of the 'sol genomics network' and a match (98% identity) was retrieved with Unigene Sequence SGN-U274975. Upon this sequence information, which was longer than the original query sequence, it was possible to design a reverse primer further downstream on the sequence to obtain an amplicon of an extended region also downstream of the InDel. Next, two mismatches were introduced at the end of the forward primer to allow cleavage of the more resistant allele with restriction enzyme *BsrI* (5'..ACTGGN▼..3')(Figure 2.7). The amplicon size of the PCR fragment prior to digestion was 660 bp. The sizes of the fragments after digestion were 560 bp for the more susceptible allele and 530 bp for the more resistant allele (Figure 2.6).

The size difference between the susceptible allele and the resistant allele in the reaction was 35 bp as expected (Figure 2.6). The 35 bp fragment was not visualized on the 3% gel due to size limitations. However, both fragments were 100 bp smaller than expected compared to the undigested control of 660 bp. The reason for this is unclear; we suggest that the enzyme cuts also at an ACTGAT site at position 591 bp, which is similar to the restriction site (ACTGGN).

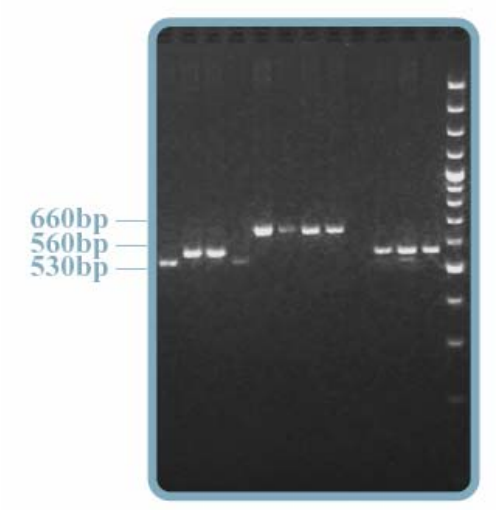

Figure 2.6 PCR products prior of marker *239E4left* prior and after digestion. The size ladder on the right is a 100 bp ladder (Fermentas, Life Sciences).

Figure 2.7 Alignment between alleles of the *239E4left* sequence, the primer sequences used, and the resulting CAPS marker sequence of the more susceptible allele. The arrows indicate the direction of the PCR primers, the position of the InDel and the two mismatches in the forward primer are surrounded by boxes, the restriction site is indicated with a bar and two dots, a second putative restriction site is indicated with a box on positions 584 bp-587 bp.

Discussion

Positional QTL mapping using distantly related potato breeding material

The main objective of this study was to detect alleles associated with resistance to *G. pallida*. In addition, association with other central agronomic qualities, such as starch content, maturity, yield, starch yield and resistance to late blight were included into the analysis. Two tetraploid potato populations have been subjected to phenotypic and genetic analysis for association mapping of QTL. The analysis showed that 43% of the PCR-based marker alleles and 38% SNPs were associated with traits under investigation. These high numbers are not surprising when taking the breeding history and pedigree information of the populations into account. Hundred years of potato breeding correspond merely to five or six meiotic generations which makes the selection of unrelated plant material difficult (Gebhardt et al. 2004). The genetic variation among individuals was high (indicated by the high level of polymorphisms detected), and linkage disequilibrium (LD) is probably strong explaining the high percentage of associated markers detected in this study (see also Chapter 4). Several markers on the same chromosomal segment (haplotype) could therefore describe the same QTL.

A significant degree of associations is of advantage for breeding companies, which benefit in their selection process from numerous markers associated with favourable traits. However, only marker alleles with a frequency > 10% are of interest because otherwise tremendous breeding efforts are needed to implement this allele within the breeding population (Jens Lübeck, personal comment). The drawback is that chances are low, that identified markers have a causal relationship with the trait, or are actually positioned in a gene involved in the expression of a protein shaping this trait. Nevertheless, the selected markers identified in this study serve as informative markers in population genetics such as this analysis.

Interaction analysis among various loci, as described by Bormann et al. (2004), would be of interest. They should be considered in addition to QTL main effects since phenotypes are the end result of a multiplicity of genomic interactions (Lynch and Walsh 1998). Molecular interactions in potato were detected for abscisic acid content (Simko et al. 1997) where at least three distinct loci on three chromosomes were associated with variation in abscisic acid content. One of the QTL was detected only as a main (single locus) effect, and two QTL were found through two-locus interaction analysis (epistasis).

Clustering of genes involved in the characterization of important agronomic traits

A number of marker alleles which were associated with different traits as well as a number of markers which were found to be associated with more than one trait were detected. Interestingly, SNPs of sequencing markers, *StPAD4-1*, *StSGT1-1*, *239E4left* and *BA81l15t3* were associated also with traits not previously tagged in this region. Most probably the detection of associations was facilitated due to the presence of clusters of different agronomic traits on genomic regions in the potato genome, which is in line with earlier findings that *R* genes and/or QTL are organized in clusters (Gebhardt and Valkonen 2001; Michelmore and Meyers 1998). Most markers (Figure 2.5) used in this study were located on the upper arm of chromosome V where different QTL for agronomic traits have previously been mapped. Therefore several markers in this region could be abundant detecting the same QTL. SSR marker *STM5130* was previously found to be linked to a QTL conferring resistance to *G. pallida* (Bryan et al. 2004) which could be confirmed by our findings in the BNA population. In addition association with the trait MCR was found in the SaRa population which has not been reported before.

BA81l15t3 is located near the resistance QTL *Gro1.2* to *G. rostochiensis* on the lower arm of chromosome X. In the present study BA81l15t3_SNP219 was associated with resistance to *G. pallida*. Similarly a locus (*Grp1*) conferring resistance to both potato cyst nematode species was previously identified by Rouppe van der Voort et al. (1998). Similarly 239E4left_SNP211 originating from BAC *239E4left* which is closely linked to the *H1* gene conferring resistance to *G. rostochiensis* (Bakker et al. 2004) was found to be associated also with *G. pallida*, AUDPC, MCR, maturity and starch.

StPAD4-1 and St*SGT1-1* are both alleles involved in defence-signalling (DS) loci in potato and previously found to be linked to markers known to detect resistance to late blight (Bormann et al. 2004; Oberhagemann et al. 1999; Leonards-Schippers et al. 1994). In the present study *StPAD4-1* was found to be associated with starch and starch yield and *StSGT1-1* was associated with *G. pallida* and maturity assuming that also in these regions on the chromosomes clusters of QTL involved in the characterization of different agronomic traits are located.

A number of markers alleles or nucleotide alleles associated with more than one trait could also be explained do to a pleiotropic effect of the same gene(s) or the genes that control the different traits are closely linked genes. These reasons have been suggested to be the cause for the correlation between the two traits plant maturity and resistance to late blight (Colon et al. 1995).

Assessment of phenotypic data in two different populations

The results for both populations are diverse, which could be explained due to diverse phenotyping methods and statistical analysis used by the two breeding companies. Also differential expression of a phenotypic trait by genotypes across environments (Genotype x Environment (GxE) interaction) is of primary importance for quantitative genetics and its application in breeding (Eberhard and Russel 1966; Tiret et al. 1993). Therefore it was not feasible to compare data of populations which were evaluated in different environments approximately 200 km distal from each other. Intriguingly the composition of the populations seemed to be comparable regarding the allele frequencies of the selected markers in this study. This substantiates the assumption that the large differences in the results are due to the diverse phenotype data. In order to counteract this problem data sets are currently being adapted and the populations will be analysed again jointly.

Development of an allele-specific marker for Marker assisted selection (MAS)

Inexpensive and easy-to-use PCR assays for the detection of marker alleles have become a common method to score favourable traits (Niewöhner et al. 1995; Zhang and Stommel, 2001, Marczewski et al. 2001; Sattarzadeh et al. 2006). A CAPS marker was designed previously in a diploid mapping population on the basis of the 239E4left sequence that mapped to a position 0.8 cM from the *H1* resistance gene against *G. rostochiensis* at the centromeric side to the long arm of chromosome V (Bakker et al. 2004).

Yet, most desirable for potato variety breeding is a combination of high late blight resistance with early plant maturity (Gebhardt et al. 2004). We were able to develop a PCR-based marker for the rapid detection of a QTL for MCR against *P. infestans* based on the *239E4left* sequence.

This assay in combination with other markers for resistance against *P. infestans* can add to a specific and efficient selection of more resistant individuals to encounter the threat of the pathogen and improve the breeding process in the future.

Marker assisted selection (MAS) is an application in which molecular genetics is integrated with traditional methods of artificial selection of phenotypes (Lande and Thompson 1990). The use of DNA markers instead of phenotypic assays, which often take several years, reduces cost and time and is a much more accurate method to accomplish breeding goals. Besides economical factors also environmental issues concerning the reduction of toxic pesticides are an important consideration. Association mapping methods

that use already existing cultivars provides a highly suitable model for direct testing of candidate gene markers with the subsequent development of markers for MAS.

Chapter 3

Identification of a PCR-based marker highly diagnostic for potato varieties with high resistance to *Globodera pallida* pathotype Pa2/3 introgressed from *Solanum vernei*

Abstract

Globodera pallida is a parasitic root cyst nematode of potato, which causes reduction of crop yield and quality in infested fields. Field populations of *G. pallida* containing mixtures of pathotypes Pa2 and Pa3 (Pa2/3) are currently most problematic for potato cultivation in middle Europe. Genes for resistance to *G. pallida* have been introgressed into the cultivated potato gene pool from the wild, tuber bearing *Solanum* species *S. spegazzinii* and *S. vernei*. Selection of resistant genotypes in breeding programs is hampered by the fact that the phenotypic evaluation of resistance to *G. pallida* is time consuming, costly and often ambiguous. DNA-based markers diagnostic for resistance to *G. pallida* would facilitate the development of resistant varieties. A PCR assay 'HC', linked with high levels of nematode resistance in a diploid mapping population, was assessed in tetraploid potato breeding material. Screening with the HC marker 33 potato varieties resistant to *G. pallida* pathotypes Pa2 and/or Pa3, and 21 susceptible varieties demonstrated that the HC marker was diagnostic for presence of high levels of resistance to *G. pallida* pathotype Pa2/3. Further it was shown that the HC marker was only present in accessions of *S. vernei*.

Introduction

Quantitative trait loci (QTL) for resistance to *Globodera pallida* and *Globodera rostochiensis* have been found in experimental populations originating from different *Solanum* species (Chapter 1). In some cases mapping data can be used for the development of PCR-based markers linked to the QTL. However, a diagnostic value of a DNA marker beyond a particular QTL mapping population has been demonstrated only for the marker SPUD1636 linked to the major quantitative resistance locus (QRL) on chromosome V (Bryan et al. 2002). In this case, an allele-specific, 226 base pair amplicon was found in accessions of *S. vernei* and in some highly resistant breeding lines that have *S. vernei* as source of resistance to *G. pallida* in their pedigree.

A PCR-based marker (Haplotype C marker) linked to individuals with high resistance to *G. pallida* pathotype Pa2/3 was developed in former work based on Single nucleotide polymorphism (SNP) genotyping of a diploid mapping population (Sattarzadeh et al. 2006). This marker mapped to the upper arm of chromosome V in the vicinity of marker SPUD1636 and was found to be significantly associated ($p < 0.01$) with *G. pallida* pathotype Pa2/3 when testing in two distantly related tetraploid breeding populations (Chapter 2).

Ross (1986) explained that different *S. vernei* accessions have been attributed to the eelworm resistance in potato cultivars. The first screening performed by Ellenby (1948) already revealed high resistance of an *S. vernei* (syn. *S. ballsii*) accession against *G. pallida*.

Based on literature and pedigree data (Hutten and van Berloo 2001), the observed resistance against *G. pallida* on the upper arm of chromosome V was likely to originate from *S. vernei* clone VTn 62-33-3 (Ross 1986) (Figure 3.1) or hybrid clone AM 78.3778 (Rouppe van der Voort et al. 1998). Most likely several genes are responsible for the resistance of *S. vernei* displaying a quantitative character and can be detected via the HC marker (Sattarzadeh et al. 2006).

In the present study the specificity of the marker was assessed in different wild *Solanum* species. Many of which have been reported in early studies to be potential sources of resistance to *G. pallida* (Deshmukh and Weischer 1970; Turner and Stone 1984; Dellaert and Hoekstra 1987; Kreike et al. 1994).

The idea was to confirm the diagnostic value of the HC marker in commercial varieties (Table 3.1) and identify the source of resistance underlying this QTL.

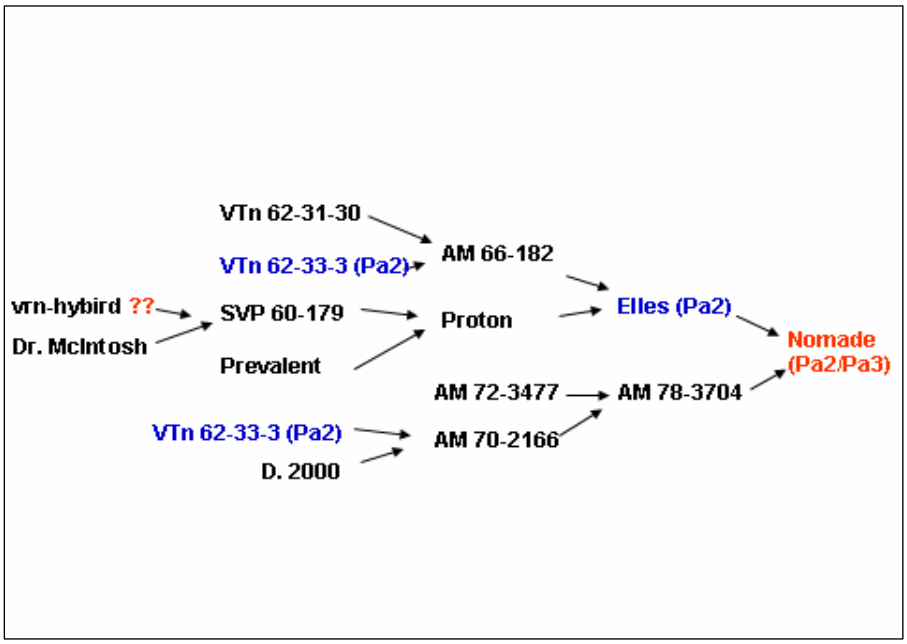

Figure 3.1 Pedigree of the highly resistant variety 'Nomade'. The source of resistance cannot be clearly assigned to one parental clone. VTn 62-33-3 was supposed to be the source of resistance but was found to be resistant only against pathotype Pa2.

Materials and Methods

Plant material: Seeds from different *S. vernei* accessions were obtained from the former German-Dutch potato gene bank of the Resource Center in Braunschweig (BGRC) and cultivated in the greenhouse.

Thirty three varieties with resistance to *G. pallida* pathotypes Pa2 and/or Pa3 (Table 3.1) were obtained from Saka-Ragis Pflanzenzucht, Böhm-Nordkartoffel Agrarproduktion (BNA), Bavaria Saat (by courtesy of A. von Zwehl), HZPC Holland B.V., Averis Seeds B.V.and B.F. Leestemaker & A. Smid (by courtesy of Jan Draaistra). DNA of 21 susceptible varieties was available (Gebhardt et al. 2004) from the collection maintained by the IPK potato germplasm bank at Groß-Lüsewitz (Germany). *S. vernei* clone 62-33-3 (Ross 1986) and the interspecific hybrid clone AM78.3778 (Rouppe van der Voort et al. 1998) were kindly provided by Björn Niere (Institute for Nematology, BBA Münster, Germany). Clone AM 78.3778 has been originally provided by Jan Draaistra.

DNA of the following *Solanum* species was available (Gebhardt et al. 2004); the number of accessions per species is given in parenthesis: *S. acaule* (3), *S. alandiae* (3), *S. andigena* (3), *S. berthaultii* (3), *S. brevicaule* (3), *S. bukasovii* (3), *S. canasense* (3), S. *chacoense* (3), *S. demissum* (3), *S. dulcamara* (1), *S. etuberosum* (2), *S. gourlayi* (3), *S. hondelmannii* (2), *S. kurtzianum* (3), *S. leptophyes* (3), *S. lignicaule* (1), *S. maglia* (1), *S. megistacrolobum* (2), *S. microdontum* (3), *S. morelliforme* (1), *S. nigrum* (1), *S. neorossii* (1), *S. oplocense* (3), *S. phureja* (3), *S. pinnatisectum* (1), *S. sparsipilum* (3), *S. spegazzinii* (3), *S. stenotomum* (3), *S. stoloniferum* (3), *S. vernei* (3), *S. verrucosum* (3). See appendix for the accession numbers.

Allele specific PCR of the HC marker: The combination of the forward primer 5' ACACCACCTGTTTGATAAAAAACT 3' with the reverse primer 5' GCCTTACTTCCCTGCTGAAG 3' resulted in haplotype *c* specific amplification of a 276 base pair DNA fragment using the following protocol: 50 ng template DNA were amplified in 15 µl PCR-mix (20 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 50 mM KCl, 100 µM dNTP, 200 nM of each primer, 2% v/v DMSO, 1 unit *Taq* DNA polymerase) and the PCR touchdown conditions: 5 min at 94°C, one cycle of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C, six cycles of 30 sec at 94°C, 30 sec at 65°C decreasing the annealing temperature to 60°C by 1°C per cycle and 30 sec at 72°C, 30 cycles of 30 sec at 94°C, 30 sec at 60.5°C and 30 sec at 72°C, finally 5 min at 72°C. PCR products were separated on standard agarose gels and visualized with ethidium bromide staining. The marker SPUD1636 was amplified as described (Bryan et al. 2002). The BA213c14t7 (snp139 and snp 274) primers were as followed f-CAATTGATTCATTTTATGTAGCGAG, r-TCTTGACGCAAACCTCTGCGAG, the amplicon was sequenced with the following primer: fn-AATATAAGATATAACTAAATTAAC.

