The Lily Yearbook
of the
North American Lily Society

NUMBER SIXTY-THREE

2011 - 2012

CALVIN HELSLEY
JAAP M. VAN TUYL
EDITORS

Published by the Society

Contents

An Introduction to the Lily Breeding Research at Wageningen University and Research Centre 5-10
Jaap M. van Tuyl

Easter Lily Research in Southern Japan 11-19
Eisuke Matsuo

The Development of Storage Methods for Clonal Material of Lily (*Lilium* L.) 20-26
Frans Bonnier & Jaap van Tuyl

Micro Propagation of Lily: History, Obstacles and Advancements on the Horizon 27-38
Geert-Jan de Klerk

Avoidance of Cross-Contamination During the Initiation Step in Lily Tissue Culture 39-44
Naser Askari & Geert-Jan de Klerk

A Cytogenetics Lesson from Lilies 45-54
Rodrigo Barba-Gonzalez

How to Obtain Unreduced Gametes 55-59
Rodrigo Barba-Gonzalez

Fertility Recovery and Polyploidization of Interspecific Hybrids 60-67
Ki-Byung Lim & Jaap M. van Tuyl

Overcoming Crossing Barriers in Hybridisation with OT-hybrids 68-77
Jianrang Luo, Paul Arens & Jaap M. van Tuyl

Cytological Maps Based on Recombination Sites Detected by GISH in Interspecific Lily Hybrids 78-85
Nadeem Khan, Agnieszka Marasek-Ciolakowska, Munikote Ramanna, Paul Arens, Alex van Silfhout & Jaap M Van Tuyl

The Use of Chromosomal Markers for Interspecific Hybrids Verification in *Lilium* *86-90*
Agnieszka Marasek-Ciolakowska & Teresa Orlikowska

Meiosis in Interspecific Lily Hybrids 91-94
Songlin Xie

Different Ways to Create Triploid Lilies 95-99
Shujun Zhou & Jaap van Tuyl

The Potential of Aneuploids for Selecting New Lily cultivars 100-105
Shujun Zhou

Molecular Markers as a Tool for Parental
Selection for Breeding in Lilium 106-112
Arwa Shahin, Paul Arens, W. Eric van de Weg & Jaap M. van Tuyl

The Effect of Sugar and ABA on the Longevity of Lily Flowers 113-119
Arwa Shahin, Alex van Silfhout, Francel Verstappen,
Harro Bouwmeester, Jaap M van Tuyl & Paul Arens

The Correlation of Lily Saponins Content and Resistance
to *Fusarium oxysporum f.sp. Lilii* *120-125*
Hongzhi Wu, Sixiang Zheng, Yufen Bi & Li Yi

The Outcome of Lily Breeding Research at RDA, Rep. of Korea 126-134
Ju Hee Rhee & Kang Yun-Im

Lilium pumilum DC. on the Roof of the World 135-138
Nan Tang, Daocheng Tang & Jaap van Tuyl

Lily Breeding in Nanjing Forestry University 139-143
Mengli Xi & Jisen Shi

Development of Lily Production in Northeast China 144-147
Lianwei Qu

Studies on *Lilium lancifolium* in China *148-151*
Zhigang Wang & Huihua Zhang

Development of Lily Production in Yunnan, China 152-155
Xue Wei Wu, Li Hua Wang, Jing Hong Yang, Ji Wei Ruan,
Su Ping Qu & Ji Hua Wang

Lily Genetic Modification at Wageningen UR 156-163
Frans Krens & Bernadette van Kronenburg-van de Ven

Plant Cytogenetics: Terms and Techniques 164-173
Nadeem Khan

NALS Officers Directors and Committees *174-176*

An Introduction to the Lily Breeding Research at Wageningen University and Research Centre

Jaap M. van Tuyl, Plant Breeding, Wageningen University and Research Centre (WUR), The Netherlands

At wageningen university (WUR), lily breeding research was started more than 40 years ago. It started with the building of a *Lilium* species collection (De Jong, 1974) and actual breeding research was initiated in 1977 (Van Tuyl, 1980). A large number of projects were carried out and reported in the last 30 years in the NALS Lily Yearbooks. These articles were focussed on the breeding of Asiatic lilies for low light conditions (Van Tuyl & Van Groenestijn, 1983), interspecific hybridization between *Lilium longiflorum* and the white Asiatic hybrid 'Mont Blanc' (Van Tuyl et al, 1988), the ability of *Lilium longiflorum* to be grown in the Netherlands (Van Tuyl, 1988) and on polyploidization of lilies (Van Tuyl, 1986 and Van Tuyl and Kwakkenbos, 1989). The 1990 volume was completely filled with research reports of Wageningen UR: Survey of research on mitotic and meiotic polyploidization at CPRO-DLO (Van Tuyl, 1990); The use of oryzalin as an alternative for colchicine in *in vitro* chromosome doubling of *Lilium* (Van Tuyl et al. 1990); Breeding for *Fusarium* resistance in lily (Löffler et al. 1990); Breeding for resistance against *Fusarium* in tetraploid *Lilium* (Straathof and Van Tuyl, 1990); *In vitro* selection for resistance against *Fusarium oxysporum* in lily: prospects (Löffler et al.); Application of *in vitro* pollination techniques in breeding and genetic manipulation of lilies (Bino et al. 1990); Preliminary examination of some factors causing variation in flower longevity of *Lilium* cut flowers (Van der Meulen-Muisers and Van Oeveren, 1990); Development of a culture system for microspores of lily (Van den Bulk et al., 1990); Wide interspecific hybridization of *Lilium:* Preliminary results on the application of pollination and embryo-rescue techniques (Van Creij et al., 1990). In the last 20 years only one article was published in the Lily Yearbook: Introgression with *Lilium* hybrids: Introgression studies with the GISH method on *L. longiflorum* x Asiatic, *L. longiflorum* x *L. rubellum* and *L. auratum* x *L. henryi* (Van Tuyl et al., 2002). Therefore in this volume we will catch up and give an overview of recent developments in lily research within Wageningen UR Plant Breeding.

In the last 15 years eight PhD-students finished their study in lily. Frans Bonnier was a researcher in the Urgency Program for Bulb diseases and Breeding Research from 1989-1993 and focussed on long term storage of bulb crops and defended his PhD-thesis entitled "Long term storage of clonal

material of lily (*Lilium* L.)" on 22-9-1997. In this volume of the lily yearbook he summarized his work: The Development of storage methods for clonal material of lily (*Lilium* L.). Ki-Byung Lim, a graduate of Kyungpook National University (South Korea) received his PhD of Wageningen University with a thesis "Introgression breeding through interspecific polyploidisation in lily: a molecular study" in 2000 (November 27). He is an NALS-member and contributes in this volume with a paper on "Fertility of interspecific hybrids and recovery of fertility in Lilium Interspecific hybrids". With him also Munikote Ramanna, retired cytogeneticist joined our group and encouraged students to study the cytogenetics of lily. He was responsible for the elucidation and understanding of mechanisms of 2n-gamete formation and intergenomic recombination in a range of interspecific *Lilium* hybrids. Rodrigo Barba Gonzalez studied in Mexico (Universidad Guadalajara) and came in 2002 to Wageningen for his PhD-study. He graduated in 2005 (13 September) on a thesis with the title: "The use of *2n* gametes for introgression breeding in Oriental × Asiatic lilies". He is a member of the NALS and wrote two papers for you, one about "How to obtain unreduced gametes" and another one called "A cytogenetics lesson from lilies". Shujun Zhou studied at Beijing University in China to obtain his master degree in Botany in 1992. In 2004 he came to Wageningen for his PhD-study. On March 27 2007 he defended his thesis: "Intergenomic recombination and introgression breeding in Longiflorum x Asiatic lilies". He contributed 2 articles one on breeding with triploids and another one about aneuploids. Nadeem Khan studied Biochemistry, Botany and Plant Physiology at different universities in Pakistan and came in 2006 for his PhD to The Netherlands. June 3 2009 he obtained his degree on a thesis: "A molecular cytogenetic study of intergenomic recombination and introgression of chromosomal segments in lilies (*Lilium)".* You can find 2 articles from his hand, one about terminology in cytogenetics and one about chromosomal recombination sites, in detailed description in his thesis. Arwa Shahin studied at Damascus University in Syria. In January 2008 she started her PhD program at Plant Breeding of Wageningen University. Her thesis "Development of Genomic Resources for Ornamental lilies (*Lilium* L.)" was completed in 2012 and defended in public June 19, 2012. She wrote an article about the main subject of her thesis: "Molecular markers as a tool for parental selection for breeding in *Lilium"* and one about vase life in lilies. Songlin Xie studied at Northwest A&F University in China and became a PhD-student in 2006 and came to Wageningen in 2007, where he worked for 2 years on chromosome behaviour in lily hybrids. He returned to China for one year to finish his Chinese PhD and came back in 2011 to receive his PhD-degree in Wageningen on

June 6, 2012 with a thesis: "A molecular cytogenetic analysis of chromosome behaviour in *Lilium* hybrids". In his contributed article he explains meiotic processes in interspecific hybrids. Now he works as head of the Group of bulbous flower breeding, Sino-Europe Agricultural Development Centre in Zhangzhou, China, where he is secretary of the third Symposium on the genus Lilium to be held in April 2014. Jianrang Luo is researcher at the College of Forestry, Northwest Agricultural and Forestry University. He came in 2010 for a sandwich PhD to Wageningen. In 2013 he obtained his PhD-degree in China on a dissertation called "Analysis of chromosome behaviors and gamete fertility of OT (Oriental x Trumpet) lily hybrids". For the Yearbook he produced an article based on his PhD: "Overcoming crossing barriers in hybridization with OT-hybrids". Nan Tang is a PhD-student of Northwest A&F University in China, now in Wageningen working on tulip, but in China she worked with her father Daocheng Tang from Quinhai University on the distribution of *Lilium pumilum* at the Qinghai-Tibet plateau. In this Yearbook she describes this project. Naser Askari is a PhD-student from Iran and works on a dissertation in lily tissue culture under supervision of Geert-Jan de Klerk senior researcher in Plant Breeding. His article describes a technique to prevent contamination in tissue culture of lily. The title of his article is "Avoidance of Cross-Contamination during the Initiation Step in Lily Tissue Culture".

Besides the work of PhD-students also guest researchers participated during the years in our lily research. One of the first was Eisuke Matsuo. When I visited Japan in 1981 he guided me to Okino-Erabu, the native island of *Lilium longiflorum*. In 1983 he was our guest researcher for one year and worked mainly with *L. longiflorum*. Ju-Hee Rhee (before Hye-Kyung Rhee) worked for many years at RDA, Korea on breeding of lily and was a guest researcher in 1997 and 2003. In 2002 she finished her dissertation from Seoul National University on interspecific hybridization of lilies in South Korea. She reports about her work carried out at RDA in Korea. Agnieszka Marasek-Ciolakowska is a researcher at the research institute of Horticulture in Skierniewice , Poland. In 2002 she received her PhD-degree in lily. For the yearbook she wrote an article about this research: "The use of chromosomal markers for interspecific hybrids verification in *Lilium*". From 2007 till 2011 she was a guest researcher in our group and worked mainly on tulip. Hongzhi Wu received her dissertation from Yunnan Agricultural University in 2008 on a dissertation with the title: "*Lilium* oriental breeding via 2n gametes and an analysis on resistance of their progenies to *Fusarium* bulb rot disease". For the yearbook she reported on the relation between saponin content in the bulb and the Fusarium resistance of a genotype. Mengli Xi is

a researcher at Nanjing Forestry University, China and guest in our lab in 2012. She established in Nanjing a lily breeding group ten years ago and exploited native Lilium species as she summarized in her contribution. Lianwei Qu studied at Shen-Yang Agricultural University in China and worked for one year in our group (2012-2013). He has written his story with lilies and the production of lilies in North-east China. Zhigang Wang is researcher in the Flower Institute of the Liaoning Agricultural Academy of Sciences (LAAS) and head of the lily breeding group. He has written an article on "Studies on of *Lilium lancifolium* in China". Xuewei Wu works at the Flower Institute in Kunming (Yunnan) and worked there on lily breeding for almost 10 years. I visited the flower Institute in Kun Ming several times. He obtained his PhD thesis recently (May 2013) on "Studies of bulb harvest date and vernalization on growth and flowering of lily" from Dankook University, South Korea. He describes the development of lily production in his home province Yunnan.

Geert-Jan de Klerk is senior researcher in Plant Breeding and worked for many years on optimisation of tissue culture of flower bulbs. He is an expert and shows that in his paper: "Micro propagation of Lily: History, Obstacles and Advancements on the Horizon". Frans Krens is group leader in Plant Breeding and expert in Genetic transformation in many plants. Also in lily genetic transformation is already for more than 25 years an important research topic, as can be seen from his review article.

Finally I mention Paul Arens who joined the Wageningen lily group six years ago to take over my work step by step. You can find him as co-author in several papers here presented. I am grateful to all contributors for their willingness to summarize their work for the members of the North American Lily Society and fill a full Yearbook. I hope you enjoy it!

References

De Jong, P.C. (1974). Some notes on the evolution of lilies. *The Lily Yearbook of the North American Lily Society* 27: 23-28.

Löffler, H.J.M., J.R. Mouris and M.J. van Harmelen, (1990). In vitro selection for resistance against *Fusarium oxysporum* in lily: prospects. *The Lily Yearbook of the North American Lily Society* 43: 56-60.

Löffler, H.J.M., Th.P. Straathof, R.P. Baayen, and E.J.A. Roebroeck, E.J.A. (1990). Breeding for *Fusarium* resistance in lily. *The Lily Yearbook of the North American Lily Society* 43 : 51-55.

Straathof, Th.P. and J.M. van Tuyl, (1990). Breeding for resistance against *Fusarium* in tetraploid *Lilium*. *The Lily Yearbook of the North American Lily Society* 34: 23-27.

Van den Bulk, R.W., H.P.J. de Vries-Van Hulten and J.J.M. Dons, (1990). Development of a culture system for microspores of lily. *The Lily Yearbook of the North American Lily Society* 43: 45-50.

Van Creij, M.G.M., L.W.D. van Raamsdonk and J.M. van Tuyl, (1990). Wide interspecific hybridization of *Lilium*: preliminary results of the application of pollination and embryo-rescue methods. *The Lily Yearbook of the North American Lily Society* 43: 28-37.

Van der Meulen-Muisers, J.J.M. and J.C. van Oeveren, (1990). Preliminary examination of some factors causing variation in flower longevity of *Lilium* cut flowers. *The Lily Yearbook of the North American Lily Society* 43: 61-66.

Van Tuyl, J.M. (1980). Lily breeding research at IVT in Wageningen. *The Lily Yearbook of the North American Lily Society* 33: 75-82.

Van Tuyl, J.M. and J.E. van Groenestijn, (1982). Breeding Asiatic lilies for low light requirement. *The Lily Yearbook of the North American Lily Society* 35: 106-111.

Van Tuyl, J.M. and A.A.M. Kwakkenbos, (1986). Some examples of meiotic polyploidization in *Lilium*. *Quart. Bull. of the North American Lily Society* 40(2): 10-11.

Van Tuyl, J.M. (1988). Dutch-grown *Lilium longiflorum* a reality. *The Lily Yearbook of the North American Lily Society* 41: 33-37.

Van Tuyl, J.M., C.J. Keijzer, H.J. Wilms and A.A.M. Kwakkenbos, (1988). Interspecific hybridization between *Lilium longiflorum* and the white Asiatic hybrid 'Mont Blanc'. *The Lily Yearbook of the North American Lily Society* 41: 103-111.

Van Tuyl, J.M. and A.A.M. Kwakkenbos, (1989). Research on polyploidy in interspecific hybridization. *The Lily Yearbook of the North American Lily Society* 42: 62-65.

Van Tuyl, J.M. (1989). Research on breeding polyploid lilies at IVT Wageningen. *Quart. Bull. of the North American Lily Society* 43(2): 23-28.

Van Tuyl, J.M. and H.M.C. van Holsteijn, (1990). An introduction to the lily breeding research at CPRO-DLO. *The Lily Yearbook of the North American Lily Society* 43: 6-8.

Van Tuyl, J.M. (1990). Survey of research on mitotic and meiotic polyploidization at CPRO-DLO. *The Lily Yearbook of the North American Lily Society* 43: 10-18.

Van Tuyl, J.M, B. Meijer and M.P. van Diën, (1990). The use of oryzalin as an alternative for colchicine in in-vitro chromosome doubling of *Lilium*. *The Lily Yearbook of the North American Lily Society* 43: 19-22.

Van Tuyl, J.M., Mi-Young Chung, Jae-Dong Chung and Ki-Byung Lim, (2002). Introgression with *Lilium* hybrids: Introgression studies with the GISH method on *L. longiflorum* x Asiatic, *L. longiflorum* x *L. rubellum* and *L. auratum* x *L. henryi*. *The Lily Yearbook of the North American Lily Society* 55: 17-22.