Results

Evaluation of potato varieties for markers HC and SPUD 1636

Thirty three varieties with resistance to *G. pallida* pathotypes Pa2 or Pa2/Pa3, the differential *S. vernei* clone 62-33-3 (Pa2) (Ross 1986), AM 78.3778 (Pa2/3) (Rouppe van der Voort et al.1998) and 21 susceptible varieties as control group were tested for the presence of the HC marker. With the exception of cv 'Karakter', all varieties with high resistance to *G. pallida* pathotype Pa2/3 and clone AM78.3778 were HC positive. The six

varieties with resistance to pathotype Pa2 only, the variety 'Sante' with resistance to pathotypes Pa1 and Pa2, the *S. vernei* clone 62-33-3 and all susceptible varieties were HC negative. From five varieties with reported intermediate or partial resistance to *G. pallida* (Amado, Kantara, Kardent, Katinka and Pallina), two were HC positive and three were negative (Table 3.1). In addition the set of potato varieties and *S. vernei* differential clones were tested for presence or absence of the diagnostic SPUD1636 marker, which is linked to a major QRL to *G. pallida* in the same region on potato chromosome V (Bryan et al. 2002). All susceptible varieties tested lacked the SPUD1636 marker, but SPUD1636 was present in only 12 of the 22 highly resistant varieties, in 8 of the 12 varieties with incomplete resistance, and in both *S. vernei* differential clones (Table 3.1).

Table 3.1 Population test of markers HC, BA213c14t7-snp139, BA213c14t7-snp274 and SPUD1636 (Bryan et al. 2002) in varieties susceptible or resistant to *G. pallida* according to passport data.

Variety or clone	Breeder	Resistance to *G. pallida* pathotypes[1]	HC	snp274	snp139	SPUD 1636
Angela	BNA	Susceptible	0	AAAA	GGGG	0
Arkula	NORIKA	Susceptible	0	AAAA	GGGG	0
Assia	Uniplanta	Susceptible	0	AAAA	GGGG	0
Christa	KWS Saat (N: Ragis)	Susceptible	0	AAAA	GGGG	0
Clarissa	BNA	Susceptible	0	AAAA	GGAA	0
Desiree	Lange, W.	Susceptible	0	AAAA	GGGG	0
Gloria	Saatzucht Soltau- Bergen	Susceptible	0	AAAA	GGGG	0
Grata	Stader Saatzucht	Susceptible	0	AAAA	GGGG	0
Hela	Vereinigte Saatzuchten	Susceptible	0	AAAA	GGGG	0
Karat	NORIKA	Susceptible	0	AAAA	GGAA	0
Karlena	NORIKA	Susceptible	0	AAAA	GGGG	0
Koretta	NORIKA	Susceptible	0	AAAA	GGGG	0
Lyra	BNA	Susceptible	0	AAAA	GGGG	0
Maxilla	NORIKA	Susceptible	0	-	-	0
Milva	Saatzucht Berding	Susceptible	0	AAAA	GGGG	nt
Nora	BNA	Susceptible	0	AAAA	GGGG	0
Selma	Bavaria Saat	Susceptible	0	AAAA	AAAA	0
Tempora	BNA	Susceptible	0	AAAA	GGAA	0
Toccata	BNA	Susceptible	0	AAAA	GGAA	0
Tomensa	BNA	Susceptible	0	AAAA	GGGG	0
Ute	Bavaria Saat	Susceptible	0	AAAA	GGGG	0
Avano	Karna	Pa2/3	1	TTTT	AAAA	0
Avarna	Karna	Pa2/3	1	TTTT	AAAA	1
Aveka	Karna	Pa2/3	1	AATT	GGAA	1
Averia	Karna	Pa2/3	1	TTTT	AAAA	1
Aviala	Karna	Pa2/3	1	TTTT	AAAA	1
Brisant	Bavaris Saat	Pa2/3	1	AATT	GGAA	0

Variety or clone	Breeder	Resistance to *G. pallida* pathotypes[1]	HC	snp274	snp139	SPUD 1636
Festien	E.J. Feunekes	Pa2/3	1	AAAA	AAAA	1
Florijn	Hoiting	Pa2/3	1	ATTT	GGAA	0
Goya	J. Goosen	Pa2/3	1	ATTT	GAAA	0
Innovator	HZPC	Pa2/3	1	AAAT	AAAA	0
Karakter	Averis Saatzucht	Pa2/3	1/0 [2]	AAAT	GGGG	0
Kartel	Karna	Pa2/3	1	ATTT	GAAA	1
Melanie	H.K.Kroeze & G.M. Bunte	Pa2/3	1	AATT	GGAA	1
Menco	J.H. Mencke	Pa2/3	1	AATT	GGAA	1
Mercator	J.H. Mencke	Pa2/3	1	AATT	GAAA	0
Mercury	J.H. Mencke	Pa2/3	1	AATT	GGAA	0
Nomade	Matschaap Boerhave VOF	Pa2/3	1	AATT	GGAA	0
Seresta	R.H. Sloots	Pa2/3	1	ATTT	GAAA	1
Siamero	Agrico	Pa2/3	1	AATT	GGAA	1
Stabilo	R.H. Sloots	Pa2/3	1	TTTT	AAAA	1
Starga	R.H. Sloots	Pa2/3	1	AATT	GGAA	1
Valiant	H. Kuipers	Pa2/3	1	TTTT	GAAA	1
AM78.3778		Pa2/3	1	-	-	1
Elles	B.F. Leestemaker & A. Smid	Pa2	0	AAAA	GGGG	1
Feska	E.J. Feunekes	Pa2	0	AAAA	AAAA	0
Karida	Karna	Pa2	0	AAAA	GGGG	0
Karnico	Karna	Pa2	0	AAAA	GGGG	1
Producent	Kweekbedrijf Prummel	Pa2	0	AAAA	GGGG	0
S.vernei 62-33-3		Pa2	0	-	-	1
Sante	J. Vegter	Pa1/2	0	AAAA	GGGG	1
Amado	BNA	Pa2/3 partial	1	AATT	GGAA	0
Kantara	Karna	Pa2/3 partial	0	-	-	1
Kardent	Karna	Pa2/3 partial	1	AATT	GGGA	1
Katinka	Karna	Pa2/3 partial	0	AAAA	AAAA	1
Pallina	Bavaris Saat	Pa2/3 partial	0	AAAA	GGGG	1

[1]Resistance according to '81e rassenlijst Landbouwgewassen 2006' and 'Beschreibende Sortenliste Kartoffeln 2005'.
[2] This variety gave inconsistent results for the HC marker in repeated tests.
nt: not tested

Evaluation of the diagnostic value of the HC marker

In an earlier study, a group of SNP markers linked to the resistance QTL were co-segregating and found to be located on the same haplotype (Sattarzadeh et al. 2006). The group included, snp139 and snp274 in amplicon BA213c14t7, and one SNP used to develop the HC marker, BA87d17t3 snp212 (Table 3.2). In order to determine the diagnostic value of the HC marker, the 54 varieties were scored additionally for the markers BA213c14t7 snp139 and snp274 by amplicon sequencing (Table 3.1). The haplotype *c* specific allele *T* of snp274 was in nearly complete linkage disequilibrium with the HC marker, the only exception being cv 'Festien' (Table 3.1). Based on the dosage of

snp274 allele *T*, the *G. pallida* resistant varieties were mostly duplex, triplex or even quadruplex for the resistance allele. In contrast, the haplotype *c* specific allele *A* of snp139 was less diagnostic, as it occurred in all varieties with resistance to *G. pallida* pathotype Pa2/3, but also in five susceptible varieties and in cv 'Feska' being resistant only to pathotype Pa2 (Table 3.1).

Table 3.2 Haplotype model deduced from the observed segregation of three SNP markers linked to QTL for nematode resistance on chromosome V (after Sattarzadeh et al. 2006). Haplotype specific nucleotides are shown in bold letters.

Position ►	BA87d17t3	BA213c14t7	
Haplotype▼	snp212	snp274	snp139
a	C	A	G
b	C	A	G
c	**T**	**T**	**A**

Tracking the origin of the HC marker allele

Initially the specificity of the HC marker was assessed in 76 accessions of 31 different wild *Solanum* species and the results clearly showed the presence of HC exclusively in *Solanum vernei* (data not shown). Further we were interested in the spread of the introgression within *S. vernei* and therefore tested the presence or absence of the marker in a total of six different accessions (Table 3.3). Only genotypes of accessions BGRC 17542 and BGRC 24732 were found positive for the marker and within these two accessions not all genotypes were HC positive.

Table 3.3 Test range of *Solanum vernei* accessions.

CGN[1]	BGRC[2]	Origin in Argentina	Total number of plants	HC positive plants
18110	24729	?	27	0
21315	8241	Province Tucuman	32	0
17836	15451	Province Jujuy	33	0
17995	17542	Province Tucuman	32	31
18111	24732	Province Tucuman	37	35
17992	17536	Province Jujuy	33	0

[1]Centre for Genetic Resources, the Netherlands, www.cgn.wur.nl
[2]Braunschweig Genetic Resource Center

Discussion

The results of our study show that SPUD1636 was less diagnostic for high resistance to *G. pallida* pathotype Pa2/3 than the HC marker, BA213c14t7 snp139 and snp274. Physically snp274 is closer to HC than snp139 (Table 3.1). The differences in the diagnostic values of the two markers infer a possible decrease of the QTL between those two positions. To follow up on this hypothesis a fine mapping experiment of this QTL was carried out (Chapter 4).

The HC marker is the most diagnostic DNA marker currently available for high resistance to *G. pallida*. It was detected in almost all tested german or dutch potato varieties with high resistance levels to *G. pallida* pathotype Pa2/3 and most probably derives from the wild relative *S. vernei.* The resistance allele of *S. vernei* detected by the HC marker on potato chromosome V was named *RGp5-vrnHC* because its position is identical or very similar to the major cyst nematode QRL *Grp1* and *Gpa5* described previously (Rouppe van der Voort et al.1998, 2000; Sattarzadeh et al. 2006).

The present results indicate that the allele *RGp5-vrnHC* has been widely used in resistance breeding in Germany and the Netherlands. The origin of this allele is unknown, as several *S. vernei* clones have been described as resistance sources (Ross 1986), which are not available any more for testing. Also parental lines of the varieties tested were not available for this analysis. At this point, we can only exclude as a source clone *S. vernei* 62-33-3, which is resistant to *G. pallida* pathotype Pa2 (Ross 1986) and was accordingly HC negative. On the other hand we were able to confirm the presence of the introgression in hybrid clone AM 78.3778, which is related by descent to *S. vernei*. In the future *S. vernei* accessions available in germ plasm collections can easily be screened by PCR with the HC marker.

Some varieties with incomplete resistance such as ‘Amado’ and ‘Kardent’ were HC positive. This can result from incomplete dominance of the *RGp5-vrnHC* allele in certain genetic backgrounds or by using *G. pallida* populations which partially overcome the resistance allele *RGp5-vrnHC* in the resistance assessment (Sattarzadeh et al. 2006).

Many experiments have been performed to track and characterize the introgressions from *S. vernei* in modern breeding material (Jacobs et al. 1996), and to assess the potential for future breeding programmes. In this study we were able to show the diagnostic value of the HC marker to detect varieties with high resistance to *G. pallida* Pa2/Pa3 and to show that

the resistance allele from *S. vernei* detected by the HC marker on potato chromosome V is present in two out of six accessions.

Similarly to this Gebhardt et al. (2004) were able to confirm that marker alleles associated with increased resistance to late blight and late plant maturity originated from an introgression from the wild species *S. demissum*.

For the development of molecular markers through linkage and association studies, the investigation of the materials held in potato collections, such as the Commonwealth Potato Collection or the IPK Potato Collection at Groß Luesewitz (GLKS), is a feasible starting point.

The two accessions positive for the HC marker originate from the same province in Argentina, namely Tucuman. This may be by chance alone or it could be an indication that some accessions of the *S. vernei* population in this area have evolved high levels of resistance due to strong selection pressure from the nematode.

The results of this study also reveal that the pedigree information available for potato cultivars is misleading in the sense that it does not clearly name the origin of resistance to *G. pallida*. The clone VTn 62-33-3 was regarded as the origin of Pa2/Pa3 resistance according to pedigree information (Figure 3.1) despite the fact that it is only resistant to Pa2 (Kort et al. 1977). Due to the lack of the HC marker fragment in the PCR assay we could prove that this clone was not the source of resistance.

Conclusions

The present study intriguingly demonstrates the power and diverse application of DNA based molecular markers. The HC marker assay allows a specific selection of individuals resistant to *G. pallida*. The use of DNA markers opposed to phenotypic assays, which often take several years, reduces cost and time and is a much more accurate method to accomplish breeding goals. PCR-based marker assays for the detection of favourable traits will help improving the characteristics of modern potato varieties (see also Chapter 2). This strategy is called marker assisted selection (MAS).

Chapter 4

Fine mapping of a quantitative trait locus (QTL) for resistance to *Globodera pallida* in tetraploid potato (*Solanum tuberosum*) based on population genetics

Abstract

Potato cultivars consist mainly of *S. tuberosum* ssp. *tuberosum* clones harbouring few introgressions from wild *Solanum* species, which have been repeatedly crossed with each other. To fine map a quantitative trait locus (QTL) for resistance to *G. pallida*, a collection of 38 susceptible and 41 resistant tetraploid breeding clones and varieties related by descent were genotyped using Single Nucleotide Polymorphisms (SNPs). SNPs were identified at eight loci within the interval between markers *GP21-GP179* known to flank the QTL and at two loci outside this interval on chromosome V. Based on the SNP data, linkage disequilibrium (LD) and marker-trait associations were analyzed. Marker-trait associations were identified over a genetic distance > 2 cM due to the LD that exists in this region. We were able to delimit the region harbouring the QTL between SNP markers *GP21_SNP1257* and *BA213c14_SNP274* by the deduction of haplotype(s) associated with the resistant phenotype.

Introduction

A major yield limiting factor in potato cultivation is the potato cyst nematode, *Globodera pallida* (Greco 1988; Marks RJ and Brodie BB 1998). Under poor nematode management, crop losses induced by *G. pallida* can range from 20 to 70%. The propagation of nematode resistant crops could counteract this problem and therefore breeding for resistance against *G. pallida* is one of the major goals of breeding companies and research laboratories working with the potato crop. Breeding for superior varieties could be facilitated by the development of molecular markers, which detect favourable alleles at an early stage and thus allow for a precise and fast selection of good breeding material. A PCR-based marker (HC marker) diagnostic for potato varieties with high resistance to *G. pallida* pathotypes Pa2/3 was developed based on Single Nucleotide Polymorphisms (SNPs) (Sattarzadeh et al. 2006). The HC marker is located on chromosome V in a hot spot for resistance to various pathogens between RFLP markers *GP21* and *GP179* (Leonards-Schippers et al. 1992, 1994; Caromel et al. 2005; Kreike et al. 1994; Rouppe van der Voort et al. 1997, 2000; Ritter et al. 1991; de Jong et al. 1997) and tags a major quantitative resistance locus (QRL) against the nematode. This interval was shown to have a genetic size of 3 cM (Meksem et al. 1995).

Understanding the mechanisms of resistance requires the cloning and understanding of genes involved in the characterization of these traits. Fine mapping of the QTL is a first step towards the ultimate goal of identifying the underlying gene or genes. The strength of LD in a region can be inferred by the amount of association between single nucleotide polymorphism (SNP) markers. Linkage disequilibrium (LD) is the term used for a non-random association of alleles at two or more loci (Tenesa et al. 2003). If two loci are in LD some combinations of alleles occur more or less frequently in a population than would be expected from a random formation of the haplotypes. The potential of association mapping depends on the physical size of LD.

Haplotypes are particular combinations of alleles observed in a population. When a mutation arises, it does so on a specific chromosomal haplotype (Gabriel et al. 2002). Therefore association between a mutant allele and its ancestral haplotype is only disrupted by mutation and recombination in subsequent generations and it should be possible to trace each variant allele in the population by identifying the particular ancestral segment from

which it arose. However, LD does not necessarily require a physical connection on a chromosome resulting in one haplotype; therefore it is not possible to unambiguously deduce the number of haplotypes solely from LD information.

Another caveat using population genetics is the occurrence of population structure, which can result in "spurious associations" between a phenotype and markers that are not linked to any causative locus (Lander and Schork 1994). Such associations occur when the presence of a trait varies across subpopulations, thereby increasing the probability that individuals displaying the trait will be sampled from particular subpopulations. Any marker allele that is in high frequency in the overrepresented subpopulations will then be associated with the trait (Pritchard et al. 2000).

Combining information about marker-trait associations and LD structure we acquired information about the observed region harbouring the QTL. In addition we determined haplotype(s) associated with the resistant phenotype. As a result we could map the QTL to a genetic interval of 2 cM, which equals a physical distance between 500-1000 kbp (Chapter 5).