Easter Lily Research in Southern Japan

Eisuke Matsuo (Professor Emeritus, Kyushu University)

1198-68, Tsuko, Ogori-shi, Fukuoka-ken 838-0102 Japan

1. Native Easter lily in Senkaku Retto, Ryukyu

My lily research, which continued over approximately 20 years, started in 1970 with Easter lily (*Lilium longiflorum*) bulbs which I collected at the Uotsuri-jima Island, Senkaku Retto (Pinnacle Islands), Ryukyu, in the East China Sea. These islands are parts of Ryukyu Islands that are known as a native habitat of Easter lily.

From the 6th to 15th December 1970, I stayed on this uninhabited island as one of the members of the Scientific Exploration Team of Senkaku Retto, jointly organized by Kyushu University and Nagasaki University. We observed many Easter lily populations, both near the shoreline and near the top of the hill (363m above sea level) (Photos 1 and 2). I collected samples from these populations, and took them back to Fukuoka for further study.

Photo 1. Wild Easter lilies close to the shoreline of the Uotsurijima, senkaku Retto.

Photo 2. A wild Easter lily near the top of the hill of the Uotsurijima, Senkaku Retto.

My first work on Easter lily was to examine the ability of scale propagation of these collected stocks in comparison with the typical Japanese cultivars (Matsuo, 1972). Parts of these original stocks and scale-propagated progenies were sent to Kobayashi of the Kagoshima Agricultural Experiment Station for his further investigations.

After moving to the Kagoshima University in 1974, I was engaged in the study of scale propagation and bulb production of lilies, needed for producing Easter lily bulbs which were the most important product exported at that time from Okino-erabu Island, Kagoshima-ken. As a result of this study, an article on types of leaf emergence from scale bulblets was published in the Lily Yearbook of the North American Lily Society in 1976 (Matsuo and Arisumi, 1976). (Figure 1).

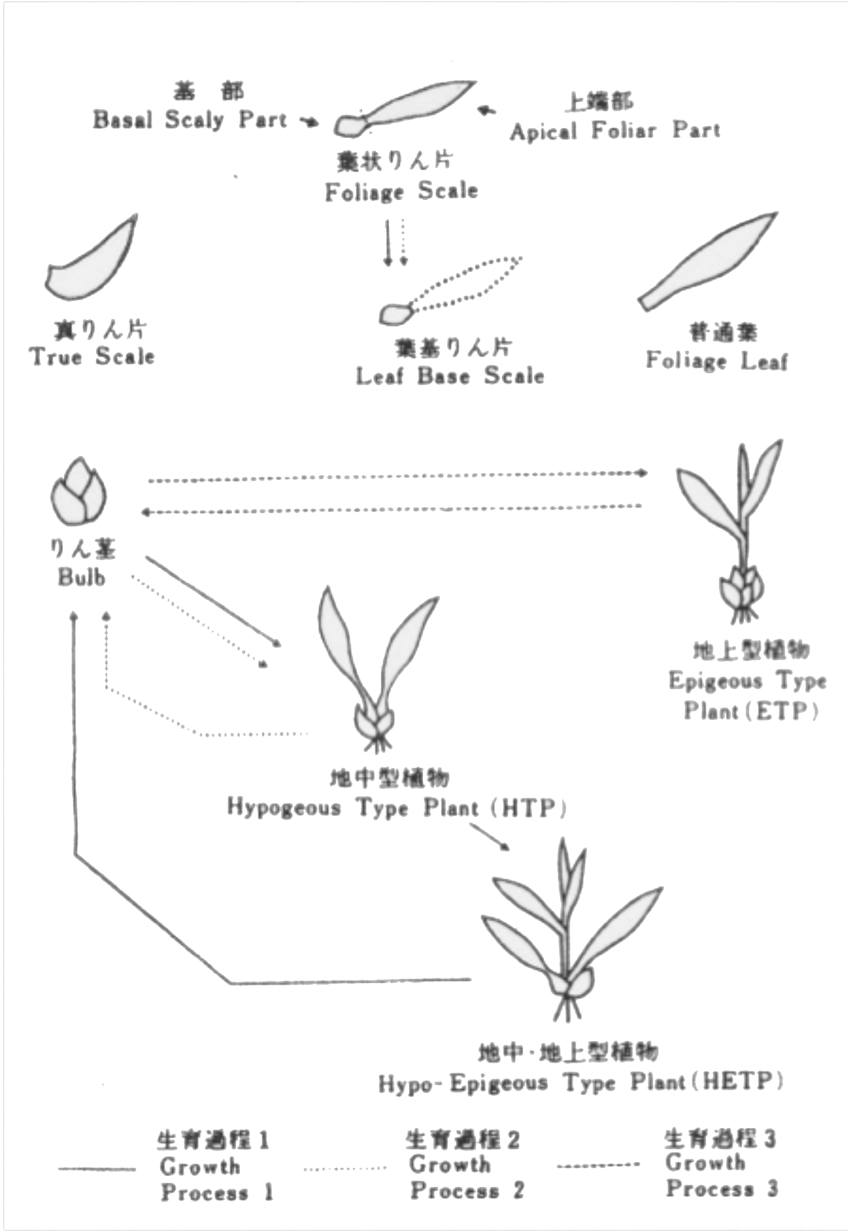

Fig. 1. Types of leaf emergence from the Easter lily bulb. (Modified after Matsuo and Arisumi, 1976).

While I was working at Kagoshima University, well-known lily researchers from other parts of the world visited Okino-erabu and/or Kagoshima for their lily research studies; for example Dr. J. M. van Tuyl (Institute of Horticultural Plant Breeding, The Netherlands; IVT) in 1981 and 1992, Dr. A.N. Roberts (Oregon State University) and his bulb growers' group in 1986 (Kii, 1991), Dr. S.M. Roh (USDA) in 1987, and Dr. H.F. Wilkins (University of Minnesota) in 1992.

2. Lily study in the southern Japan

When I started the lily research program, Okino-erabu was the most famous lily bulb producing area in the world, and there were some well-known lily growers, researchers and breeders in Kyushu.

Masayoshi Kobayashi (Kagoshima Agricultural Experiment Station) was a lily breeder and a supervisor of lily bulb production in Okino-erabu. Easter lily bulb production developed greatly under his supervision. His most striking achievement was the development of the "Oyako-rinpen ho (Mother bulb block system)" for propagation of virus-free scale bulblets. This was a method to plant the mother bulb and its scales on a line, and if the mother plant showed any virus symptom, all of scale progenies of this bulb were discarded before scale bulblets were used for bulb production. Thus, the spread of the virus was decreased, resulting in good quality bulb production in Okino-erabu, before the micro propagation method was developed.

Dr. Tokiharu Matsukawa is known for introducing the Easter lily cultivar 'Hinomoto', which was a leading cultivar for more than 30 years in Japan.

According to his personal communication and interview, he observed this *longiflorum* type in the garden of Kiemon Nakahara', in a suburb of Fukuoka-shi in 1959. The owner told him that it was collected by Higo Mokuzai Co Ltd in the Yakushima Is., Kagoshima. Dr. Matsukawa recognized its superiority for the use as a cut flower, and started trials to investigate its forcing ability.

In 1962, after three years of testings, he named this scale-propagated bulb stock 'Nippon' (Japanese name of Japan) in 1962, for examination of the Fukuoka-ken New Cultivar Judging Committee. As the nation's name is not permitted for a plant cultivar, he changed the name to 'Hinomoto' which is an older/less formalized name of Japan. The 'Hinomoto' line was registered in 1965 as a new Easter lily cultivar of the Ministry of Agriculture Plant Name Registration System.

All of the rights on 'Hinomoto' were transferred to the Okino-erabu Kyuukon Seisan Kumiai (The Bulb Growers Corporation in Okino-erabu), which resulted in the more prosperous Easter lily bulb production in Okino-

erabu.

While his forcing ability tests of Easter lily were progressing, Dr. Matsukawa noticed that "brushing plants on the top of plants" made plants dwarf. This is called "Sesshoku-Waika" (dwarfing induced by brushing) in Japanese. After some experiments with Easter lily and *L. speciosum*, he presented this fact at the Fall Meeting of the Japanese Society for Horticultural Science in 1971 (Matsukawa and Kashiwagi, 1971), and submitted the manuscript for the HortScience. He received the reviewed manuscript with some comments. He did not re-submit the revised manuscript for private reasons, resulting in this amazing finding not being published at that time. Familiar phenomena were reported by shaking the trunk of *Liquidambar* (Neel and Harris, 1971), the stem of corn plants (Neel and Harris, 1972), leaves and stem of chrysanthemum (Hammer et al., 1974) and by rubbing the internode of several plants (Jaffe, 1973), and Mitchell et al. (1975) described such phenomena as "seismomorphism" based on his precise experiments with tomato plants, but not brushing the crown or top of plants as shown by Dr. Matsukawa.