Materials and Methods

Plant material: Seventy-nine varieties and breeding clones were obtained from Saka-Ragis Pflanzenzucht, Böhm-Nordkartoffel Agrarproduktion (BNA), Bavaria Saat (by courtesy of A. von Zwehl), HZPC Holland B.V., Averis Seeds B.V., Karna, E.J. Feunekes, Kweekbedrijf Prummerl and B.F. Leestemaker & A. Smid (by courtesy of Jan Draaistra). The breeding clones obtained from Saka-Ragis Pflanzenzucht and Böhm-Nordkartoffel Agrarproduktion (BNA) were encoded with a letter ('S' for Saka- Ragis and 'B' for Böhm-Nordkartoffel) and a number.

Assessment of resistance to G. pallida: previously described in chapter 2.
The status of nematode resistance of the varieties was obtained from the variety lists of the Netherlands (81e rassenlijst Landbouwgewassen, 2006, ISSN 0168-7484) and Germany (Beschreibende Sortenliste Kartoffeln, 2005, ISSN 1430-9777).

Table 4.1 Population with presence or absence of the HC marker and the corresponding breeding company.

	HC marker positive (resistant)		HC marker negative (susceptible)	
Number	Genotype	Breeder	Genotype	Breeder
1	Avano	Karna	B01	BNA
2	Avarna	Karna	B02	BNA
3	Averia	Karna	B03	BNA
4	Aviala	Karna	B04	BNA
5	B23	BNA	B05	BNA
6	B24	BNA	B06	BNA
7	B25	BNA	B07	BNA
8	B37	BNA	B08	BNA
9	B39	BNA	B09	BNA
10	B41	BNA	B10	BNA
11	B42	BNA	B11	BNA
12	B43	BNA	B12	BNA
13	B44	BNA	B13	BNA
14	B47	BNA	B14	BNA
15	B48	BNA	B15	BNA
16	B50	BNA	B16	BNA
17	B51	BNA	B17	BNA
18	B52	BNA	B18	BNA

	HC marker positive (resistant)		HC marker negative (susceptible)	
Number	Genotype	Breeder	Genotype	Breeder
19	B55	BNA	B19	BNA
20	B80	BNA	B31	BNA
21	B96	BNA	B62	BNA
22	B108	BNA	Elles	B.F. Leestemaker & A. Smid
23	Brisant	Bavaris Saat	Kantara	Karna
24	Festien	E.J. Feunekes	Karida	Karna
25	Innovator	HZPC	Karnico	Karna
26	Karakter	Averis Saatzucht	Pallina	Bavaris Saat
27	S02	Saka-Ragis	Producent	Kweekbedrijf Prummel
28	S03	Saka-Ragis	S12	Saka-Ragis
29	S07	Saka-Ragis	S13	Saka-Ragis
30	S08	Saka-Ragis	S14	Saka-Ragis
31	S09	Saka-Ragis	S15	Saka-Ragis
32	S28	Saka-Ragis	S16	Saka-Ragis
33	S50	Saka-Ragis	S17	Saka-Ragis
34	S61	Saka-Ragis	S18	Saka-Ragis
35	S73	Saka-Ragis	S19	Saka-Ragis
36	S76	Saka-Ragis	S20	Saka-Ragis
37	S83	Saka-Ragis	S49	Saka-Ragis
38	S101	Saka-Ragis	S64	Saka-Ragis
39	S105	Saka-Ragis		
40	S108	Saka-Ragis		
41	Siamero	Agrico		

DNA isolation: Young, healthy potato leaves were harvested from each individual, freeze dried and stored in air-tight containers at -20°C. Total genomic DNA was extracted from 0.3 g – 0.4 g freeze dried leaf material according to Bormann et al. (2004).

Concentration measurement of DNA: previously described in chapter 2.

Genotyping of the population

Standard PCR reaction: previously described in chapter 2.

Purification of PCR products for sequencing: previously described in chapter 2.

Sequencing of PCR amplicons: All markers with the exception of *GP179* were sequenced as previously described in chapter 2. Sequence information which inferred a recombination

event (individuals S09, S28, and S61) used for the deduction of the haplotype(s) were sequenced forward and reverse.

Detection of single nucleotide polymorphisms (SNPs): previously described in chapter 2.

Microsatellite Analysis: Nineteen SSR-markers (Feingold et al. 2005; Milbourne et al. 1998) distributed on 11 chromosomes were scored for 2-7 different alleles and employed to analyze population structure (Figure 4.1, Table 4.2). See chapter 2 for more details on the methods.

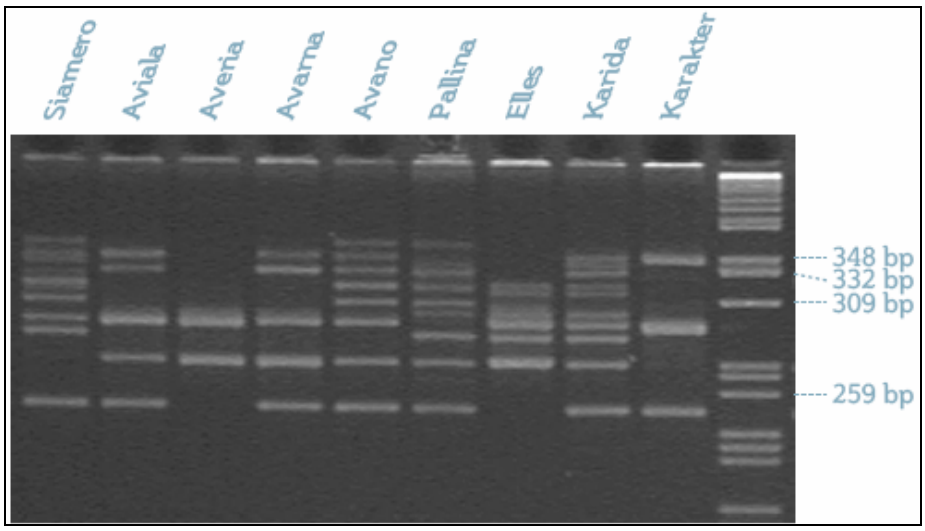

Figure 4.1 SSR loci StI013 (Feingold et al. 2005) separated on Spreadex Gel for nine different varieties, right lane size marker M1 (Elchrom Scientific, Cham, Switzerland).

Assessment of population structure: Population structure was analyzed using the program STRUCTURE version 2.1 (Pritchard et al. 2000).

Table 4.2 SSR markers scored in the BNA and SARA population to assess population structure.

SSR marker	Primer sequence 5'-3'	TA°C	Size range (bp)	LG
StI 004	f-gct gct aaa cac tca agc aga a r-caa cta caa gat tcc atc cac ag	60 – 54*	78 - 112	VI
StI 013	f-cca ctt cct cca ctt cca aa r-cca tgg ttg cac caa cta ga	60 – 54*	240 -340	III
StI 020	f-gac gca gaa ctc atc ttg ttc a r-gca aaa ttt gaa aaa cta tgg atg	60	106 - 127	IV
StI 022	f-tct cca att act tga tgg acc c r-caa tgc cat aca cgt ggc ta	63	114 - 145	VIII

SSR marker	Primer sequence 5'-3'	TA°C	Size range (bp)	LG
StI 023	f-gcg aat gac agg aca aga gg r-tgc cac tgc tac cat aac ca	60 – 54*	160 - 280	X
StI 024	f-cgc cat tct ctc aga tca ctc r-gct gca gca gtt gtt gtt gt	60 – 54*	149 - 186	II
StI 028	f-ata ccc tcc aat ggg tcc tt r-ctt gga gat ttg caa gaa gaa	60	170 - 217	XI
StI 043	f-caa tgc gaa tgt tgc tac tgg t r-atc cac caa gac ctc cag aa	60 – 54*	127 - 140	I
StI 047	f-act gct gtg gtt ggc gtc r-acg gca tag att tgg aag cat c	60 – 54*	128 - 170	VIII
StI 058	f-caa gca cgt tac aac aag caa r-ttg aag cat cac ata cac aaa ca	60 – 54*	77 - 103	V
STM 0037	f-aat tta act tag aag att agt ctc r-att tgg ttg ggt atg ata	48	75 - 90	XI
STM 0038	f-aac tct agc agt att tgc ttc a r-tta ttt agc gtc aaa tgc ata	54	108	II
STM 1043	f-att tga att gaa gaa ctt aat aga a r-cac aaa caa aat act gtt aac tca	53	226	VII
STM 1052	f-caa ttt cgt ttt ttc atg tga cac r-atg gcg taa ttt gat tta ata cgt aa	59	200 - 268	
STM 1097	f-tga ttt agt tgc ttg ttt g r-gct ttc gat cct aat aca cc	54	90 - 160	VII
STM 1104	f-tga ttc tct tgc cta ctg taa tcg r-caa agt ggt gtg aag ctg tga	57	164 - 185	VII
STM 1106	f-tcc agc tga ttg gtt agg ttg r-atg cga atc tac tcg tca tgg	60	150 - 200	X
STM 2012	f-gcg gcc gct tct cag cca a r-tct cgt tca atc cac cag atc	64	247	X
STM 3012	f-caa ctc aaa cca gaa ggc aaa r-gag aaa tgg gca caa aaa aca	57	168 - 213	IX

*Touch down PCR; StI markers: Feingold et al. 2005; STM markers: Milbourne et al. 1998

Pyrosequencing

Pyrosequencing was performed to analyze marker *GP179* according to the manufacturer's instructions (Biotage AB, Uppsala, Sweden).

All oligonucleotides were synthesized and HPLC-purified by SIGMA-Genosys, Germany.

PCR products were produced using the primer pairs GP179r1pyro-biotin, 5′-(biotin) CCC ATG GCT ACA TGC ATT ATG TAT T -3′; and GP179f1pyro, 5′- TCC TCC TTT GAA ATA TGT TTC ACT TCT A -3′ .The primer pair produced a 217-bp fragment.

PCR conditions: PCR was carried out in a total volume of 25 µl containing 4 mM Tris–HCl, pH 8.3, 20 mM KCl, 3 mM $MgCl_2$, 0.5 µM each primer, 0.2 mM dNTPs, 1 unit *Taq* DNA Polymerase (Invitrogen Life Technologies, Freiburg, Germany). After initial denaturation at 94 °C for 2 min, 50 cycles of amplification were carried out starting at 93 °C for 45 s, followed by 45 s at 57 °C and 1 min at 72 °C, with a final extension at 75 °C for 10 min. PCR products were quantified by 1.5% gel electrophoresis.

Solid-phase template preparation for single-stranded DNA template: Fifteen microliters of biotinylated PCR product was immobilized onto 5 µl streptavidin-coated Super Paramagnetic beads (Streptavidin Sepharose™ High Performance, Amersham Biosciences, Sweden) in Binding buffer (10 mM Tris–HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20; pH 7.6) for 15 min while on a shaker. The Streptavidinbead–template complex was captured using a PSQ96 Sample Prep tool (Biotage AB, Uppsala, Sweden) and single-stranded template was generated by washing in 0.5 M NaOH for 1 min followed by washing in 100 µl washing buffer (20 mM Tris acetate, and 5 mM Mg acetate). One microliters of the sequencing primer (0.25 mM) was annealed to the immobilized template in 40 µl of annealing buffer and heated at 80 °C for 2 min followed by slow cooling to room temperature.

Pyrosequencing: The sequencing reaction was performed automatically with a PSQ 96 system (Biotage AB, Uppsala, Sweden) using a SNP reagent kit according to the manufacturer's instructions and the sequencing primer GP179seq1, 5′-ACTTCTAAGTGATAATCTTGA -3′.

Pyrosequencing was performed at 28 °C in a volume of 40 µl on an automated PSQ96 instrument (Biotage AB, Uppsala, Sweden). A four-enzyme mixture of DNA polymerase, ATP sulfurase, firefly luciferase, and nucleotide-degrading apyrase; the substrate luciferin; and the four separate deoxynucleoside triphosphates were loaded into the reagent cartridge (PSQ96 SNP Reagent Kit; Biotage AB, Uppsala, Sweden). The sequencing procedure was carried out by a stepwise elongation of the primer strand upon sequential addition of the different deoxynucleoside triphosphates and simultaneous degradation of nucleotides by apyrase.

Statistical analysis

Marker-trait association: Detecting a significant association ($p < 0.01$) between resistance and SNP markers was accomplished with the Chi-square test of independence using SPSS (SPSS 13 for Windows, SPSS Inc. Chicago, IL, USA).

Linkage Disequilibrium: The statistical analysis of LD was carried out in collaboration with Dr. Joao Paulo. The amount of LD among SNPs was calculated using the Exact Test available in SAS Proc Freq (SAS Institute Inc. 2004), performed on contingency tables. The tests of independence were based on genotype frequencies for each SNP marker combination. Genotype classes were defined upon the observed combinations of 4 nucleotides (corresponding to the 4 homologous chromosomes) to form the contingency table (Table 4.3).

Table 4.3 Contingency table to test association of two loci.

locus 2 ► ▼locus 1	TTTT	TTTC	TTCC	total no. genotypes
CCCC	6	1	2	9
CCCT	9	4	1	14
CCTT	9	4	2	15
CTTT	13	2	0	15
total no. genotypes	37	11	5	53

Results

Association analysis using the HC marker: The molecular HC marker linked to the resistance QTL was used to screen tetraploid individuals to define the sample population. 41 HC positive genotypes displaying high resistance to *G. pallida* and 38 HC negative genotypes susceptible to the nematode were used.

SNPs were identified at eight loci including the interval markers *GP21-GP179* known to flank the QTL and at two loci outside this interval on chromosome V. The loci involved are spanning a genetic interval of 38 cM on an integrated genetic map (Meksem et al. 1995; Meyer et al. 2005). SNPs of markers *BA87d17t3* and *BA213c14t7* were successfully tested for resistance to *G. pallida* in prior linkage investigations (Sattarzadeh 2003).

A total of 151 SNPs were scored. 55 SNPs, located within and between markers *GP21* and *BA213c14t7*, were significantly associated with resistance to *G. pallida* (Table 4.4).

Table 4.4 Markers used in this study, number of SNPs and number of significantly associated SNPs. Association was assessed with the Chi-square test at $p < 0.01$.

Marker name	Total SNPs	Associated SNPs
GP21	18	13
Hypothetical protein (*ORF3*)	33	17
Hypothetical protein (*ORF12*)	8	1
ATPase (*ORF20*)	24	5
BA87d17t3	12	6
BA213c14t7	21	13
Protein kinase	9	-
GP179	5	-
StPto	14	-
239E4left	10	-

LD between SNP markers: Information about the genetic structure in this region was obtained through LD analysis. The q-values of the pairwise associations are shown in figure 4.2. SNPs are plotted on both axes (SNP data from *ORF3* is missing in this analysis due to technical constraints). The associations which were significant at a $q < 0.05$ are indicated in blue colour. Patterns of LD can be observed, regions with strong LD

surrounded by regions with weak LD. Strong LD persists mainly in the 3 cM region between RFLP markers *GP21* and *GP179*. Associations reveal the presence of blocks with strong LD, mainly around the diagonal. This indicates a high degree of LD among SNPs within the sequenced amplicons (500-600 bp). In general LD becomes less pronounced as the distance between SNPs increases (Figure 4.2). LD decreases between markers *GP179* and *StPto* and remains weak regarding the most distal marker *239E4left*.

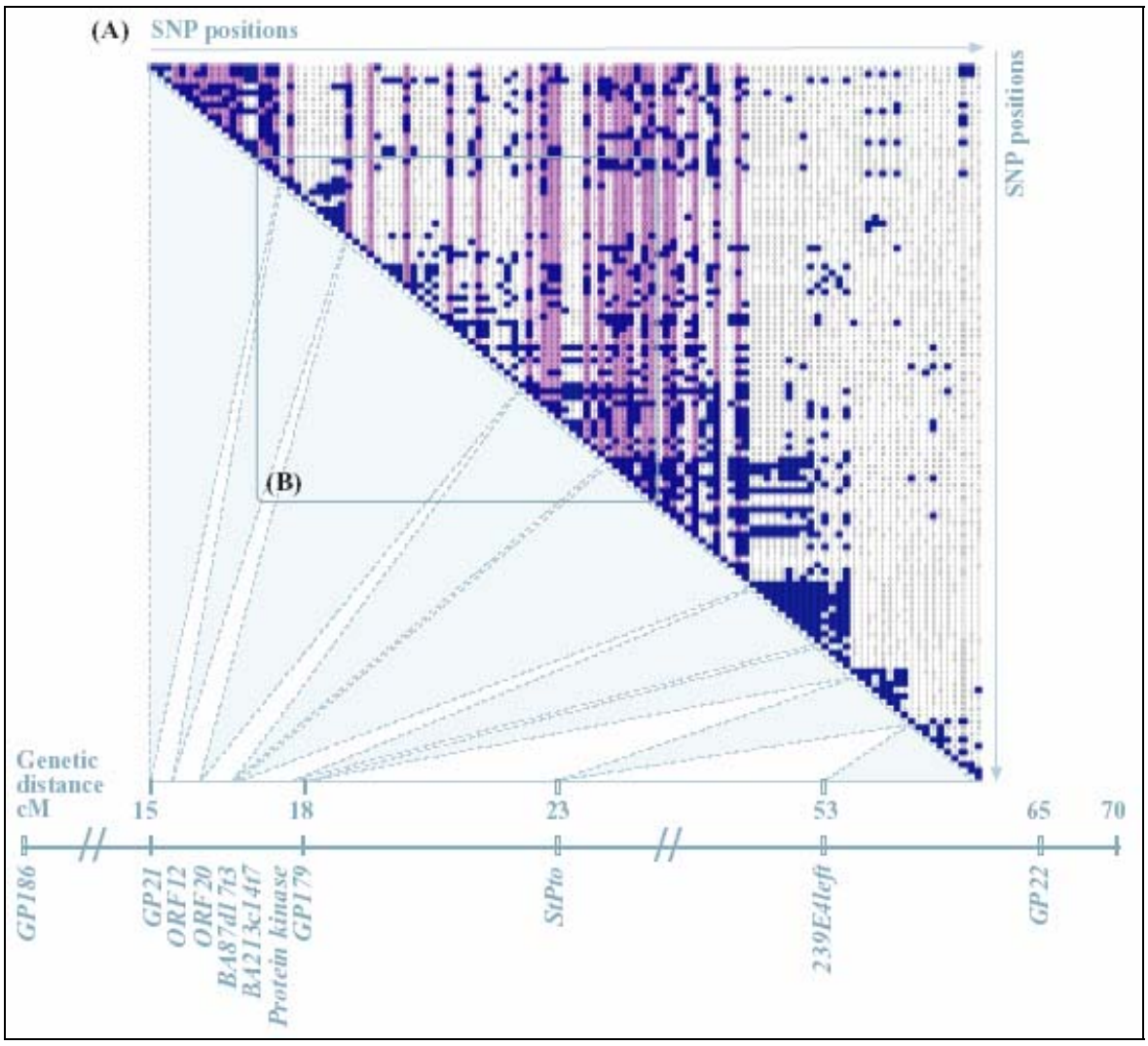

Figure 4.2 Scheme showing the linkage disequilibrium matrix and the position of the SNP markers on the genetic map. **(A)** Linkage disequilibrium matrix based on SNPdata; SNP positions are plotted on both axes. Associations which were significant at a q-value < 0.05 are indicated in blue colour. Columns highlighted in violet mark SNP positions which were associated with resistance. **(B)** Refined region of the resistance QRL. The loci involved are spanning a genetic segment of 38 cM on an integrated genetic map from (Meksem et al. 1995) and mapping experiments summarized in the PoMaMo database (http://gabi.rzpd.de, Meyer et al. 2005).