In 1999 Dr. Matsukawa was awarded "Matsushita-Konosuke Hanano-Banpaku Kinensho (Matsushita Konosuke Flower Prize)" for his achievements including his introduction of 'Hinomoto' and finding and practical application of "Sesshoku waika" (dwarfing of plants by brushing) (http:matsushita-konosuke-zaidan.or.jp/works/flowerprize/win/index.html, 2013).

Yukio Kuwahara was a bulb and cut flower grower in Kagoshima, with whom I co-worked around the 1990s to improve the bulb storage method for forcing in Japan. Until 1980s bulbs were stored at 2C in wet sawdust in a wooden box. It was a hard work to pack bulbs with wet sawdust in a wooden box by hands and to carry the box in and out the storage room. Kuwahara and I stored bulbs in a polyethylene bag without sawdust to keep them in wet condition and to decrease the box weight for easy treatment. These bulbs resulted in the same quality of cut flower as the traditional sawdust storage (Matsuo and Kuwahara, 1992). This method has become popular in Japan.

In 1990 he and I visited the lily bulb producing areas in Oregon and California, USA. He was very surprised to know that this area was rich in stones. This visit made him grow lily bulbs in stony volcanoes soils of "Mt. Kaimondake", Kagoshima.

Later Kawahara became a superior farmer of lily bulb and cut flower production, becoming a specialist supervisor of the freshmen in agricultural extension courses, and of researchers at the Kagoshima Flower Experiment Station.

3. Lily study in the Netherlands

The visit of Jaap van Tuyl to Kagoshima in 1981 gave me a chance to study lily breeding and production in the Netherlands. With partial financial support from Kagoshima-ken Ikuei Zaidan and the IAC (The International Agriculture Centre, Wageningen, The Netherlands), I studied lilies with him as a visiting researcher at the Institute of Horticultural Plant Breeding in the Netherlands (IVT) from September 1982 to August 1983. At that time he was the Head of the Lily Breeding Section, IVT, being engaged in lily breeding and releasing the Easter lily 'Gelria' (Photos 3 and 4).

Photo 3. Jaap van Tuyl and his assistant in his breeding field.

Photo 4. Easter lily 'Gelria'.

After observing Van Tuyl's research at the IVT and the techniques of the Dutch bulb growers, I predicted that the Easter lily bulb production for export in Okino-erabu would decrease sharply in several years (Matsuo, 1994). My prediction, unfortunately, came true during the 1990s (Table 1).

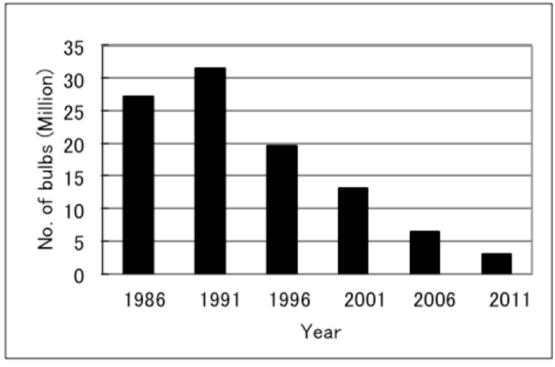

Table 1. Easter lily bulb production in Okino-erabu, Japan (Number of bulbs, million).(Data from "The Documents of Okino-erabu Yuri/Freesia Seisan Kumiai" was compiled by Mr. Oofuku.).

4. Visiting bulb producing areas in the USA

In 1986 and 1990, I visited the Easter lily bulb producing areas located at the boundary of southern Oregon and northern California along the Pacific Ocean, which was the only Easter lily bulb producing area in the USA. To visit both these locations, Prof. Dr. A.N. Roberts (Oregon State University) drove me from Corvallis, Oregon, to northern California. He was one of the famous lily researchers in the USA. He guided me to his Lily Research Center (Photo 5), which was managed by Lee Riddle, and to bulb producing farmers.

Photo 5. Dr, A.N. Roberts Lily Research Center in Brookings, Oregon. Alan N. Roberts (left) and Eisuke Matsuo (right).

At the first visit in 1986, it was amazing to observe the huge scale of the bulb production in Oregon and California, which was supported by agricultural machines and seasonal laborers (Photo 6), as compared with the small scale production managed by farmers by themselves in Okino-erabu.

Photo 6. Lily bulb production in the USA was managed under the supports of big machines and seasonal laborers.

On the second visit in 1990, Yukio Kuwahara, a lily bulb and cut flower producer in Kagoshima, accompanied me to the lily bulb producing area in the USA. He also was surprised to find that the lily bulb production fields there are rich in stones. He applied this practice in the bulb production in Kagoshima.

Moreover, in the 1990s visit we were fortunate to meet Leslie Woodriff and his daughter at his breeding farm "Fairyland Begonia Garden" in McKinleyville, California (Matsuo, 1997). Woodriff is the breeder of famous

Oriental lilies such as 'Star Gazer', 'Black Beauty', etc. (Photo 7).

Photo 7. Leslie Woodriff, Lee Riddle, Eisuke Matsuo and Yukio Kuwahara (from left to right) at Woodriff's home and/or farm in California.

5. 1992 International Lily Symposium in Okinoerabu, Japan

This symposium was organized during the 50th anniversary of Wadomari-cho on 23 April 1992. For this symposium five persons engaged in lily research and business were invited as keynote speakers, as follows (Yuri Festa '92 in Okino-erabu Jikko-iinkai et. al., 1992):

Coordinator of 1992 International Lily Symposium in Okinoerabu, Japan: Dr. Kiyoshi Ohkawa (Shizuoka University, Japan)

Problems of bulb production and forcing in *Lilium longiflorum* Thunb. : Dr. Eisuke Matsuo (Kagoshima University, Japan)

Lily production and breeding in the Netherlands: Dr. Jaap M. van Tuyl (Plant Breeding and Reproduction Centre, Netherlands)

Present situation and problems of Easter lily in the USA: Dr. Harold F. Wilkins (Nursery Exchange, California, USA). (This presentation was the co-work with Dr. John M. Dole, University of Minnesota, who was not present at the symposium).

The circulation of Japanese lily-bulbs in Europe: Frans Onings (P.F. Onings Bulb Company, Netherlands)

I thank my colleagues and informants who gave me much help and/or suggestions during my lily research and preparation of this paper.

Photo 8. Front cover of the Proceedings International Lily Symposium in Okino-erabu, 1992.

Literature cited

Hammer, P.A., C.A. Mitchell and T.C. Weiler. 1974. Height control in greenhouse chrysanthemum by mechanical stress. HortSciencce 9(5):474-475.

http:matsushita-konosuke-zaidan.or.jp/works/flowerprize/win/index.html, 2013.09.10. checked by the author)

Jaffe, M.J. 1973. Thibmomorphogenesis: The response of plant growth and development to mechanical stimulation with special reference to *Bryonia dioica*. Planta (Berlin) 114: 143-157.

Kii, R. 1991. Okinoerabushima – Erabu-teppouyuri freesia. Hana to kaori no tsuushinsha. Wadomari, Kagoshima. pp.221.

Matsukawa, T. and Y. Kashiwagi. 1971. Engei shokubutsu no waika ni oyobosu kusshokusei ni kansuru kenkyuu. 1. Sesshoku ni yoru yuri no waika ni tsuite. Shouwa 46 nendo engei gakkai shuukitaikai kenkyuu happyou youshi: 286-287. (In Japanese).

Matsuo, E. 1972. Studies on the Easter lily (*Lilium longiflorum* Thunb.) of Senkaku Retto. 1. Comparative study on growth responses of scale bulblets in 'Senkaku', 'Hinomoto' and 'Munakata'. J. Jaoan. Soc, Hort. Sci. 41:383-392.

Matsuo, E. 1986. Oranda to Nippon no Tppouyuri Seisan (*Lilium longiflorum* production in the Netherlands and Japan). Shun-endo, Kagoshima. pp257. (In Japanese).

Matsuo, E. 1992. Problems of bulb production and forcing in *Lilium longiflorum* Thunb. pp.1-34. In: Yuri Festa 92 in Okino-erabu Jikko-iinkai, Kagoshima-ken Wadomari-cho and Erabuyuri Freesia Seisan Shukka Kumiai (ed. and publish.). Proc. 1992 International Lily Symposium in Okino-erabu, Japan. pp.194.