Fine-mapping of the QTL

The HC marker was developed using allele-specific primers based on SNP loci *BA87d17t3_SNP212* and *BA87d17t3_SNP444* (Sattarzadeh et al. 2006). SNP markers associated with the HC marker (Figure 4.2, highlighted violet columns) were analyzed for the fine mapping of the QTL. For this we examined solely the SNP positions displaying nucleotides which clearly correlate with the more resistant phenotype, e.g. *ORF3_SNP142* (C/T), position 'C' is associated with the resistant individuals because only resistant individuals have a 'C' at this position. Table 4.5 gives an example for sixteen SNP positions for six resistant (1) and six susceptible (0) individuals. For these sixteen SNP positions, the nucleotides which are associated with resistance are displayed in the second row of table 4.5. The HC positive individuals S09 at position *GP21_SNP1257*, and S21 and S61 at position *BA213c14t7_SNP274* don't harbour the nucleotide associated with resistance (indicated with an asterisk). Consequently we propose that recombination events must have occurred resulting in *GP21_SNP1257* and *BA213c14t7_SNP274* to be the two most distal positions flanking the QTL.

Table 4.5 SNP data of associated, informative markers: (1) HC positive; (0) HC negative genotypes. The second row displays nucleotides present in the resistant haplotype(s); SNP data with asterisks (*) mark the positions which differ from the SNP data of the other resistant individuals

1	Pheno type	Geno type	GP21_ SNP 1090	GP21_ SNP 1165	GP21_ SNP 1170	GP21_ SNP 1257	ORF3_ SNP 92	ORF3_ SNP 142	ORF3_ SNP 332	ORF3_ SNP 337	ORF3_ SNP 371	ORF3_ SNP 419	ORF3_ SNP 497	ORF20 _ SNP 449	BA87d 17t3_ SNP 212	BA87d 17t3_ SNP 444	BA213c1 4t7_ SNP 274	BA213 c14t7_ SNP 139
2	Haplotype (s)		C	C	C	G	T	C	C	C	T	G	A	C	T	C	T	A
3	1	S08	AAAC	CGGG	CTTT	GTTT	CCCT	CTTT	AAAC	CGGG	AAAT	AAGG	ATTT	CCGG	CCCT	CCTT	AAAT	AGGG
4	1	S09	AAAA*	CCGG	CCTT	TTTT*	CCCT	CTTT	AAAC	CGGG	AAAT	AAGG	ATTT	CCCG	CTTT	CCCC	ATTT	AAAG
5	1	S28	AAAC	CCGG	CCTT	GTTT	CCCT	CTTT	AAAC	CGGG	AAAT	AAGG	ATTT	CCCC	CCTT	CCCC	AAAA*	AAAA
6	1	S61	AAAC	CGGG	CTTT	GTTT	CCCT	CTTT	AAAC	CGGG	AATT	AAGG	ATTT	CCGG	CCTT	CCCC	AAAA*	AAAA
7	1	B48	AAAC	CGGG	CTTT	GTTT	CCCT	CTTT	AAAC	CGGG	AAAT	AAAG	ATTT	CCGG	CCTT	CCTT	AATT	AAGG
8	1	S73	AAAC	CCGG	CCTT	GTTT	CCCT	CTTT	AAAC	CGGG	AAAT	AAGG	ATTT	CGGG	CCTT	CCTT	AAAT	AGGG
9	0	S14	AAAA	GGGG	TTTT	TTTT	CCCC	TTTT	AAAA	GGGG	AAAA	AAAA	TTTT	CCGG	CCTT	TTTT	AAAA	GGGG
10	0	S15	AAAA	GGGG	TTTT	TTTT	CCCC	TTTT	AAAA	GGGG	AAAA	AAAA	TTTT	CGGG	CCTT	CCTT	AAAA	GGGG
11	0	S16	AAAA	GGGG	TTTT	TTTT	CCCC	TTTT	AAAA	GGGG	AAAA	AAAA	TTTT	GGGG	CCCC	CTTT	AAAA	AGGG
12	0	S17	AAAA	CGGG	CTTT	TTTT	CCCC	TTTT	AAAA	GGGG	AAAA	AAAA	TTTT	GGGG	CCCC	TTTT	AAAA	GGGG
13	0	S18	AAAA	GGGG	TTTT	TTTT	CCCC	TTTT	AAAA	GGGG	AAAA	AAAA	TTTT	GGGG	CCCC	CCCC	AAAA	GGGG
14	0	S19	AAAA	GGGG	TTTT	TTTT	CCCC	TTTT	AAAA	GGGG	AAAA	AAAA	TTTT	GGGG	CCCC	CCCC	AAAA	GGGG

Table 4.6 Markers genotyped in this study.

Marker name	BAC clone	Primer sequence (5'- 3')	Amplicon size (bp)	TA (C°)
GP21	BC256o1	f-CCC TGA ACC TCC TTA TCC CC[4]	754	61
		r-ACT ATT ATG TCT ATG AGG AAG TGG TC[4]		
ORF3[1]	BA47f2	f-TTG CTT GAA CAT GAT CCA CAC[4]	750	59
		r-GCT TAG TTT CCT CTG GCA CC		
		fn-CGA GAA GAG GAA AAA GAT GTC		
		rn-GCT ATG AAC TTC ATC AAC ATG		
ORF12[1]	BA27c1	f-CGA TGA ATA TGA AGA TGA AGC[4]	426	57
		r-ACC TTT TTA GCT TTG ATA CGT TG[4]		
ORF20[1]	BA122p13	f-ACA AGT ACA GCT AAT AGA CCC[4]	560	55
		r-GGC TTA TCG TCT TCA CTA CC[4]		
BA87d17t3[1]	BA87d17	f-GTA GTA CAT CAA CAT ACA TTT TGC GG	510	56
		r-CTC AGA ATT CAG AGC TTC AAC TGA TG		
		fn-AAC AGG CTT AAT CCT CAT CCG C		
BA213c14t7[1]	BA213c14	f-CAA TTG ATT CAT TTT ATG TAG CGA G	650	56
		r-CT TGA CGC AAA CCT CTG CGA G		
Protein kinase	BA151m8[6]	f-ACT TGA AAT GAT AAC AGG TAG GAG	550	58
		r-CCC TTT TGC CAG TTC ATC C		
GP179[2,5]	BA151m8	f-TCC TCC TTT GAA ATA TGT TTC ACT TCT A	188	56
		r-(BIO[7])CCC ATG GCT ACA TGC ATT ATG TAT T		
		s-ACT TCT AAG TGA TAA TCT TGA		
239E4left[3]	239E4	f-GGC CCC ACA AAC AAG AAA AC	340	56
		r-AGG TAC CTC CAT CTC CAT TTT GTA AG		
StPto[2]	BC76f14	f-TCA CAT TGG ATT GGG TGG C[4]	670	55
		r-CGA GTC CAC TGC CCA TTC[4]		

(f) forward primer, (r) reverse primer, (fn, rn) nested primers used for the sequencing reaction
Primers were designed upon sequence information deduced from: [1]Ballvora et al. 2007; [2] PoMaMo database (Meyer et al. 2005);
Primers were designed from: [3]Bakker et al. 2004; [4] Ilarionova 2005;
[5] SNPs scored using pyrosequencing
[6] BAC was sequenced, accession number AY 730335
[7] biotinylated

Statistical analysis

Population structure:

Nineteen simple sequence repeats (SSRs) or microsatellite markers were used as underlying markers for random genotyping of the population.

The bar plot in figure 4.3 illustrates the distribution of fractions (vertical bar) adding to 100% (1.00) of each single individual (horizontal line), if 5 subpopulations were present. The observation of the distribution of individual genotypes over the subpopulations shows that all individuals are strongly admixed, containing loci originating from all the subpopulations. This suggests the absence of population substructure; hence the remaining analysis was performed as for a single population (Joao Paulo, personal comment).

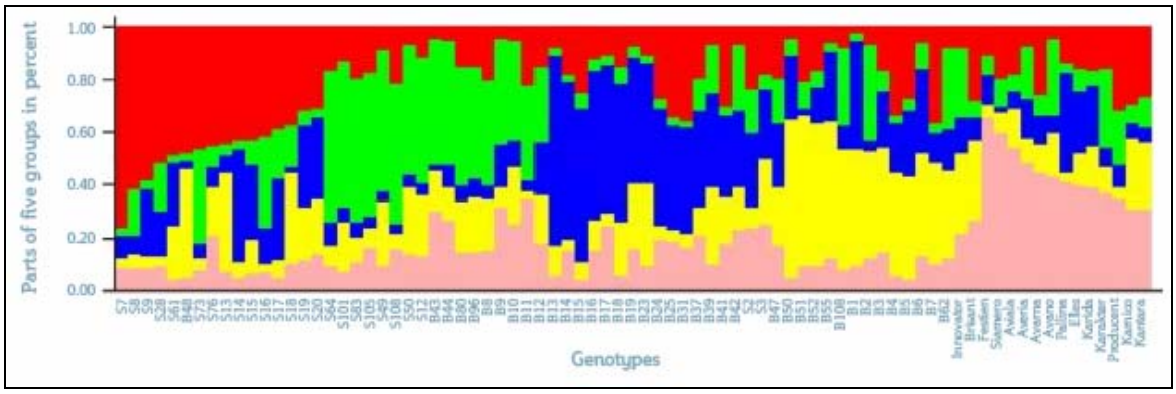

Figure 4.3 Bar plot. Each individual is represented by a single vertical line broken into 5 coloured segments. The numbers on the y-axis are the parts of the 5 groups in percent. All 79 individuals examined are plotted on the x-axis.

Discussion

Marker-trait associations, analysis of LD structure and haplotyping were used to increase the mapping resolution of the QTL for resistance to *G. pallida*.

The diagnostic value of the HC marker with high resistance to the nematode suggests that the trait can be traced back to an allele or a few alleles at one single locus which was introgressed from *Solanum vernei* (Chapter 3). When genotyping numerous markers in the vicinity of this PCR marker, markers at a genetic distance of 2 cM corresponding to a physical distance between 500 and 1000 kb (Chapter 5) were found associated with the HC marker. This suggests that several alleles or haplotypes in this region might be physically linked. Apparently, since the time point of the introgression of the allele(s), insufficient recombination events have occurred to reduce linkage in this region. The assessment of LD in this region confirmed this assumption. The LD matrix (Figure 4.2) shows the LD between polymorphic sites of SNP marker loci along chromosome V. For a more comprehensive picture of LD in this section on chromosome V, more SNP data is needed between markers *GP179* and *239E4left*.

Haplotypes are defined as a set of closely linked genetic markers present on one chromosome which tend to be inherited together. The potato population used in this study comprised of tetraploid breeding clones and varieties, which are separated by a few generations according to information in the 'Potato Pedigree database' (Hutten & van Berloo 2004). Therefore a relatively small number of haplotypes may be present reflected by the overall strong LD. We were able to assign haplotypes to the resistant phenotype. The number of haplotypes cannot be defined because several haplotypes could be present which are identical at the SNP positions we examined. Variation of SNP data in the susceptible group on the other hand could be explained by the fact that more diverse haplotypes which are not associated with resistance are present in the genome, which are not associated with resistance.

However divergences in SNP data of resistant individuals infer that a recombination event must have occurred separating the haplotype(s) present in the resistant group. A possible event is illustrated in figure 4.4. A part of chromosome V is shown, blue chromsomes represent *S. tuberosum* and the red chromosome originates from the donor of the resistant haplotype, *S. vernei*; the letters indicate the haplotypes which derived from table 4.5. A double recombination event could have occurred resulting in an introgression between marker loci *GP21_SNP1257* and *BA213c14t7_SNP274* harbouring the QTL conferring the resistance to *G. pallida.*

Scoring of additional SNPs in the vicinity of the HC marker are required to further narrow down the region of the QTL.

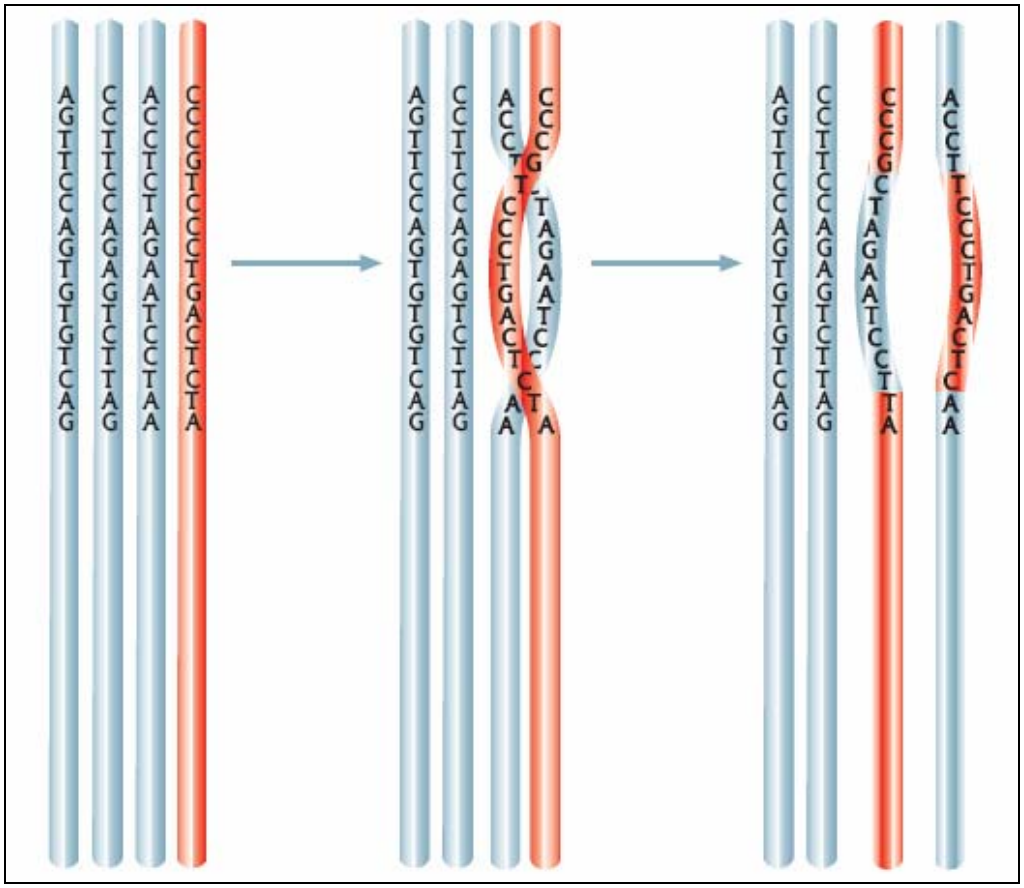

Figure 4.4 Scheme of a possible meiotic recombination event on chromosome V. Blue chromosomes originate from *S. tuberosum* and the red chromosome originates from *S. vernei*. The black letters are different haplotypes derived from the SNP data.

Regarding our results it appears that large segments of the chromosome were subjected to recombination, spanning a genetic region of > 3 cM (Figure 4.2). *Solanum vernei*, the species from which this QTL was originally introgressed, should be included in the analysis. A population segregating for this QTL could display enough polymorphism to facilitate the fine mapping. A similar approach was followed by Kaloshian et al. (1998) who combined data from the cultivated tomato (*L. esculentum*) and the wild relative (*L. peruvianum*) to localize the root-knot nematode resistance gene *Mi* to a region spanning less than 65 kb.