Matsuo, E. 1994. Oranda (The Netherlands) to Nippon no teppouyuri seisan. Shun-endou, Kagoshima, Japan. pp.257. (In Japanese).

Matsuo, E. 1997. 'Star Gazer' umino oya, chomeina yuri no ikushuka Woodriff shi no omoide. Engei Shin-chishiki Hana no go 52(8):18-22. (In Japanese).

Matsuo, E. and K. Arisumi. 1976. A glossary of lily terminology. The Lily Year Book North America 29:103-105. (Translated by S. Paschild).

Matsuo, E. and Y. Kuwahara. 1992. Sokusei-yo teppouyuri kyuukon no kan-i reizouhou 2. Kiribana saibai nouka ni okeru polyethylene-dzume kyuukon reizou no jissai. Nougyou oyobi engei (Agriculture and Horticulture) 67(9):1010-1014. (In Japanese).

Mitchell, C., C. Severson, J. Wott and P. Hammer. 1975. Seismomorphogenic regulation of plant growth. J. Amer. Soc.

Hort. Sci. 100:161-165.
Neel, P.L. and R.W. Harris. 1971. Motion induced inhibition of elongation and induction of dormancy in *Liquidambar*. Science 173:58-59.
Neel, P.L. and R.W. Harris. 1972. Tree seedling growth: Effects of shaking. Science 175: 918-919.
Yuri Festa 92 in Okino-erabu Jikko-iinkai, Kagoshima-ken, Wadomari-cho and Erabuyuri Freesia Seisan Shukka Kumiai (ed. and publish.). 1992. Proc. 1992 International Lily Symposium in Okinoerabu, Japan. pp.194.

The Development of storage methods for clonal material of lily (*Lilium* L.)

Frans Bonnier and Jaap van Tuyl,

Wageningen University and research Center, Plant Breeding

Germplasm collections are important for crop improvement and research. The lily gene bank at Wageningen University has maintained several thousands of lily genotypes for more than 40 years and has been successfully used for both crop improvement and research (Van Tuyl et. al., 2011)(Fig 1, 2). Also in China, the main gene centre for *Lilium*, lily germplasm is collected, described, conserved and distributed (Yuan et al., 2011).

Fig 1. A part of the lily collection at Wageningen University.

Lily genotypes must be preserved vegetatively as clones, because the genotypes are unique and heterozygous. Using seeds would affect the unique genetic combinations. Collections of bulb crops are usually maintained by yearly planting, harvesting, and storing of the bulbs. Eliminating one or more seasons of bulb growing by long term bulb storage would reduce costs for maintaining a lily collection. Therefore, research was started to develop techniques for long term storage of lily bulbs (Bonnier, 1997). The objectives of the experiments were:

Fig 2. Lily breeders visiting the lily collection in Wageningen.

1) The development of methods to measure viability.

2) The development of techniques for long term storage.

3) The development of techniques to increase freezing tolerance.

4) The determination of

the involvement of oxidative stress in the loss of regeneration capacity during storage of lily bulbs in moist peat at -2 °C.

Viability of lily scales and scale bulblets

In order to develop optimal storage methods, it was necessary to be able to measure the effects of different storage conditions on the viability of the lily material. Most useful was a fast and easy test for viability. Lily bulbs can be regenerated by the formation of bulblets at the bottom of detached scales (Griffiths, 1933), however this is time consuming. Therefore, ion leakage of lily scales in distilled water was tested as a criterion for viability of lily scales. Ion leakage was measured either by conductivity of the external solution or potassium content of the external solution after 1,5 h after placing the scales in 150 ml of distilled water. Bulbs were artificially damaged by severe cold, heat, rising temperatures or drying out. In all instances, severe damage or death of the material was accompanied by high values of conductivity and potassium leakage. Ion leakage measured by conductivity and potassium content of external solution after 1.5 h leakage of scales gave similar results (Bonnier et al., 1992). Also after storage of lily bulbs at -2°C during 2,5 years, ion leakage could be used as indicator for loss of viability (Bonnier et al., 1994).

Techniques for long term storage

Storage of bulbs in moist peat

Bulbs of Asiatic hybrids, Oriental hybrids and *L. longiflorum* can be stored in moist peat at -2 °C for year-round forcing of lily bulbs (Beattie and White, 1993). The maximum storage duration of *Lilium* bulbs stored by this method was determined for 'Avignon', 'Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc' (Asiatic hybrids), 'Star Gazer' (Oriental hybrid), 'Gelria', and 'Snow Queen' *(L. longiflorum).* The viability was determined by the percentage of bulbs with at least one regenerative scale (bulb regeneration), the proportion of regenerative scales (scale regeneration), and ion leakage of white inner scales. Maximum storage duration based on bulb and scale regeneration varied between 2.9 and 4.0 years for the Asiatic hybrids and between 2.0 and 2.4 years for the other cultivars. Ion leakage of inner scales was increased for all cultivars at a storage duration of 3 years except for 'Enchantment' and 'Mont Blanc'. It was concluded that a lily collection can probably be effectively stored for 2 years at -2 °C in moist peat (Bonnier et al., 2000).

Storage of bulblets from scales in polyethylene bags

Modified atmosphere (MA) packaging in polyethylene film bags has been used to extend the storage life of many crops including pre-cooled tulip bulbs (Prince *et al.,* 1986) . An atmospheric equilibrium develops in the bags, which is enriched in Co_2 and diminished in 0_2. The equilibrium is dependent on the respiratory rate of the material and the gas-permeability of the bags. This method was investigated for lily scale bulblets at different temperatures.

Scale bulblets of 10 lily genotypes, including Asiatic hybrids, Oriental hybrids, *Lilium longiflorum,* and *L. henryi,* were disinfected and stored either dry, sealed air-tight in polyethylene bags (0.05mm thick), or in moist vermiculite in open polyethylene bags for a period of 2 years at -2 °C, 0 °C and 17 °C.

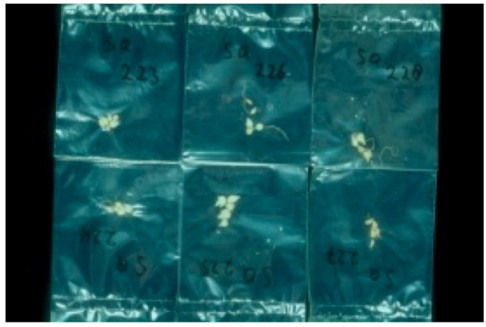

Fig 3. Storage in polyethyleen bags

Storing scale bulblets air-tight in polyethylene bags at -2 °C resulted in the smallest decrease in mass, the least ion leakage and the highest sprouting proportion after 2 years of storage (Fig 3). All genotypes survived 2 years of storage this way (Bonnier et al., 1996).

Storage *in vitro* culture

In vitro storage has several advantages. It requires small amounts of space and the composition of the medium gives an extra opportunity to create conditions of slow growth, for instance osmotic stress or a low concentration of nutrients. The medium and the sealed tubes prevent the bulblets from drying out and make it possible to store the bulblets at a dormancy inducing temperature of 25 °C.

In vitrot regenerated bulblets of 10 lily genotypes (Asiatic hybrids, Oriental hybrids, *L. longiflorum* and *L. henryi)* were stored for 28 months at -2 °C and 25 °C on

Fig. 4. In vitro culture of lily is a standard procedure.

Fig 5. In vitro storage: effect of variations in MS and sucrose in the medium (from left to right: 1/4MS + 9% sucrose , 1/4MS + 6% sucrose, MS + 9% sucrose, MS + 6% sucrose)

four different media: a quarter or standard concentration MS-nutrients with 9 % (w/v) or 6 % sucrose (Fig. 4/5). The combination of a quarter of the MS-nutrients and 9 % sucrose gave the highest reduction in sprout and bulb growth, the highest viability and the highest percentage of regrowth after 28 months of storage. At 25 °C, all lily genotypes survived 28 months of storage under these conditions. At -2 °C, genotypes of *L. longiflorum* and *L. henryi* died during prolonged storage (Bonnier and Van Tuyl, 1997).

Also Godo and Mii (2001) stored 21 lily species in vitro at 25°C for more than one year using callus cultures. However, one of the species, *L. davidii*, could not be regenerated from the callus after the prolonged storage.