Many factors, such as population mating patterns and admixture, can strongly influence LD. Generally, LD decays more rapidly in outcrossing species as compared to selfing species (Nordborg et al. 2000). Apparently recombination is less effective in selfing species, where individuals are more likely to be homozygous, than in outcrossing species. Nevertheless, reduction in recombination at our interval could be correlated to the presence of an alien segment derived from the introgression of the wild relative *S. vernei*. Studies of the tomato clearly show the suppression of recombination depending on the source and size of the introgression (Liharska et al. 1996, Kaloshian et al. 1998). Admixture is gene flow between individuals of genetically distinct populations as result of intermating. It results in the introduction of chromosomes of different ancestry and allele frequencies. Often the resulting LD extends to unlinked sites, even on different chromosomes, but breaks down rapidly with random mating (Pritchard et al. 1999). This aspect could also explain a similar observation in our data where LD extends partly even as much as 38 cM. Moreover selection for or against a phenotype controlled by two unlinked loci (epistasis) may result in LD despite the fact that the loci are not physically linked (Flint-Garcia et al. 2003). However it should be noted that the appearance of LD between distant loci may also be due to statistical limitations or by chance alone.

A higher reduction in recombination could also be assigned to the underlying chromosome structure. Recombination in the heterocentromeric region is known to be suppressed as opposed to the eurchromatic regions. In order to exclude this as the prevailing reason for our observations a Fluorescent *in situ* experiment was carried out and the segment was clearly located in the euchromatic region (Chapter 5).

Linkage disequilibrium may vary between and within different species (Reich et al. 2001, Long et al. 1998). Sugarcane exhibits extensive long-range LD, approximately 10 cM

(Jannoo et al. 1999) which is comparable to potato chromosome V with a minimum of 3 cM between markers *GP21* and *GP179* (Figure 4.2). There are several explanations. Firstly sugarcane and the potato are both outcrossing mating types but are propagated in a vegetative manner restricting crosses solely to the breeding process of new varieties (Jannoo et al. 1999). The majority of modern sugarcane cultivars were derived from the interspecific cross between *Saccharum officinarum* and *S. spontaneum*, followed by multiple backcrosses to *S. officinarum*. The resulting cultivars generally originated from fewer than 10 meioses since the first interspecific cross. Similarly, modern potato varieties were derived from *Solanum tuberosum* ssp. *tuberosum* harbouring few introgressions from wild *Solanum* species producing 5-6 meiotic generations within the past 100 years (Gebhardt et al. 2004).

Simko et al. (2006) assessed LD in *Solanum tuberosum* and surveyed both coding and noncoding regions of 66 DNA fragments from 47 accessions for SNPs. The estimate from their data indicates that LD, measured as r^2, declines below 0.10 at a distance of ~10 cM but fragments show relatively fast decay of LD in the short range (r^2 = 0.208 at 1 kb). In the present study all loci are located on the same chromosome spanning a region of approximately 38 cM. As opposed to the results of Simko et al. (2006), our results show strong LD in the short range of the sequenced amplicon (500-600 bp). This divergence shows that LD is highly influenced by the origin of the population. Simko et al. (2006) used different accessions of diploid and tetraploid *S. tuberosum* together with accessions of other *Solanum species*. In the present study solely tetraploid breeding material was included and thus the larger extent of LD could be expected. Hyten (2005) made a similar observation when comparing four different soybean populations on level of LD decline. In the domesticated Asian *Glycine max* population LD did not decline along the 500 kb sequenced region but the wild *G. soja* population had large LD decline averaging 12 kb.

R1.3 as a candidate for a QTL conferring resistance to *G. pallida*

Ballvora et al. (2007) identified 48 ORFs on a 417 kb contig within our region of interest including plant-specific leucine-rich repeat proteins highly similar to the potato *R1* resistance gene for *Phytophthora infestans*. This results in an average of 1 ORF every 9 kbp. Accordingly we can expect about 55 to 111 ORFs in the 500 – 1000 kb region under investigation. The *RGp5-vrnHC* allele, detected with the HC marker, is defined through the

SNPs *BA87d17t3_SNP212* and *BA87d17t3_SNP444* (Sattarzadeh et al. 2006). These SNPs are located in the *R1* homologue *R1.3* displaying 74.7% identity to the *R1* gene. *R1.3* is a member of the leucine zipper/NBS/LRR class of plant resistance genes and it was shown to represent a putative functional resistance gene (Ballvora et al. 2007). It was felt that the diagnostic marker positioned on a putative resistance gene is strong circumstantial evidence for *R1.3* as a candidate for the resistance QTL. Currently transgenic knock-out plants for the entire *R1* resistance gene family are assessed for their resistance to *G. pallida* Pa2/3. For this, varieties highly resistant to *Globodera pallida* were transformed using *Agrobacterium* mediated transformation.

This experiment serves to identify a gene involved in the resistance. The analysis of resistance gene homologues as candidates at QTLs for resistance to plant diseases has been used in several earlier studies (Pfleger et al. 2001; Geffroy et al. 2000).

Statistical constraints

The basic component of all LD statistics is the difference between the observed and expected haplotype frequencies. With our data the calculation was limited due to the lack of information about the gametal phase of the SNPs. The calculation of association was based on genotype combinations, each genotype consisting of 4-nucleotides. There are software packages available able to analyze the LD for tetraploids (Buckler 2003; Schneider et al. 1997). However they assume that the gametic phase is known or they use methods that assume random mating to compute LD. In this study we worked with a highly heterozygous population due to inbreeding of wild accessions and asexual reproduction in local areas. To address this limitation a method was developed to analyze SNPs at the tetraploid level that does not make any assumption on random mating (Joao Paulo, personal comment). However the difficulty, when applying the Exact test, is that there are many possible genotypic arrays (9025 pairwise comparisons) to consider. In the case of *ORF12* we might observe that the detection of rare haplotypes is not in LD with more abundant haplotypes in the other region. However for this ORF, 30% of the data could not be evaluated due to technical reasons, which could be a simple reason for the lack of LD at this locus.

The successful construction of an LD map on chromosome V revealed no sufficiently distinct region of LD to fine map the QTL detected by the HC marker.

Future prospects

Linkage disequilibrium plays an integral role in association mapping, and determines the resolution of an association study (Flint-Garcia et al. 2003). LD-based approaches of association mapping will be used extensively also in *Solanum* populations when the genome-wide sequences become available. First sequence information about the tomato via the International Tomato sequencing project (http://www.sgn.cornell.edu/about/tomato_sequencing.pl) is already available. Information about the potato will become accessible via the Potato Genome Sequencing Consortium (http://www.potatogenome.net/). With the availability of whole genome sequences in the *Solanaceae* family we will be able to exploit further the structures of the potato genome.

Chapter 5

Fluorescent *in situ* hybridization (FISH) mapping of the *GP21* - *GP179* region on chromosome V of potato (*Solanum tuberosum*)

Abstract

The upper arm of chromosome five is known to harbour genes involved in the characterization of important agronomic traits, such as resistance to *Globodera pallida* and *Phytophthora infestans*. This interesting region is located between RFLP markers *GP21* and *GP179*. In the present study we aimed at defining the physical size of this segment using Fluorescent *in situ* hybridization (FISH) on pachytene chromosomes. FISH is a powerful cytogenetic mapping tool which allows reliable determination of physical distances between regions of interest. Three BAC clones, corresponding to marker loci *GP21*, *GP179* and *StPto*, were selected and labelled as probes for FISH. The markers were discovered in the euchromatic region on the long arm of chromosome V spanning a region of approximately 5.8 µm, which was estimated to correspond to 3.5 Mb.

Introduction

New cultivars are being developed in Europe by more than 300 potato breeders growing about eleven million first year seedlings each year (Swiezynski 1987). The breeding objectives are diverse. Cultivars have to be adapted for various types of utilization: direct human consumption; industrial processing; or animal feeding. Of major importance is resistance to nematodes, viruses, late blight, and storage diseases (Swiezynski 1987). Knowledge of the number and genomic position of factors controlling resistance has been obtained through analysis of diploid mapping populations using DNA-based markers (reviewed in Gebhardt and Valkonen 2001). One major objective in potato production is the establishment of resistance to the potato cyst nematode, *Globodera pallida*. This resistance trait shows a continuous variation and is assumed to be controlled by several genetic loci which act collectively on the expression of resistance (Meyer et al. 1998). These loci are referred to as quantitative trait loci (QTL). However, information on the extent and the molecular basis of these QTL is far from complete. The genetic distance between the RFLP markers *GP21* and *GP179* flanking one major QTL for resistance against *G. pallida* was estimated to be 3 centi Morgan (Meksem et al. 1995; Ballvora et al. 2002).

In early reports about the construction of genetic linkage maps between the potato and the tomato, the close relationship between the tomato and the potato was shown (Bonierbale et al. 1988). For seven chromosomes, the order of loci appears to be identical in tomato and potato, while for the other five - including chromosome V - intrachromosomal rearrangements are apparent. These rearrangements are paracentric inversions with one breakpoint at or near the centromere (Bonierbale et al. 1988, Tanksley et al. 1992). According to these findings markers *GP21* and *GP179* are included in an inversion that differentiates the tomato from the potato whereas marker *Pto* was placed distal on chromosome V on the opposite side of the centromere (Pillen et al. 1996). However, comprehensive physical data to confirm these findings is lacking to date. Work on the construction of a close contig in this region could not yet answer the question about the size of the complete region (Ballvora et al. 2007; Kuang et al. 2005).

In situ hybridization is a technique that allows highly sensitive detection of specific nucleic acid sequences on chromosomes fixed on a microscope slide. Use of fluorescent labels for *in situ* hybridization is referred to as Fluorescence in situ hybridization (FISH) (for review

see de Jong et al. 1999; Jiang and Gill 1994, 2006). It is an effective and accurate cytogenetic tool for mapping single copy and repetitive DNA sequences on chromosomes. The hybridization of Bacterial Artificial Chromosomes (BACs) on pachytene bivalents has become a frequent tool to facilitate physical mapping in many different plant species, such as rice (Cheng et al. 2001a; 2001b, 2006), *Medicago* (Schnabel et al. 2003), tomato (Zhong et al. 1999), maize (Saddler et al. 2000; Wang et al. 2006), and *Arabidopsis* (Lysal et al. 2001). The pachytene chromosome-based FISH mapping shows a superior resolving power compared to the somatic metaphase chromosome-based methods. The length of the chromosomes at meiotic pachytene is longer than at mitotic metaphase; for the tomato it was found to be about 15-times larger (de Jong et al. 2000). Pachytene chromosomes of the potato are quite comparable to tomato chromosomes indicating a close evolutionary relationship of these plants (Brown 1949; Barton 1950; Yeh and Peloquin 1965).
Especially the digital imaging system using CCD (charged coupled device) cameras now enables cytogeneticists to collect and process large amounts of FISH data, thus allowing application of the FISH technique to large-scale physical mapping projects (Jiang and Gill 2006). Using pachytene bivalents has the other advantage that the chromosome structure, such as euchromatin and heterochromatin, can be distinguished. In the potato the heterochromatic regions are confined to the regions around the centromere; telomeres are not characterized through distinct heterochromatic blocks (Ramanna and Wagenvoort 1976).

Also chromosome identification of *S. tuberosum* is generally based on pachytene analysis. Ramanna and Wagenvoort (1976) described chromosome V with a median centromere position and the chromatic part on the long arm being 1.5-2 times that of the short arm using dihaploid *S. tuberosum* ssp. *tuberosum* derived from cv. ‘Gineke’.
We present data on the physical localization of the molecular markers *GP21*, *GP179* and *StPto* located on chromosome V of the potato. Marker *StPto* was included in the experiment since previous experiments indicated the presence of additional QTL in this region beyond the interval *GP21-GP179*. In addition we wanted to position the markers with respect to the centromere and the telomere. Eventually, we aim to obtain valuable complementary information regarding size and chromosomal position of the marker loci to assist the map-based cloning efforts regarding QTL in this region.

We aimed to determine the physical size of a region on chromosome V which has been shown to harbour resistance factors (see chapter 4 for more details).

Materials and Methods

Plant material, BACs and primers: The diploid genotype P6/210 used for constructing the BAC libraries (Ballvora et al. 2002, 2007) was used for the *in situ* hybridization experiments. The BAC BA151m8 for marker *GP179* was obtained from A. Ballvora (unpublished results). Primer sequences of the probes from markers *GP21* and *GP179* and BAC names are summarised below (Table 5.1).

Table 5.1 Markers and their corresponding BAC, sizes, and primer sequences of the probes used for filter hybridization.

Marker (BAC)	Reference primers	Primer sequence (5'- 3')	Amplicon size (bp)	TA (C°)
GP21 (BC256o1)	Ilarionova 2005	f-CCC TGA ACC TCC TTA TCC CC r-ACT ATT ATG TCT ATG AGG AAG TGG TC	754	61
StPto (BC76f14)	Ilarionova 2005	f-TCA CAT TGG ATT GGG TGG C r-CGA GTC CAC TGC CCA TTC	670	55

Standard PCR reaction: previously described in chapter 2.

Purification of PCR products for sequencing: previously described in chapter 2.

Sequencing of PCR amplicons: previously described in chapter 2.

Detection of BACs for FISH: Filter hybridization was performed using a standard protocol (Sambrook et al. 1989). Radioactive labelled [a-^{32}PdATP] (Amersham Biosciences) probes for markers *GP21* and *StPto* were prepared using nick translation according to the description of the manufacturer (Invitrogen Life Technologies, Freiburg, Germany).

Probes for filter hybridization were generated by PCR from 50 ng genomic DNA template of *S. tuberosum* in 20 µl buffer (20 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl), including 200 nM of each primer (Table 5.1), 100 µM of dNTP and 1.0 unit *Taq* DNA polymerase (Invitrogen Life Technologies, Freiburg, Germany). Standard cycling conditions were: 3 min initial denaturation at 94°C, followed by 40 cycles of 45 sec denaturation at 94°C, 45 sec annealing at the 56°C and 1 min extension at 72°C. Reactions

were finished by 8 min incubation at 72°C. PCR products were examined for quality on 1.5% ethidium bromide stained agarose gels.

DNA labelling for filter hybridization: 200 ng of PCR product was added to 2.5 µl buffer including pre-mixed nucleotide solutions (dNTP Mix (minus dATP): 0.2 mM each of dCTP, dGTP, dTTP, 500 mM Tris-HCl (pH 7.8), 50 mM MgCl2, 100 mM 2-mercaptoethanol) (Invitrogen Life Technologies, Freiburg, Germany); 2.5 µl DNA Polymerase I/DNase I (Invitrogen Life Technologies, Freiburg, Germany) and 30 µCi α-32PdATP] filled with sterile water to a final volume of 25 µl and incubate for 1h at 16°C.

Purification of labelled DNA: A Sephadex™ G-50 column (Amersham Biosciences) was used to clean unincorporated radiolabelled nucleotides from the probe using gravity-flow chromatography. The probe and 400 µl TE buffer were added to the column and the flow-through was discarded. 400 µl TE buffer were added and the purified probe was collected in a 1.5 ml Eppendorf tube and incubated for 5 min at 95°C.

Filter hybridization: High-density colony filters of the *S. tuberosum* P6/210 BAC library were pre-hybridized at 65°C for 1 h in glass tubes containing hybridization buffer (5 x SSPE, 5 x Denhardts' solution (100 x Denhardts': 2 g BSA (Bovine serum albumin), 2 g Ficoll® 400; 2 g PVP Poly-Vinyl-Pyrrolidon) and 0.08% SDS) adding 5 mg Herring Sperm DNA as a blocking agent to minimize non-specific binding of hybridization probe to the filter. The hot probe was added to the tubes and hybridized over night at 65°C.

Washing and detection: Filters were washed three times for approx. 10 min at 65°C in 2x SSPE+ 0.1%SDS, 1x SSPE+ 0.1%SDS and 0.2%SSPE+ 0.1%SDS respectively. Following 10 min in Southern wash buffer (50 mM NaH_2PO_4*2 H_2O, pH 7; 10 mM EDTA Na_2* 2 H_2O; 2% SDS) at room temperature and they were then exposed for one day to filter (Kodak X-OMAT AR FILM).

Potato genomic libraries: BA library: The BA BAC library was supplied by LION Bioscience AG (Heidelberg, Germany). The library was constructed from *Hin*dIII partially digested high-molecular-weight genomic DNA of the diploid potato genotype P6/210 in the binary vector pCLD04541 (Jones et al. 1992). The BAC library consists of approximately 100,000 clones with an average insert size of 70 kb. The colonies were stored in 264 384-microtitre plates (Genetix, Oxford, UK) in 2YT medium (Sambrook

et al. 1989) with freezing buffer (5.5% w/v glycine, 7 mM (NH_4) SO_4, 1.5 mM sodium citrate, 0.3 mM $MgSO_4$, 13 mM KH_2PO_4, 27 mM K_2HPO_4).

BC library: The potato *S. tuberosum* P6/210 genotype was used for the BC BAC library using the pECBAC1 vector (Fijter et al. 1997). The library is composed of 100,608 BAC clones with an average insert size of 80 kb, equivalent to ten times the genome coverage of the haploid *S. tuberosum* genome.

Plant Material: *S. tuberosum*, P6/210 were grown in the greenhouse at 20°C with 16 h light until flowers started to develop.