Techniques to increase freezing tolerance

Effects of freezing duration, previous storage duration of bulbs at -2 °C, and partial dehydration of scales on freezing tolerance of lily scales were studied for a series of cultivars. Freezing tolerance of scales was estimated by measuring ion leakage and recording scale bulblet regeneration. Both methods gave similar results. Freezing tolerance decreased with freezing exposure. A longer previous storage duration of the bulbs at -2 °C tended to reduce freezing tolerance of the scales. Dehydration of the scales to 10-20 % loss of water content significantly increased freezing tolerance. Further dehydration to 30-40 % loss of water content did not further increase freezing tolerance. Nucleation temperatures, temperatures during crystallization and melting temperatures of the scales were recorded for the cultivar 'Enchantment'. Nucleation occurred at higher temperatures after a longer previous storage duration of bulbs, indicating a reduced capacity to remain super cooled. The increased freezing tolerance of dehydrated lily scales could partly be explained by a decreased melting temperature of the scales. It was concluded that long term storage of lily bulbs at -2 °C was safer after partial dehydration to 10-20% loss of the original water content (Bonnier et al., 1997a).

The involvement of oxidative stress

Possible involvement of oxidative stress in the loss of regeneration capacity was tested for 'Enchantment' scales from bulbs stored for 0 to 5 years at -2 °C in moist peat. Regeneration ability decreased after more than 1 year of storage and was completely lost after 5 years. White (i.e. with no visual damage) scales were used to test whether breakdown of membranes by oxidative stress was an early event in this storage-induced viability loss of lily bulbs. Estimates of changes in ion leakage, the content and oxidation state of glutathione, the content of phospholipids, the content of neutral lipids, the content of free fatty acids, and the degree of unsaturation of fatty acids in phospholipids during storage, gave no indication that oxidative stress is a major factor associated with the loss of regeneration capacity of lily bulbs during cold storage (Bonnier et al., 1997b).

Perspectives

The developed storage methods facilitate the maintenance of a lily germplasm collection. Storage of bulbs needs a lot of space, but has the advantage that plants can quickly be regenerated and used for breeding or research. Storage of scale bulblets in polyethylene bags or in vitro has the advantage that a large collection can be preserved in a relative small place. However, it takes at least one to three years to raise a small scale bulblet into a flowering plant. The possibility to increase freezing tolerance by partial dehydration, and the probable absence of oxidative stress during cold storage give good prospects for the development of techniques providing a further increase in the maximum storage duration of lily germplasm.

Cryopreservation could also be a suitable storage method, as it allows storage for almost unlimited periods. It has been used successfully for apical meristems of some lily genotypes (Bouman and De Klerk, 1990; Matsumoto et al., 1995). However, the preparation of meristems is time consuming, meristems are easily damaged during freezing and thawing and it takes even more time to raise a flowering plant form a meristem than from a scale bulblet.

References

Beattie, D.J. and J.W. White, 1993. *Lilium* - hybrids and species. In: De Hertogh, A.A. and M. Le Nard (eds.). The physiology of flower bulbs. Elsevier, Amsterdam, The Netherlands: page 423-454

Bonnier, F.J.M., 1997. Long term storage of clonal material of lily (*Lilium* L.). PhD-thesis, Wageningen University, 111 pp.

Bonnier, F.J.M. and J.M. van Tuyl, 1996. Freezing of vegetative germplasm of lily for 0 to 4 yr. Acta Hort. 414: 169-173.

Bonnier, F.J.M., J. Keller, and J.M. van Tuyl, 1992. Conductivity and potassium
leakage as indicators for viability of vegetative material of lily, onion and tulip. Acta Hort. 325: 643-648
Bonnier, F.J.M., J. Keurentjes and J.M. van Tuyl, 1994. Ion leakage as a criterion for
viability of lily bulb scales after storage at -2 °C for 0.5, 1.5 and 2.5 years. HortSci. 29: 1332-1334.
Bonnier, F.J.M., R.C. Jansen and J.M. van Tuyl, 1996. Long term lily scale bulblet
storage: effects of temperature and storage in polyethylene bags. Ann. Appl. Biol. 129: 161-169.
Bonnier, F.J.M., R.C. Jansen, and J.M. van Tuyl, 1997a. Freezing tolerance of bulb scales of lily cultivars: effects of freezing and storage duration and partial dehydration. J. Plant Physiol. 151: 627 -632.
Bonnier, F.J.M., F.A. Hoekstra, C.H.R. De Vos, and J.M. van Tuyl, 1997b. Viability
loss and oxidative stress in lily bulbs during long-term cold storage. Plant Sci. 122: 133-140.
Bonnier, F.J.M. and J.M. van Tuyl, 1997. Long term *in vitro* storage of lily: effects of
temperature and concentration of nutrients and sucrose. Plant Cell Tiss. Org. Cult 49:81-87.
Bonnier, F.J.M., J.M. van Tuyl, and A. Tribulato, 2000. Long term storage of lily bulbs at -2 °C. Acta Hort. 508: 329 – 334.
Bouman, H. and G.J. De Klerk, 1990. Cryopreservation of lily meristems. Acta Hort. 266: 331-336
Godo, T., and Mii, M., 2001. In vitro germplasm preservation of lily species utilizing callus cultures at low temperature. Acta Hort. (ISHS) 560:153-155.
Griffiths, D., 1933. Vegetative propagation of the lily. The Lily Yearbook of the Royal Horticultural Society 2: 104-118.
Matsumoto T., A. Sakai and K. Yamada, 1995. Cryopreservation of *in* v/fro-grown apical meristems of lily by vitrification. Plant Cell Tissue and Organ Culture 41: 237-241.
Prince, T.A., R.C. Herner and J. Lee, 1986. Bulb organ changes and influence of temperature on gaseous levels in a modified atmosphere package of precooled Tulip bulbs. J. Amer. Soc. Hort. Sci. I 11 : 900-904.
Van Tuyl, J.M., P. Arens., M.S. Ramanna, A. Shahin, N. Kahn, S.

Xie, A. Marasek-Ciolakowska, K. Lim and R. Barba-Gonzalez, 2011. Lilium: page 161 - 184. In: Kole, C. (editor). Wild crop relatives: Genomic and breeding resources plantation and ornamental crops. DOI: 10.1007, isbn. 97836422120 17. Springer-Verlag, Berlin, Heidelberg, 330 p.

Yuan, L.I., Liu, Q. and Liu, Q., 2011. Conservation evaluation and enhancement of wild lily germplasm in China. Acta Hort. (ISHS) 900:53-57.

Micropropagation of Lily: History, Obstacles and Advancements on the Horizon

Geert-Jan de Klerk

Wageningen UR Plant Breeding,

PO Box 16, 6700 AA Wageningen, The Netherlands

History

Tissue culture of lily started off in the early 1950s with the research of Sheila Robb, a PhD student at Massey University in New Zealand (Robb, 1954, 1957). Because tissue culture of monocotyledons was at that time still poorly developed, her main objective was "to culture excised monocotyledon tissue *in vitro*" (Robb, 1954). She expected that tissue excised from scales of lily would prove responsive to culture *in vitro* because "it is well-known that lily bulb scales, when isolated from the parent bulb, readily regenerate bulbils basally, and this behaviour is made use of by horticulturalists in the propagation of this plant". The use of tissue culture as an indispensable way of vegetative propagation was still far away, and for Robb, unimaginable. Soon, however, researchers aimed at the use of tissue culture for propagation. Initially propagation was done in one step only without subculturing: scales from field grown bulbs were cultured *in vitro* under such conditions that they regenerated as much bulblets as possible. So actually researchers transferred conventional scaling to the *in vitro* environment and used new possibilities like sucrose and plant hormones in the medium. To this end, Stimart and Ascher (1978) examined scale culture in Easter lily and reported that an average bulb with 100 scales provides 8000 or more bulbs in 6 weeks.

At the same time, progress was made with virus removal by meristem culture. It was recognized that virus free plants are neither resistant nor immune to virus infection and that when cultured in the field they are again virus infected within a few years. Multiplication *in vitro* was recognized as a means to avoid re-infection (Allen, 1974) and because only a few tiny virus free plants were available for multiplication, the one step method discussed in the preceding paragraph was not useful and was replaced by a multistep procedure. Initially researchers did target at multiplication via callus proliferation (Sheridan, 1968; Stimart et al., 1980), but soon scales taken from *in vitro* regenerated bulblets were used for subculturing (Anderson, 1977; Takayama and Misawa, 1979). In the early 1980s, Novak and Petru (1981) and Takayama and Misawa (1983) published a micropropagation scheme

that is still predominantly used today (Fig. 1). Ever since adjustments were proposed like culture in liquid medium / bioreactors (Thakur et al., 2006), the use of temporary immersion bioreactors (Goo et al., 2005), propagation via somatic embryogenesis (Kim et al., 2003) and the use of thin cell layer explants (Van Le et al., 1999) or roots (Kumar and Choudhary, 2005) as explants, but as yet none of these modifications were successful commercially.

Needs and Opportunities

Tissue culture of lily seems to run smoothly and has reached now an estimated production of 50 – 100 million bulblets per year. The tissue-cultured bulblets are planted in the field and during a few years additional bulblets are produced by conventional scaling. After some time, the level of virus infection becomes serious and a new batch of tissue-cultured bulblets is planted and used for scaling. In addition to producing virus free plants, micropropagation is also a major aid in breeding. Because of the rapid propagation *in vitro* newly bred cultivars can be introduced on the market in 7-8 years (Langens-Gerrits, 2003). In spite of these successes there are some major problems and there are also major opportunities.

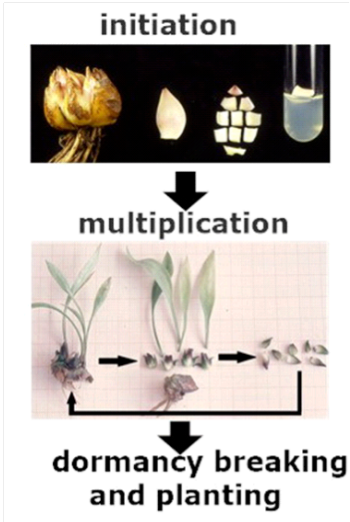

Figure 1. The standard micropropagation protocol of lily

Price

Bulblets produced in tissue culture are expensive which necessitates additional propagation in the field. It is generally believed that propagation via somatic embryogenesis in liquid medium will solve the high-price problem in most crops but as yet this technology cannot be used routinely in lily (as in almost all other crops). There are various major problems, among others contamination related to the growth in liquid medium. This problem should be solved first.

Contamination

Since contamination is a major problem when culturing in liquid medium and as propagation in liquid medium, especially in temporary immersion bioreactors, is for the micropropagation of many crops a breakthrough

(Paek et al., 2005) the problem of contamination will be dealt with below in detail. It should be noted that contamination is anyway a problem tissue culture of lily (like in many other geophytes).

Growth

A second major biological problem in lily tissue culture is the relatively slow growth of lily *in vitro*. Micropropagation is much faster than *ex vitro* propagation, but this is not related to faster growth, but to the performance of several propagation cycles per year, the possibility to culture small explants (scale fragments instead of scales) and the additions in the nutrient medium. Slow growth will also be dealt with in more detail below.

Recalcitrance to regeneration.

Finally, micropropagation of lily involves repeated adventitious regeneration: the formation of new organs (in the case of lily, new bulblets) from somatic cells. In this respect, two major problems occur. First the produced plantlets may not be true-to-type, especially after an intermediate period of callus growth. Lily though seems very stable after an intermediate callus phase (Van Aartrijk et al., 1990). Propagation by adventitious regeneration from scale fragments will even be less prone to genetic instability and only few, poorly documented cases have been reported. The second major problem is the recalcitrance to regenerate. To solve this problem as yet the proper choice of explant (tissue type, physiological and ontogenetic age) is the major solution (De Klerk, 2003), but when the underlying mechanisms of regeneration have been revealed, new procedures will emerge.

Contamination - The problem

Contaminants are introduced in the tissue culture environment because of their association with the explant, by inadequate manipulations in the laboratory and/or by micro-arthropod vectors. In general, the explant is the major source. The use of underground storage organs as source of explants is often associated with heavy contamination (Ziv and Lilien-Kipnis, 2000). Microorganisms may inhabit the epidermis but often also occur within the tissue e.g. in the vascular tissues and in the intercellular spaces. External contaminants can usually be effectively dealt with by a treatment with NaOCl solution, but submergence in a solution with decontaminants is not effective for microorganisms living within the tissue: the disinfectants cannot reach the endophytes in insufficient amounts. The reason for this is the same as for translocation of medium components in tissues and will be dealt with in the next section 'Growth'. Some laboratories add antibiotics to

the medium but their effect is just that bacterial growth in the medium is inhibited so that the cultures seem to be uncontaminated. However, when the plant material is transferred to medium without antibiotics, bacteria originating from the plant tissues will soon flourish on the nutrient medium.

In this respect, one finding is of utmost importance namely the inability of bacteria to grow on plant nutrient media and perhaps even on bacteria media. It is a widespread misunderstanding that most, if not all bacteria species grow abundantly on MS supplemented with sucrose. Leifert and Waites (1992) inoculated liquid MS-sucrose medium with ten bacterial species. Four died off and five survived but only when there were also plants growing on the medium. Just one species showed significant growth. This suggests that many endophytes do not grow readily on plant nutrient media. Epstein (2009) goes much further and believes that very many bacterial species cannot grow on the conventional bacteria medium so that we are actually unaware of their existence (the "Great Plate Count Anomaly"). It has recently been suggested that plants cultured *in vitro* almost ubiquitously harbour endophytic microorganisms (Thomas, 2010). Latent bacteria include bacteria species that are unable to flourish on plant nutrient media but also the generally uncultivable bacteria (Epstein's bacteria). After transfer to tissue culture, these endophytes likely survive within the plants as latent contamination and may become capable of growing on plant nutrient medium after some time by epigenetic or genetic changes. This may be the source of the contamination that appears after some months or years. Alternatively, it may also be that such contamination is introduced during handling when subculturing.

The solutions

The previous paragraphs indicate that attempting to solve the endophyte problem is fighting a losing battle. However, things are not as bad as they look. When contaminants stay within the plants, they cannot overgrow the cultures even though they may reduce growth. Only few crops suffer so much from endogenous contamination that a continuous supply of antibiotics is required to prevent flourishing of the microorganisms on the nutrient medium. Because of the relatively high costs involved, instead of antibiotics a low concentration of NaClO may be used. To my knowledge this has not been used to allow tissue culture of crops notorious for contamination but it has been used as a more general procedure. It was found that microplants did not suffer from low NaClO concentrations (Teixeira et al., 2006). It should be noted again that the endophytes stay alive within the tissue and may be unfavorable for growth (Long et al., 1988; Barberini et al., 2012), so

this solution is not preferable.

Additional progress can be made in three other ways that have not, or only little been dealt with, in research.

Selection of starting material. It is well known that some tissues are more inhabited by microorganisms than others, although the only proof for this are differences in percentage of contaminated cultures in tissue culture. Bulb tissue is supposedly highly contaminated. Meristems are free from most viruses so one would expect that they harbor only few other microorganisms. However, meristem culture involves other problems, among other that the plantlets may stay tiny. It has not been examined whether cultures originating from meristems are PCR-positive or PCR-negative to bacterial primers. Here it should also be noted that for the other treatments described below, different tissues have different suitability. For a hot water treatment bulb tissue is more suitable as it is robust. For vacuum infiltration bulb tissue may be less suitable as the amount of air is low, only 4-5 %, so that only little fluid can be vacuum infiltrated.

Hot water treatment. A powerful treatment known for *ca.* 125 years is the hot water treatment (HWT). It was first used for seeds (1880s), later for bulbs (1920s), and more recently for fruit and vegetables. Vase life of lily is extended by a short (5 min) HWT at 50 °C applied to leaves on cut lily stems (but not flowers) by reducing leaf yellowing (Woolf et al., 2012). Hol and Van Der Linde (1992) used a HWT because of relatively high contamination rates in daffodil. Contmination was reduced from 45% to less than 5% by 1 h HWT of 54 °C. In lily, a lower temperature (less than 45 °C) should be used (Langens-Gerrits et al., 1998). Figure 2 shows the effect of a range of temperatures in lily. For an extended HWT (1h or more), it seems crucial that the tissue has an increased capacity to withstand stress. In tissue culture this opens many possibilities because there are various ways to increase stress resistance during tissue culture (De Klerk and Pumisutapon, 2008). However, like other stresses, a HWT may

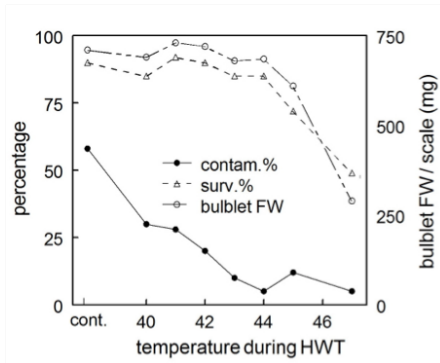

Figure 2. The effect of a 1h hot water treatment of bulbs on contamination, survival and performance of scale explants cut from these bulbs (regeneration of new bulblets determined as the total FW of the regenerated per scale).

activate dormant spores or increase release of micro-organisms resulting in increased visible contamination (Staikidou et al., 2011). Finally it should be noted that it is not known how the HWT acts. The microorganisms may not be able to survive the high temperature or in the tissue a stress reaction is evoked leading to abundant synthesis of oxygen radicles that in turn kill the bacteria.