Preparation of pachytene chromosomes: We used essentially the method of Zhong et al. (1996) with small modifications. Immature flower buds of 1.5–1.8 mm in length were fixed in ethanol/acetic acid (3:1) for at least 3 h and stored in this fixative at −20°C for several months. For cell-wall digestion the buds were rinsed three times for 1 min in deionised water and transferred to a pectolytic enzyme mixture [0.3% (w/v) pectolyase Y23 (Sigma-Aldrich, St. Louis, MO, USA), 0.3% (w/v) cytohelicase (Sepracor, Jaures, France) and 0.3% (w/v) cellulase RS (Sigma) in citrate buffer (10 mM sodium citrate buffer pH 4.5)] at 37°C for 2 h. After digestion the anthers were rinsed with deionised water, and each flower bud was transferred to a droplet of water on a microscope slide. Anthers were dissected from flower buds with fine needles and transferred to a grease-free slide. The resulting cell suspension was spread on a clean glass slide with 30 µl 60% acetic acid at 45°C for 1 min. Finally, 1 ml ice-cold ethanol/acetic acid (3:1) was added in a circle around the suspension before leaving the slides to dry.

Fluorescence in situ *hybridization*: FISH experiments were carried out in the Laboratory of Genetics in the group of Hans de Jong at Wageningen University, The Netherlands. BAC DNAs were isolated using the QIAGEN Plasmid Midi Kit according to the manufacturer's instructions (QIAGEN GmbH, Hilden, Germany). BACs were labelled with biotin-16-dUTP and/or digoxygenin-11-dUTP (Roche Diagnostics, USA) by standard nick translation, based on the replicational incorporation of modified nucleotides. The BAC clones were labelled using the manufacturer's protocol (Roche Diagnostics, USA). 1 µg of DNA was labelled as follows: BC256o1 with biotin, BC76f14 with digoxigenin and BA151m8 with biotin as well as digoxigenin. Selected BACs were poor in repeats and no

Cot100 (a fraction of excess amount of unlabeled repetitive DNA) was needed to block the hybridization of repetitive DNA. Slides were incubated with RNase (100 µg/ml in 2x SSC) for 1 h and subsequently with Pepsin for 7 min (5 µg/ml in 0.01 M HCl). After each treatment slides were rinsed in 2x SSC three times for 5 min. The preparations were then fixed in 1% formaldehyde in PBS for 10 min washed again in 2x SSC three times for 5 min, and dehydrated in ethanol series (70%, 90%, 100%, each 3 min) and air dried. The hybridization mix (20 µl/ per slide) contained labelled probes (2 ng each) in 50% (v/v) formamide, 2x SSC, 10% (w/v) sodium dextran sulphate and 50 mM phosphate buffer. The probe mix was denatured for 10 min at 100°C, chilled on ice and applied to the microscope slides. The slides were covered with a coverslip 23 x 50 mm and baked at 80°C for 2 min. Then preparations were incubated in a moist chamber at 37°C over night. Post hybridization washes were performed in 50% formamide, 2x SSC for 5 min at 42°C followed by washing in 2 x SSC for 5 min. Before the detection and amplification the slides were washed in 4T buffer (4 x SSC, 0.05% Tween 20) for 5 min. Then 100 µl of TNB (0.1 M Tris- HCl pH 7.0, 0.15 M NaCl, 0.5% blocking reagent (Roche Diagnostics, USA) was added, covered with a coverslide (24 x 50) and incubated at 37°C for 30 min. Between all further incubation steps coverslides were removed in 4T buffer and slides washed 3-times in TNT buffer (0.1 M Tris- HCl pH 7.0, 0.15 M NaCl, 0.05% Tween 20) for 5 min. The first detection step was carried out with Texas Red conjugate avidin (2 µg/ml, Roche) in TNB. The second detection step was performed with biotynilated anti-avidin in TNB and sheep anti-dig-FITC in TNB. The third detection step was carried out with Texas Red conjugate avidin (2 µg/ml, Roche) in TNB and rabbit anti-sheep-Alexa 488 in TNB. All detection steps were incubated in a moist chamber at 37°C for 1 h. Subsequently slides were washed twice in 2x SSC for 5 min and dehydrated in an ethanol series (70%, 90%, 100%, 3 min each).

Imaging: Chromosomes were counterstained with 5 µg/ml DAPI in Vectashield anti-fade (Vector Laboratories). Slides were examined under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescence illumination, filter sets for DAPI, FITC and Texas-Red/Cy3.5 fluorescence. Selected images were captured by a Photometrics Sensys 1,305×1,024 pixel CCD camera and processed with Genus Image Analysis Workstation software V 2.7 (Applied Imaging Corporation). Fluorescence images were displayed in

grey value (DAPI) or pseudo-coloured and further improved for optimal brightness and contrast with Adobe Photoshop image processing software.

Results

Isolation of loci-specific BAC clones

Specific probes for markers *GP21* and *StPto* were used to identify corresponding BAC clones through filter hybridization in the available BAC libraries. In total we observed ten putative positive filter hybridization signals for marker *GP21* and 19 putative positive signals for marker *StPto*. The BAC for marker *GP179* was supplied by Agim Ballvora. Several selected BAC clones were confirmed via PCR and sequencing of the amplicons. One BAC for each marker was selected and used for the FISH experiment.

Fluorescent *in situ* hybridization

Plasmid DNA was isolated and hybridized to pachytene chromosomes. Hybridization signals of digoxigenin-dUTP-labelled BAC BC76f14 (*StPto*), biotin-dUTP-labelled BAC BC256o1 (*GP21*) and digoxigenin/biotin-dUTP-labelled BAC BA151m8 (*GP179*) were detected using

sheep anti-dig-FITC and rabbit-anti-sheep Alexa 488 for the digoxigenin labeled BAC; Avidin Texas Red and biotynilated anti-avidin for the biotin- labeled BAC and together for the digoxigenin/biotin-labeled BAC. The three BACs appeared as green (*StPto*), red (*GP21*) and yellow (*GP179*) fluorescent dots on the chromosomes. The yellow signal is visible as a yellow-green-red dotted pattern probably due to a suboptimal transparency setting in Applied Imaging software (Hans de Jong, personal comment). BACs can produce multiple foci and even big ones as target sequences may contain local tandem arrays or duplicated single copy sequences.

Signals with varying intensity and size could be observed among several chromosomes in different preparations. However, the pattern of three strong BAC signals on chromosome V co-localized in numerous nuclei in the euchromatic region. Two representative hybridization results are shown in figure 5.1.

Physical mapping on chromosome V

In addition to the position of the markers, *in situ* hybridization also reveals the approximate position of the target region relative to the centromere and the physical distance between markers *GP21*, *GP179* and *StPto*. Clear pachytene hybridizations were selected and photographed for further analysis. The distances between the centres of hybridization signals were measured in micrometers (µm) using the software program Image J, version 1.36 (freely available on the internet at http://rsb.info.nih.gov/ij/). The values shown in table 5.2 are the average of measurements of four to six nuclei. At pachytene chromosome V has an average length of approximately 41 µm. The distance between *GP21* and *GP179* was measured to be 1.6 µm and the region between *GP179* and *StPto* to have a size of 4.2 µm (Table 5.2).

Table 5.2 Cytogenetic, genetic and physical distances on chromosome V.

Segment	**Number of observations**	**Length of the segment (SD) [µm]**	**Molecular size based on FISH (SD) [Mb]**	**Genetic size [cM]**
Complete chromosome V	4	41.0 ± 4.2	-	70
telomer-*GP21*	6	4.6 ± 0.6	2.76 ± 0.36	15
GP21-GP179	6	1.6 ± 0.4	0.96 ± 0.24	3
GP179-StPto	6	4.2 ± 0.5	2.52 ± 0.3	5

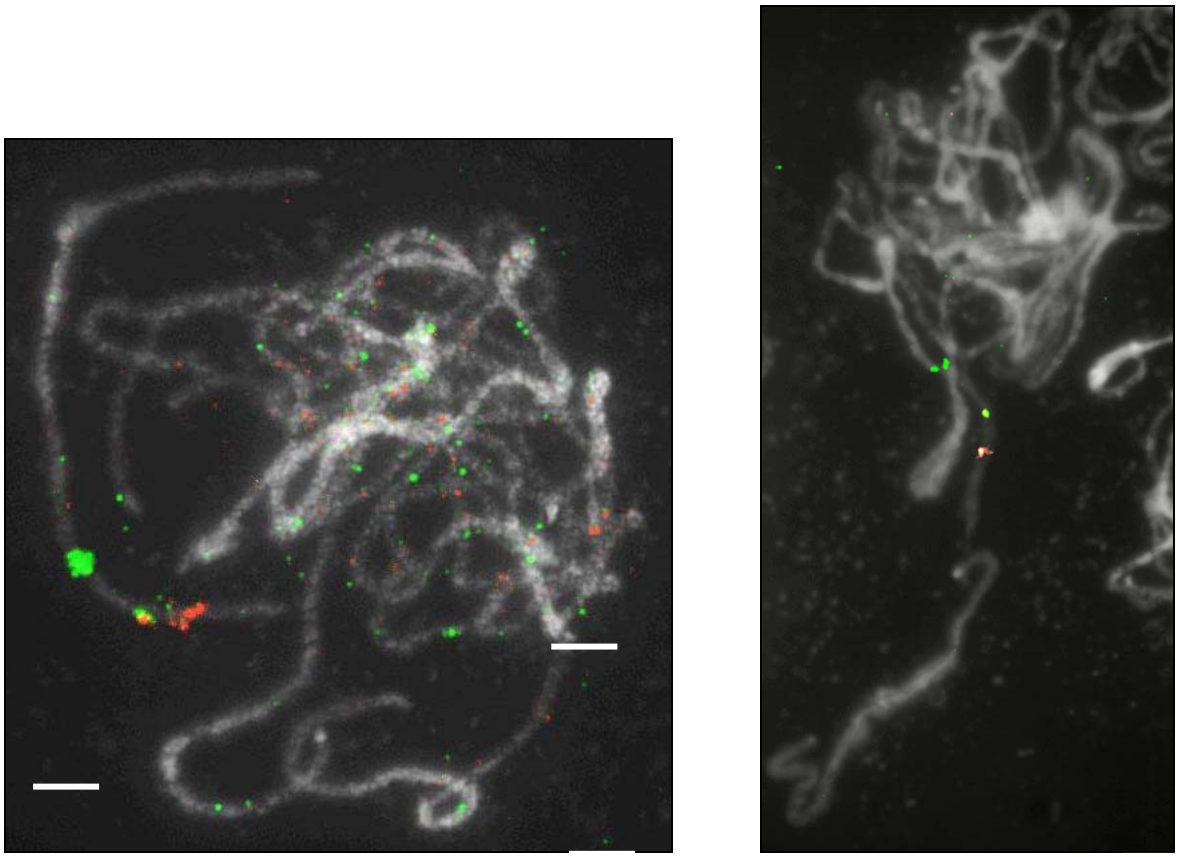

Figure 5.1 Mapping of BAC clones on pachytene chromosomes, each BAC is anchored by a genetically mapped marker. The three colours show the BACs hybridized to chromosome V, BC256o1 (*GP21*) in red, BC76f14 (*StPto*) in green, and BA151m8 (*GP179*) in yellow. The yellow signal is visible as a yellow-green-red dotted pattern. Scale bar 5 µm.

We estimated the ratio between physical distance and cytogenetic distance according to Zhong et al. (1999) with 0.6 Mb/µm in the euchromatic region (table 5.2). The genetic distances were obtained from the potato function map published online in the Gabi Primary Database (Meyer et al. 2005).

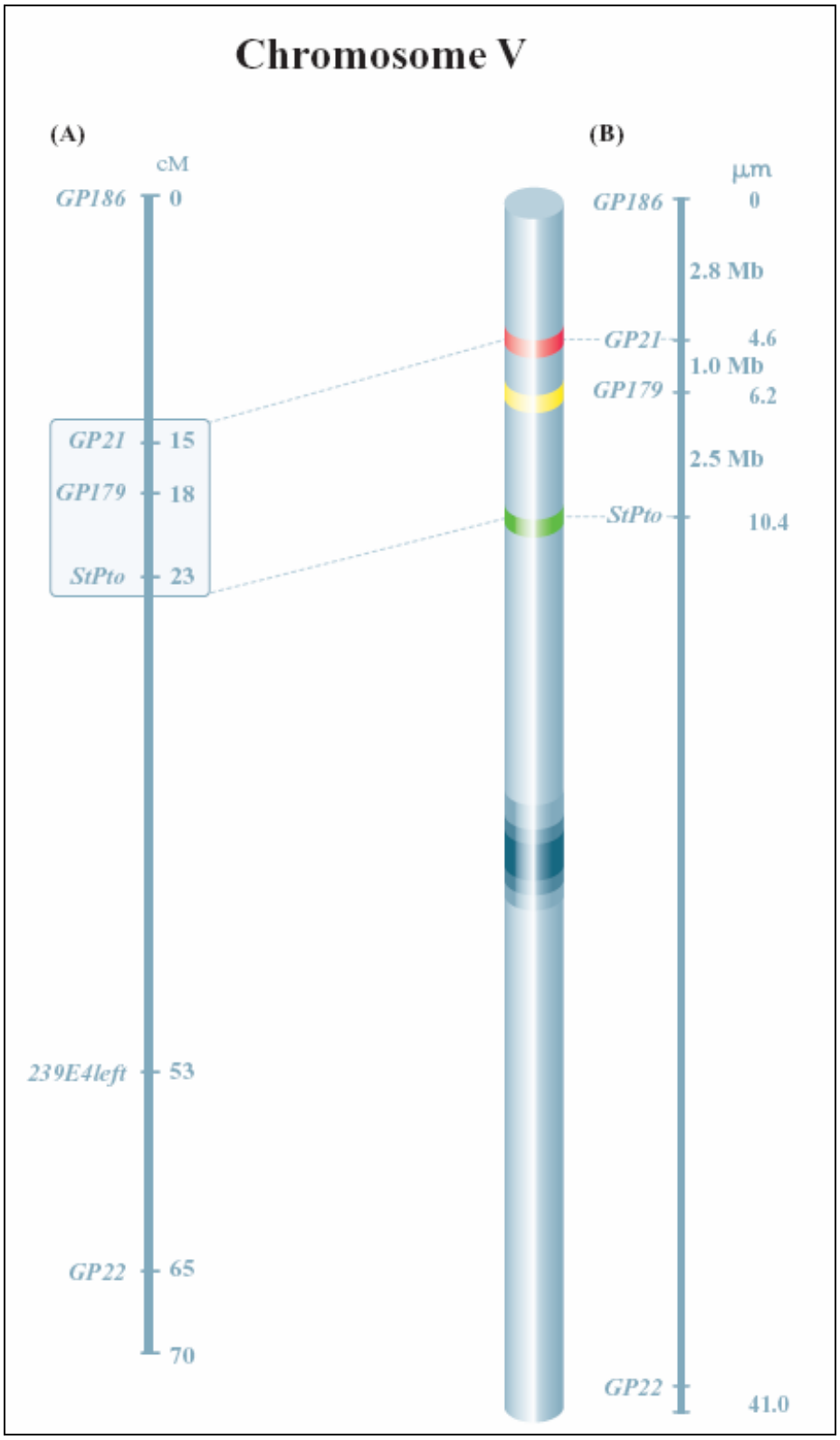

Figure 5.2 (A) Genetic map of potato chromosome V; (B) An integrated physical and cytogenetic map with an illustration of the FISH results. Marker loci *GP21*, *GP179* and *StPto* are shown in red, yellow and green colour, respectively.

Discussion

The appearance and length of the chromosomes vary during pachytene and the amount of stretching in the preparation of the slides adds to this variation (Yeh and Peloquin 1965). The size of chromosome V was described as ranging from 33-40 µm in *S. tuberosum* ssp. *andigena* (Yeh and Peloquin 1965) which is comparable to our finding of approximately 41 µm in *S. tuberosum* ssp. *tuberosum*. Using pachytene chromosomes as targets for FISH we were able to distinguish euchromatic regions from the heterochromatic regions. However, we were not able to locate clearly the centromere within the heterochromatic region.

Localization of *Pto* on chromosome V

Pachytene FISH revealed the correct position of *StPto* on the long arm of chromosome V approximately 4 µm from *GP179*. We observed the three markers located on the same chromosome arm with markers *GP21* and *GP179* in an inverse orientation compared to tomato chromosome V (Figure 5.3). Tanksley et al. (1992) hypothesized that the inversion resulted a breakpoint near or at the centromere of the chromosome, resulting in the inversion of the entire chromosome arm. This is not in accordance with our results and we could show that the inversion on chromosome V did not affect the entire arm but occurred between *GP179* and close to *Pto*. Further evidence for this hypothesis would be the physical distances between telomere and *GP21* and the two markers *GP179* and *StPto*, which are almost identical (2.5 Mb and 2.8 Mb) (Figure 5.2). Consequently an inversion would not result in differences in distances between markers when comparing the potato and the tomato chromosome V.

Nevertheless the order of the genetic loci remains identical and genetic maps may be considered as frameworks for the analysis of interesting loci.

In general, the distances between loci differ greatly between chromosome maps and genetic maps, which is a result of the non-random distribution of crossover events along the chromosome (de Jong et al. 1999; Saddler et al. 2000). Despite the mapping of the two loci *GP21* and *GP179* 15 years ago (Leonards-Schippers et al. 1992) and the improved characterization of the region, no knowledge on the physical distance was available prior to this study. Our study shows the necessity of using cytogenetic tools, such as BAC FISH, to

unambiguously determine the position of markers on chromosomes and to obtain information about the approximate physical distance between loci.

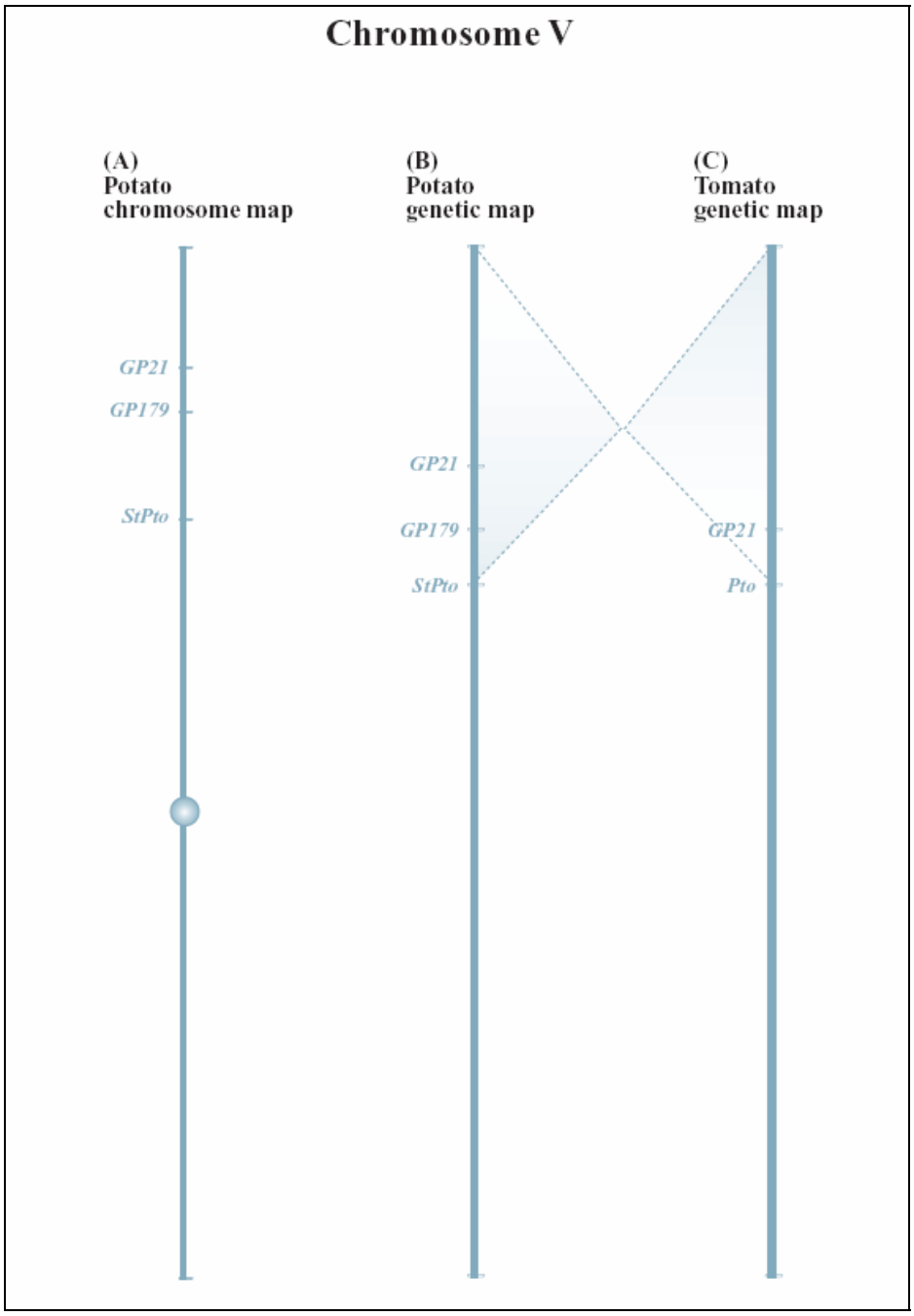

Figure 5.3 The genetic linkage maps of the tomato (C) and the potato (B) together with the potato chromosome map (A). The centromere is indicated with a circle. Markers *GP21*, *GP179* and *Pto* are shown on the left next to the chromosome.

Markers *GP21, GP179* and *StPto* map on the long arm of chromosome V

In our experiment, the three markers *GP21*, *GP179*, and *StPto* were mapped on the long arm of chromosome V (Figures 5.1, 5.2). This result is opposed to the work of Dong et al. (2000) who hybridized a BAC harbouring the RFLP marker *GP22* at the distal end on the long arm of chromosome V using somatic metaphase chromosomes via FISH. This discrepancy can be explained due to the fact that mitotic metaphase chromosomes of the potato are about 1-3.5 µm in size (Dong et al. 2000) and with a maximum optical resolution of the light microscope of about 0.2 µm too small for precise chromosome mapping.

Regarding the genetic linkage map (Figure 5.2), marker *GP22* is located on the opposite arm of markers *GP21* and *GP179* and thus, according to our results, is located on the short arm.

Outlook

The tomato FISH map is being generated as part of the Tomato Genome Sequencing project (www.sgn.cornell.edu/about/tomato_sequencing.pl). The aim is to verify the genetic and physical maps and to locate more precisely the euchromatin / heterochromatin boundary on tomato chromosomes.

The potato has not yet been subject to elaborate cytogenetic studies and comprehensive sequencing information is not available. But several cytogenetic studies on the potato are being carried out currently. Among these a Fibre FISH experiment using BACs in the region investigated in this study is in progress. The aim is to anchor the physical contig available from a segment in this region (Ballvora et al. 2007) to the exact position on the chromosome relative to the markers used in this study. In parallel, the potato BACs will be cross-hybridized on tomato chromosomes for direct comparison of the positions of the three marker loci between both species.

Conclusions

The work for this thesis was done in the department of plant breeding and genetics in the Max Planck Institute for Plant Breeding Research. The experiments were carried out in the potato genome analysis group in collaboration with two potato breeding companies.

The potato (*Solanum tuberosum*) is one of the most important food crops worldwide with major production areas in middle and Eastern Europe, Russia, India, China and the USA (Graf 2003). In Europe the infestations with the root cyst nematode *Globodera pallida* lead to severe yield reductions. Therefore high levels of genetic resistance to nematodes are an important goal in breeding new potato varieties. We were interested in the identification and characterization of quantitative trait loci (QTL) for resistance. The polygenic nature of QTL impedes the adaptation of the pathogen. Therefore this type of resistance is claimed to be more durable (Alemayehu and Parlevliet 1996; Wastie 1991) and consequently of greater interest to the potato community.
The basis of the study was the current knowledge on genomic positions of candidate genes having putative functions in pathogen recognition, defence signalling and defence responses as summarized in the 'SOLanaceae Function Map for Pathogen Resistance' (http://gabi.rzpd.de/projects/Pomamo/SolFunctionMap.html).
Starting point of this work were results obtained during the preceding project. In this project 1498 Single Nucleotide Polymorphisms (SNPs) and 127 Insertion-Deletion (InDel) markers were identified that are physically linked to candidate genes for pathogen resistance (NBS-LRR type genes). The markers tag most regions of the potato genome known to harbour resistance QTL (Rickert et al. 2003). Furthermore a physical contig of 413 kb was constructed and the genomic sequence was obtained (Ballvora et al. 2007). The contig was located in a resistance hot spot on chromosome V, where a major QTL for resistance to *G. pallida* as well as QTL for other important agronomic traits are located (Ballvora et al. 2007). SNPs located on the contig were found to be linked to a QTL for resistance to the potato cyst nematode *G. pallida* and two of them were used to develop a haplotype specific marker (HC marker) assay (Sattarzadeh et al. 2006).

In the present project screening 33 potato varieties resistant to *G. pallida* pathotypes Pa2 and/or Pa3 and 21 susceptible varieties with the HC marker demonstrated that the HC

marker was diagnostic for presence of high levels of resistance to *G. pallida* pathotype Pa2/Pa3. Further it was shown that the HC marker was exclusively present in accessions of *S. vernei*.

Identification and characterization of genes controlling quantitative pathogen resistance combined the application of different genotyping methods and extensive phenotyping, followed by association mapping. Association mapping for QTL analysis is a more powerful tool to study complex traits due to its capability to more precisely locate them on the genetic map as compared to linkage studies (Risch and Merikangas 1996). We discovered 43% of PCR-based marker alleles and 38% of SNPs significantly associated with different agronomic traits, focussing mainly on chromosome V. After collecting related data from previous studies, we were able not only to confirm known QTL but also to detect new loci of interest and develop a PCR-based marker for applied research and breeding purposes. This is a valuable achievement and serves the purposes of marker assisted selection (MAS) of the collaborating breeding companies.
The information obtained during this part of the study is compiled in the PoMaMo database which enables future studies to validate our findings, as well as to continue with additional molecular analysis of the present breeding material.

Based on the SNP data, linkage disequilibrium (LD) and marker-trait associations were analyzed. Marker-trait associations were identified over a genetic distance > 2 cM assisted by strong LD that exists in this region. The strong LD found on the upper arm of chromosome V explains the difficulties in increasing the mapping resolution of the QTL in this region and is thought to be responsible for similar difficulties in other regions. One explanation for the strong LD is the fact that the potato population used in this study is separated only by a few generations according to information which we were able to obtain via the ‘Potato Pedigree database’ (Hutten and van Berloo 2004).

As our next step, we inferred haplotypes by comparison of susceptible and resistant genotypes homozygous at defined SNP positions. But due to the lack of the complete sequence information and the heterozygous state of the potato, we could not determine the number of haplotypes present in this region. For future studies the first software program (Pothap) is available for haplotype identification in autotetraploids (Walaschewski 2005). This software was verified experimentally using nineteen tetraploid potato breeding clones

(Gyetvai 2007). Information on number and extent of haplotypes in the genome could increase the efficiency of association mapping studies to avoid tagging numerous markers to the same QTL.

In order to determine the physical size of the QTL region on chromosome V, we used Fluorescent *in situ* hybridization (FISH). The application of FISH in potato research is rare to date and we were able to demonstrate the potential of this technique in precisely locating genetic markers on chromosomes. Three BAC clones, corresponding to marker loci *GP21*, *GP179* and *StPto*, were selected and labelled as probes for FISH. The markers were found to be located in the euchromatic region on the long arm of chromosome V spanning a region of approximately 5.8 µm, which was estimated to correspond to 3.5 Mb. Markers flanking the QTL span a physical region of approximately 1000 kbp.
Cross-hybridization experiments using the potato BACs on tomato chromosomes is expected to confirm the present results and allow a more reliable comparison between molecular markers located on tomato and potato chromosome V. At present also BAC hybridizations on potato fibres are carried out with the goal to determine the position of the contig within the interval *GP21 – GP179*.

The present association study constitutes part of a genome-wide approach, intended to genotype markers that cover the whole genome at regular intervals.
This information is valuable for population genetics and will assist future map-based cloning efforts.
To date the majority of QTL cloned in plants has been identified using a positional cloning approach where candidate genes were selected for further evaluation (reviewed in Salvi and Tuberosa 2005). By means of QTL mapping, the position of each QTL is initially assigned to a genetic interval of 10 to 30 cM which usually includes several hundred genes (Salvi and Tuberosa 2005). In the potato, the question remains as to how we find candidate genes if QTL fine mapping is restricted to a size of several hundred kilo base pairs, a segment, which is very expensive to sequence.
However first sequence information about the tomato via the International Tomato sequencing project (http://www.sgn.cornell.edu/about/tomato_sequencing.pl) is public. Information about the potato will become accessible via the Potato Genome Sequencing Consortium (http://www.potatogenome.net/). With the availability of whole genome

sequences in the *Solanaceae* family we will be able to accelerate our efforts to determine genes underlying QTL.

The use of DNA markers instead of phenotypic assays, which often take several years, can reduce cost and time and is a much more accurate method to accomplish breeding goals. At present MAS in the potato is progressing slowly due to the lack of applicable PCR assays. The HC marker (Sattarzadeh et al. 2006) diagnostic for high resistance against *G. pallida* Pa2/3 is applied in current breeding programs.

The ultimate goal is 'Breeding by Design', a concept that aims to control all allelic variation for all genes of agronomic importance (Pelemann and van der Voort 2003). This work is a step in that direction, providing a first DNA based molecular marker for improving potato varieties which have been assessed in tetraploid breeding material. This achievement and future knowledge gained from unravelling research in the potato genome will enable breeders to develop a continually more sustainable agriculture.

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APPENDIX A

Solanaceae species, *Nicotianum*, *Capsicum* and *Petunia* tested for the HC marker study (Chapter 3).

Abbrev.	Species	Accession	Accession
acl a	*acaule*	BGRC 018627	EBS 2664
acl b	*acaule*	BGRC 016835	EBS 3052
acl c	*acaule*	BGRC 024555	
aln A1	*alandiae*	BGRC 018521	EBS 3090
aln E2	*alandiae*	BGRC 031187	
aln 028489	*alandiae*	BGRC 028489	
adg A3	*andigena*	BGRC 007464	
adg B3	*andigena*	BGRC 007506	
adg 007768	*andigena*	BGRC 007768	
ber a	*berthaultii*	BGRC 010063	EBS 1846
ber b	*berthaultii*	BGRC 018548	EBS 1271x1288
ber D2	*berthaultii*	BGRC 028033	
brc A1	*brevicaule*	BGRC 008207	
brc B3	*brevicaule*	BGRC 024571	
brc D3	*brevicaule*	BGRC 028023	
buk A1	*bukasovii*	BGRC 007993	
buk C1	*bukasovii*	BGRC 015424	EBS 2152
can 007165	*canasense*	BGRC 007165	EBS 1896
can 024572	*canasense*	BGRC 024572	
can 7166	*canasense*	BGRC 007166	EBS 1921
chc A3	*chacoense*	BGRC 008025	
chc b	*chacoense*	BGRC 016979	
chc C3	*chacoense*	BGRC 027357	
dms a	*demissum*	BGRC 010022	
dems 256	*demissum*		GLKS 256
dems 325	*demissum*		GLKS 325
dul 2	*dulcamara*		
tomato	*esculentum*		
tomato	*esculentum (Heir fein)*		
etb a	*etuberosum*	BGRC 28476	
etb b	*etuberosum*	BGRC 53007	
grl 007185	*gourlayi*	BGRC 007185	
grl 024600	*gourlayi*	BGRC 024600	
grl a	*gourlayi*	BGRC 007180	EBS 3048
hdm B3	*hondelmannii*	BGRC 024710	
hdm D1	*hondelmannii*	BGRC 027317	
kur b	*kurtzianum*	BGRC 017585	
ktz B3	*kurtzianum*	BGRC 017580	
ktz 017620	*kurtzianum*	BGRC 017620	
lph D1	*leptophyes*	BGRC 027269	
lph E3	*leptophyes*	BGRC 008211	
lph 018582	*leptophyes*	BGRC 018582	EBS 3096
lignica A2	*lignicaule*	BGRC 008106	
maglia A3	*maglia*	BGRC 023571	EBS 1059
meg a	*megistacrolobum*	BGRC 008113	

Abbrev.	Species	Accession	Accession
meg b	*megistacrolobum*	BGRC 027262	
eggplant	*melongena*		
mcd 027354	*microdontum var. gigantophyllum*	BGRC 027354	
mcd A3	*microdontum*	BGRC 024644	
mcd C3	*microdontum*	BGRC 007197	EBS 3202
morelli A1	*morelliforme*	BGRC 007200	EBS 3026
ngr	*nigrum*		
nrs B2	*neorossii*	BGRC 050197	
opl A2	*oplocense*	BGRC 016868	
opl C1	*oplocense*	BGRC 024650	
opl 027345	*oplocense*	BGRC 027345	
phu 7907	*phureja*	BGRC 007907	
phu 7915	*phureja*	BGRC 007915	
phu 51240	*phureja*	BGRC 051240	
pnt a	*pinnatisectum*	BGRC 008168	
sis	*sisymbrifolium*		
spl B1	*sparsipilum*	BGRC 024678	
spl 018595	*sparsipilum*	BGRC 018595	EBS 3089
spl 027229	*sparsipilum*	BGRC 027229	
spg b	*spegazzinii*	BGRC 016929	
spg D3	*spegazzinii*	BGRC 024694	
spg 8220	*spegazzinii*	BGRC 008220	
stn A3	*stenotomum*	BGRC 051242	
stn C2	*stenotomum*	BGRC 053633	
stn 027167	*stenotomum*	BGRC 027167	
sto a/2	*stoloniferum*	BGRC 007229	EBS 2942
sto b	*stoloniferum*	BGRC 007230	EBS 2626
sto 55189	*stoloniferum*	BGRC 055189	
vrn A1	*vernei*	BGRC 008241	
vrn C1	*vernei*	BGRC 024729	
vrn D3	*vernei*	BGRC 017536	
verru B2	*verrucosum*	BGRC 008250	
verru C3	*verrucosum*	BGRC 008255	EBS 2664?
verru 008245	*verrucosum*	BGRC 008245	
tobacco	*N. tabaccum*		
pepper	*C. annuum*		
petunia	*P. blau*		

APPENDIX B

Sequences of markers scored for SNPs in:
[1]Chapter 4 (significantly associated SNPs are indicated with yellow background)
[2]Chapter 2
Primer sequences are indicated in blue, sequencing primer is underlined,
SNPs are marked blue, bold and underlined.

GP21[1]

```
AAGACGACAT TGTCTATGAT TGCAGCCGGC TCTTCCCTGA ACCTCCTTAT CCCCGAGAGG  660
TAAAATTTTG TGTGCACCAT TAAAATAGAT CATTTGTCTA TGCATATTAT AAATTTCTTT  720
TTGTTTTTTA CCTGCATGGG CTCCATTTTG TGAAATGACT CAGCTGAATA TACTCTGAAG  780
GAAACAATTC AATGTCAATT GATACTCAAA CTTGTTAAAT GACAAATTCA TATATACAGC  840
AAGTGCAGGT GGTTGGTGTG AGTGATAGAG TATGTTTAAT CTTGTTGCCT TCTATGGGTG  900
ATGCTTCGAC CACCACGTTA TGAAGTAAAT TAGGCTGACT TGAGCTGATA TAAGCTATGC  960
TATCTGTTAA ATCTTTCATA TACTAAGACA CTTGCTGTTT TAAATTTCTT ATTAGTCTTT 1020
TCTATTCATA ATCTCTGACT GAAAGTAACA TGTATTATCA GCAACGGAGT AACAAATTCA 1080
TCAGTAGTTT AATACCCTAT ATTTCTTTTG CAGGTTGAAT CTATTTTTTG TCTCTCTCAT 1140
TTGATTTATA CTAGTAAACG TAAACTTTCA TACTTAATTT AAGTATAAGT GACTTCAGTG 1200
ATACTCGTCA AAAAACAAAC TTCAGTGTGT CAAATTTACA GATTTCTGAT ACTATTAGAG 1260
CAGTGCATTT TAATTCAGTT CTTTCTATCA AATACGTGGC CATTTAGAAA GTCAATGAGC 1320
AAGCAAGTAA TGCTATGCTG GCTCACTAAT GTGGTGAACT ATGACCACTT CCTCATAGAC 1380
ATAATAGTAT GAGTTTTAAA AGCTGGAGGT AAAAACACTT GACCAATTGA AAACCTTCCG 1440
```

ORF3 (REVERSE)[1]

```
GCTTAGTTTC CTCTGGCACC AAACCAAATG ATCTCTCCTT AATCTTGGTA AACTTAGCAC   40
TCTTGATACC AATTTTAGCT CCACGAGTGA CGCGAATAAT GAATGACTCA ATCGACGTTC  100
TTGTTGTTTT TGGAACTTCT TCACTATGCA ATTGCCACTT CTTTGTACAT CTTTCAACTT  160
CCTCTCCTTC TGCATAGAGA ATATCAAGTG ATTCACAAAG TTTAGAAACT AAAATCGCTA  220
CAAAAAAACT GGAATTAGCT AAAAACTTCA TCAACATGTT GTTGTTGTTG TGAAAGGCGT  280
GGCATATCTA TGAATAAAGA TAGACTAACC TTTTGAAGGT CTAACTTGAC ATTCATGAAA  340
AGCTCGATTT CTGCATTGTC CATTTGAGTT CTTTGAACAC CCTTTAAGGC CAAATTAGCT  400
TCACGTTTAT CAGCACGAAG AAACTCCCAG AAAACTTGCA TTGTCTCCTT AATAACTTCC  460
CCCAATTTCA CAATGGAAAT GACATCTTTT TCCTCTTCTC GTCCGCCCTT CTTTCCCTTA  520
AAACGATCAT CTGCTAGAAA GTCAAAGAAT TCCTCGTTAC AGTGAGTCAA GGTATTTTAA  580
TAACAACTCA ATGAAAAAAT GCATACCTCT TATGCTCGGG ACTTGAAAAA AGATACCGAG  640
AATGCACCTC TTCCTGACAT AATTTTGGAC TCGTGGTCCT TGAAATGGCT CATCCTCGAC  700
AAATCTTTGT AAAAGTACTT GAAATTGCTG ATATTCTCCG GCCACTTGGT TATATGTGTG  760
ATACTCGTGT GGATCATGTT CAAGCAA
```

ORF12[1]

```
CGATGAATAT GAAGATGAAG CTAGTCATGG ACCTAATGCG CCTACTGAAG AAGCTGAAAA   39
TACTGATACA AGACATAATT TTACTCAAAC AACTGAAAAT GAATATGCTC GAGGATCTCC   99
TCGTGAACAT ACTGGACCAT CTGAAAAGCA AGGTGAATAT GCTAAAACAT CTTCTTCTAG  159
TGTCAATGAA AAAGAAAAAG GCAAGAAAAG AAAGAGGGTT GTGGAAGATG TTAATGAAAC  219
ATTTCTCAAG AGTATGGCGG AAGTTATGAA AATTTTTACT GAAAGCCAAG ATAAAAGAAT  279
TGGTTCCTTG ATCGAAAAGA TTGGAAATCG TGACCACTCT GATATGCGTG GTCAAATTTA  339
TTCCATCATT GAATCTCCTA CATTTGATTT GTACACCATA GAGCAACGTA TCACAGCTAA  399
AAAGGT
```

ORF20[1]

```
ACAAGTACAG CTAATAGACC CAATATTGTA GAAAACATAA AAGCTAAAAC ACCCCTAAGT   39
AACTTTCATA TATGTGTGTT CATCAATCTT TGCTAACATC ACCATTTTCC TTAACACCAT   99
TATCTTTTTC GTTTACACCG TCAGCATTCT TGGCTTCTTC AGTAGCTGTT ACTTCTTTCT  159
TTTCCTCGCG ATCTTTCTCT TCCTTCTCCT TTTCAGCCGC CTTTGCTCTC TCTTCTTCCT  219
CAGCCTTCAG TTTCGCTTCC TCTTTTGCAG TTTCAAGAGC TTTAATCAAT CTCTCCAAAC  279
AAGTGTCTGC ATTTTCCTTT GAAGACTTAG GCATCAAATT CTCAGCAATA TCAGCAGGAG  339
TCATATTAGT TTCCTCCAAT AAACGACGAA TCTCAGGAAA GTGAACATGA GATTCAACGA  399
CGTCAAGATA GTTATGTGCA AGAACTTTGA ATGACTCAAA GCAACAGTAG GATAGGACAA  459
TATGTTTATC CATCCTCCCC CTCCTAATTA GAGCAGGATC AAGCTTTTCC ACGTAGTTGG  519
TAGTGAAGAC GATAAGCC
```

BA87d17t3[1]

```
GTAGTACATC AACATACATT TTGCGG
ATGCACGGAA TAACAGGCTT AATCCTCATC CGCACGAGAT AAGAAAGCAA GAAATTCATC   60
TCACCAGGAA CCAAATCCTG ATCTCTGTTA TTGTCATAGC TGGGCATGTA CAACCAGACA  120
ACCATTGCTG CAAACCCAGC CACAACTAGA ACATGACNGA AGAAAGTACG TACGCTCTGA  180
GGCTTCAGCAC ACCACCTGTT TGATAAAAAG CCTACAAGAC TCTTCAACAA CTTCAGCTCT  240
TTTAAAACCT CCTGTATTTC GCGAGAACAG AGATCATCAA TCTCTGCTAA CTCGTGAAGA  300
TTCTGTACAA CACCATTGAT GAATTTCATT ACAAACTTGG AATTATCAAC CCCACTCTTT  360
TCGAGCGAAG GCAATTTCAA TGAAAATTCCG GAGAAGAAGG AGTAATTAGC TCTGATTTCC  420
ATCTTAGTCA CCCGAATTTT GTTCTGCAGC AGGGAAGTAA GGCGATTGAG ATTTTGATAT  480
ATTTTTGATA CATCAAATGC GGAATCTTGA AACAGAGCAT GAACTTTCTG AGTGACAGTT  520
AGCATGTTGG GTTCATCAGT TGAAGCTCTG AATTCTGAG
```

BA213c14t7[1,2]

```
CAATTG ATTCATTTTA TGTAGCGAG
TTTATGCAAT GACTAGAAAA TATAACATAT AACTAAATTA ACAAGAACGC CGAAGACATA   60
CTTACATACT TTGACCGCTT TCTGAGAAAC TCAAATAGCT AACTGATGTA TAATGAGAAC  120
CTTATAGGTC ATGCATATAT TAATTAGTAT GTACCAGGTA AATTTATGTC ACGGCAAAAC  180
AGCAATAAGC AGAATATACT ATTGAATGAA TAACGTCCAC ATTACGATAT CCAAACTAAT  240
AGGTTGAGTT CCTACATGTA CTTTATGTGC AAGAAATATG CAAGTATTGG AGATTAAACG  300
TCTGCATATA AGGATAATCT AACCTGGGCA GTCAGAGACT TGATTACGTG TTTCGCAGCT  360
CTTCTTTTCT CTGCTTCAAC AGCTGCTTTT GCAGTTGCAT CCATCAGCTG CTTTGTCTTT  420
CTCCCAAGTT CAGCTTCTAG AAGTTGAGAT TTGCTTGCAA GCTCTTCCAG CTACAAATAA  480
TTTTTGAGTC AAGCTTAGCA GTGTGAAACT TTTGTGATCA CTTTACTTAG TTGCTCGCAG  520
AGGTTTGCGT CAAG
```

Protein kinase[1]

```
ACTTGAAATG ATAACAGGTA GGAGATCGAT GGACAAGAAC CGACCAAATG GGGAACACAA   39
TCTTGTTGAA TGGGCACGAC CTCATCTTGG TGAAAGAAGA AGGTTTTACA GATTGGTAGA   99
TCCTAGACTT GAAGGCCATT TTTCAATAAA AGGTGCTCAG AAAGCTGCAC AGTTGGCCGC  159
TCGTTGCCTT AGCCGTGATC CCAAAGCTAG ACCTATGATG AGTGATGTGG TTGAAGCCTT  219
GAAGCCATTA CCAAATCTTA AAGACATGGC CAGCTCATCC TACTATTTCC AGACAATGCA  279
AGCAGACCGA GTTGGATCAA GTCCAAGTAC CAAAAATGGC GTTAGAACAC AGGGATCGTT  339
CTCGAGGAAT GGACAACAAC ATCCTAGAAG TCTTTCAATC CCAAATGGTT CTCATGCTTC  399
TCCATACCAT CAGCAATTTC CCTCAGAACT CACCAAAACC AAACGGCAAA ACTTAGTATT  459
ATTGGATTGA CAAGTAATCT GTTTCTACCA TTCTTTTCGT TTTCTCCCCA GCTATGAATA  519
TATTTTGTTG GCCACCTCCC GTTTTGTCGT TGGATGAACT GGCAAAAGGG              579
```

GP179[1]

```
CTGCAGTGGT TTTAGTGATT GTGCTGCTCT TTCTCTTGTT TTGGTTTTTC TCTTTAAAAC   60
ATTTCAATGG TGTATCAAGT CAAATGTGGT TCTTTAGAGT ATCAACTGCC TGCAAACCAA  120
GTACAAATAA TGCTTAAATA GTGAGACGTA CTAGATGTTC CTCCTTTGAA ATATGTTTCA  180
CTTCTAAGTG ATAATCTTGA TAAGTGATGA TGTTTGCTGT ATATAACTCA AAACTTCAGG  240
```

```
TTCAACTTCT GTTTTTTTGG GAGATTGTAC TAATTTGAGC TCGGCCCTGA CCCCAGGCAT 300
CCAGATTCTC TACTACACAA TACATAATGC ATGTAGCCAT GGGCTGGAAA GATGAAAGTG 360
```

***StPto*[1]**

```
TCACATTGGA TTGGGTGGCT TTGGGAAAGT TTACAGGGGT GTTTTGCGTG ATGGGACAAG  41
GTGGCCCTGA AGAGGTGTAA GCGTGAGTCC TCACAAGGTA TTGAAGAGGT TCCAAACAGA 101
AATTGAGATT CTCTCTTTTT GCAGCCATCC GCATTTGGTT TCATTGATAG GATACTGTGA 161
TGAAAGAAAT GAGATGATTC TANTTTATGA CTACATTGAG AATGGGAACC TCAGGAGCCA 221
TTTGTATGGG TCAGATCTAC CAAGTATGAG CTGGGAGCAG AGGCTGGAGA TATGCATCGG 281
GGCAGCCAGA GGTCTGCATT ATTCTTCATAC TAGCGCAGTT ATACATCGTG ATGTCAAGTT 341
TATAAACATA TTGCTTGATG AGAATTTTGT GGCAAAAATG ACTGATTTTG GACTATCCAA 401
GAAAGGGACT GAGCTTGATC AAACCCATCT TAGCACCCTT GTGCAAGGAA CTATAGGCTA 461
CCTTGACCCT GAATATTTTA ANAGGGGACA ACTGACAGAA AAATCTGATG TTTATTCTTT 521
CGGTGTTGTT TTATTCGAAG TTCTTTGTGC TAGGCCTGCC ATAGTTCAAT CTCTTCCAAG 581
GGAGATGGTT AGTTTAGCTG AATGGGCAGT GGACTCG                          641
```

***239E4Left*[1,2]**

```
GGCCCCACAA ACAAGAAAAC AGAGAAAGGA AACTTAAGAG ATAGAGAGAG ACCACATTAT  40
TCGCCCATTT CAATATGAAA TTGTTATGCT GAAAGAATCT TTTTATATTT ATTTGAAGTC 100
CTCTGAGGAG GGTTTCCTGG GAAGGGTACC TCGTCGAACA AGAATGTCCC ATTTGGATAT 160
TGCTCTTGTT TCAAGTAGTT GAGGAATTTT ATCTCATTGT TTGAACTTTT TGGGGAGCAT 220
ACGAGTGTTT GTTGATCCTG ACACTGCAAG AAATCTGTTT TTTTCCAGGA ATTGGTGCAA 280
GGATTTGATC AAGAGACTGC TGCAGTTGTA GTGGCTAGAT TAGCTTCTTA CAAAATGGAG 340
ATGGAGGTAC CT                                                     400
```

***StPAD4-1*[2]**

```
ATGGAATCGG AAGCTTCATC GTTCGTAAAC ACTAACTTTT CAACAACTTT ATTAGAATTT  60
TATGCAATTT GAATTTTCTT TCACTTAATT AAAGGGAACT CATGATGCCT GCAGGTTCGA 120
GTCTAGTGAG ACTTTGGCAG CTCTTGTGGC ATCGACGCCG TTGCTGGAGG AGTCATGGAA 180
GGTTTGTGGC GTCGCCGATG CATCGGTCGA TAGCAATTTC GCCGTCAATC GAGTTGGTGG 240
GACAGCCTAT GTGGGATTCT CCGGCGTAAA ATTGGGCGCC GGAGTGGACC AAAGTTGCCG 300
GAATTTAGTG CCGCTTCCGG ATGAACTTTT CTCTTCGTTG TGCTTGGATG GGGCGGATCC 360
GGCAATGGTC CATGCCG                                               400
```

***StSGT1-1*[2]**

```
ATGGCGTCCG ATCTGGAGAC TAGGGCTAAA GAGGCGTTCA TCGACGACCA CTTTGAACTC  60
GCCGTTGACC TCTACACTCA AGCCATAACG ATGAGCCCTA AGAACCCTGA ACTTTTCGCC 120
GACCGTGCTC AGGCCAATAT CAAACTCAAC TACTTCACTG GTATCATCTT TTTTTAAACC 180
TAGTTAATCC GTTAATTTGA TTATTTGTAA GAAACGGCGC TGATTGTATT ATTTTGCTAT 240
GTTATTGCGT TCCGCGATTT ATAGAATTGG TTAAAGGTTT TGTGGATTTG TGCTTAGCTT 300
TATGCTTTTG GCATACTTGT TCTGTGCTTG GTTACGAAAG TTAACAACCG CTATCTGCTA 360
CTTGACTGCG TGGGTTTTTG GTGAATAGTT TGTTAAGCCT TTTTTGGATT ACGATTTTAA 420
TGTGTCGTTG GTACACTTTT ATTAGATCCG GTGTTTTTTT TAGGTCCTGT GTTTCTGTTA 480
ATTGGT
```

***BA81l15t3*[2]**

```
TTTAAAATCT GTTGGGTCTT CCTATAAGTT GGCCTTTTGT CGTATAAGGT GTCCCTTAGA  60
TTTCGATGGT ATAAGTGATG AAACTGGGGC ACATTCATTC TATCTCCAGT TTGTAAGTTG 120
TTATTTATGC TGGCTTGGTG GATATCATCT TTGGACTCGT CGTCCTTGTT CTTTCCCAAT 180
CTCTCTTTTT CGTTTTTTTCA TACAGTGCAG AACGACTATC CATTGATAAT TTTAAAAAAA 240
AGAAAAACAA AATGTCATGT TTAGTGGTTT CA                               260
```

Partners involved in different parts of the work

Laboratory of Genetics: Hans de Jong

Jens Lübeck and Josef Strahwald

Eckhard Tacke and Hans- Reinhard Hofferbert

Birgit Kersten, Svenja Meyer and Rico Basekow

Agim Ballvora, Birgit Walkemeier, Joao Paulo, Christiane Gebhardt and Maarten Koornneef

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Gabi: **G**enom**a**nalyse im **bi**ologischen System Pflanze

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Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschliesslich Tabellen, Karten und Abbildungen - die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Unversität zur Prüfung vorgelegen hat; dass sie - abgesehen von den unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, das ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

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03. Juli 2007

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Datum Unterschrift

Publications

Sattarzadeh A, Achenbach U, Lübeck J, Strahwald J, Tacke E, Hofferbert RH, Rotstein T, Gebhardt C (2006) Single nucleotide polymorphism (SNP) genotyping as basis for developing a PCR-based marker highly diagnostic for potato varieties with high resistance to *Globodera pallida* pathotype Pa2/3. Mol. Breed. 18: 301-312