Vacuum Infiltration. In the intercellular spaces, plants contain large amounts of air that functions in the gas exchange of cells within the tissue (Raven, 1996). When tissue is submerged and the water and tissue are subjected to vacuum, the air in the tissue is replaced by water. When decontaminants have been added to the water, the decontaminants may enter deeply in the tissue. Even though this technique seems promising, it has been used only incidentally (Miyazaki et al., 2010). An important issue with vacuum infiltration is whether the plant material survives the treatment as flooding of the intercellular spaces is detrimental after some time (Van Den Dries et al., 2013). A complicating factor is that in tissue culture the humidity is very high so that normal removal via evaporation is reduced. After vacuum infiltration of Arabidopsis seedlings, they first had a severe hyperhydric appearance but recovered after *ca*. one week (N. van den Dries en G.J. de Klerk, unp data). This indicates that vacuum infiltration does not kill the tissues.

Growth

In tissue culture of bulbous crops, large bulblets should be produced. Bulblets are much easier to handle and acclimatize than shoots. Large bulblets grow faster after planting in soil (Langens-Gerrits et al., 1997) and also perform better during subculturing. Furthermore, the phase change from juvenile to adult is promoted by high weight of the bulblets (Langens-Gerrits et al., 2003). Juvenile bulblets sprout as a rosette and adult ones with a stem. The latter grow much faster after planting (more than twice as fast). The phase change is also promoted by a low concentration of inorganics, especially P, and a high level of sucrose (Langens-Gerrits et al., 2003) and by cytokinins (Ishimori et al., 2007).

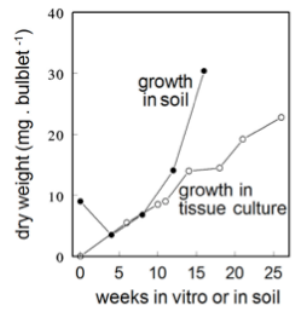

Figure 3. Growth of lily bublets in tissue culture (standard conditions, among others 3% sucrose) and in a growth chamber in soil.

As indicated in the preceding paragraph, the size of the bulblets that are being produced

is crucial. However, apart from the concentration of sucrose, the factors that determine bulblet growth are largely unknown. As a matter of fact, we even don't know in general how plantlets cultured *in vitro* achieve growth (De Klerk, 2010). Such statement may look strange at first but an analysis of the conditions *in vitro* reveals that the tools by which *ex vitro* growing plants deal with nutrient transport between tissues is distorted partly or almost fully in tissue culture (see following paragraphs). Obviously understanding the mechanisms by which plantlets do achieve growth *in vitro* in spite of this will help to improve the conditions for optimal growth in tissue-cultured plants, including lily.

First, I will show that growth *in vitro* is not fast. When comparing growth of lily bulblets *in vitro* with growth in soil, it gets clear that growth *in vitro* is slower (Fig. 3). In the experiment presented in Figure 3, the *ex vitro* culture was in a growth chamber set at 17 °C with artificial light of *ca*. 25 $\mu E.m^{-2}.sec^{-1}$, so unlikely under optimal conditions. Intuitively, one would have expected that growth in tissue culture would be fastest: temperature and water are favorable and there is plenty of organic and inorganic nutrients. The conclusion from the slower growth *in vitro* is that the tissue culture conditions are in some way(s) adverse. Probably, there are two major obstacles.

Transport of nutrients in the explants. There are two ways by which solutes (compounds dissolved in water) are translocated, namely by diffusion and by hitching a lift in the water flow. According to Fick's law, diffusion is too slow for long distance transport. Therefore plants use the water flow in the vascular tissues for transport. Even in a tissue-cultured plantlet the distances are too large. Therefore vascular tissues have also a main role in the transport of solutes in *in vitro* cultured plants.

First I will deal with the situation in shoot cultures, the vast majority of micropropagation systems. In both vascular systems, the flows are adversely affected and possibly almost erased by the *in vitro* conditions. The flow in the xylem is normally driven by transpiration from the leaves, but because of the very high relative humidity *in vitro,* transpiration will be very low. This results in decimation of the water flow in the xylem. The flow in the phloem is normally driven by uploading of photosynthesis-derived sucrose in the leaves using specialized collection phloem and unloading of sucrose in the growing organs. This leads to differences in osmotic values in the phloem which causes water flow. In tissue culture, sucrose from the medium should be uploaded instead of photosynthesis-derived sucrose and the tissue adjacent to the medium is unlikely suitable for that end, so the water flow in the phloem seems to be wiped out. Somehow, however, tissue-cultured plants can deal with this.

In lily tissue culture the situation is different from shoot cultures. The most likely pathway for transport of medium compounds in lily tissue culture is that the medium compounds diffuse into the scale explant and are then loaded into the phloem. The flow in the phloem is expectedly driven by osmotic differences brought about by uploading of sucrose at the source (= scale explant) and unloading at the sink (= regenerating bulblets). The main argument for this mechanism is that the growth of bulblets increases with the size of the scale explant (Fig. 4) so increases with the amount of phloem available. Furthermore, as yet there are no other conceivable ways of transport. It should be noted again that in other tissue culture systems (e.g. shoot cultures) a loading organ like the scale explant is not present and in these cases phloem loading must occur in another way. In the case of lily major growth improvements can be achieved when phloem loading is increased.

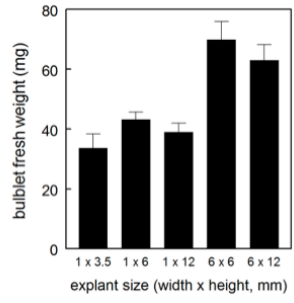

Figure 4. The FW of bulblets depends on the size or actually the height of the explant.

Wound periderm. The uptake of medium components occurs via the wounding surface especially when explants are cultured on solid medium because they are usually positioned in such way that the cut surface contacts the nutrient medium. In the case of lily, though, scale explants are cultured with the abaxial surface on the medium so with the epidermis in touch with the medium. The epidermis is relatively impermeable because of a wax layer. However, physical adhesive forces between the liquid and the cut surface act to lift the liquid and enable uptake via the cut surface. In freshly cut apple stem explants the uptake per mm^2 is *ca*. 20 times larger than the uptake via the epidermis (Guan and De Klerk, 2000). However, the wound is being healed soon after cutting and uptake is reduced. Surprisingly, the effect of wound healing on uptake (in this case uptake of the auxin naphthaleneacetic acid by tobacco tissues) has been studied only once and found to decrease rapidly (Smulders et al., 1990). There are, however, ways to reduce the formation of wound periderm (Soliday et al., 1979).

Conclusions

For bulbous crops, successful micropropagation protocols are very important. If they are not available, propagation must be carried out in soil for many years. Soil-propagation is slow and results often in heavily diseased

bulbs. In lily, researchers have been able to develop a highly successful protocol. This resulted in extensive application and boosted lily as an ornamental. However, additional major progress may be achieved. One major restraint in tissue culture of lily is our very limited knowledge about the underlying physiological processes. Increasing this knowledge will undoubtedly lead to major advancements.

References

Allen, T.C., 1974.
Production of virus-free lilies. Acta Hortic. 36, 235-240.

Anderson, W.C., 1977.
Rapid propagation of *Lilium* cv. Red Carpet. In Vitro 13, 145.

De Klerk, G.J., 2003.
Organogenesis. In: Encyclopedia of Applied Plant Sciences, Thomas, B., Murphy, D., Murray, B., eds., Academic Press. Elsevier Science Ltd., London, pp 1364-1371.

De Klerk, G.J., 2010.
Why plants grow in tissue culture? Prophyta Annual 2010:42-44.

De Klerk, G.J. and Pumisutapon, P., 2008.
Protection of in-vitro grown Arabidopsis seedlings against abiotic stresses. Plant Cell Tissue Organ Cult. 95:149-154.

Epstein, S.S., 2009.
General model of microbial uncultivability. In: Epstein, S.S., ed., Uncultivated Microorganisms, Springer, Berlin and Heidelberg, pp 131-159.

Goo, D.H., Lim, J.H., Cho, H.R., Kim, Y.J. and Kim, K.W., 2005.
Rapid enlargement of lily bulblet by bioreactor culture. Acta Hortic. 673:633-637.

Guan, H. and De Klerk, G.J., 2000.
Stem segments of apple microcuttings take up auxin predominantly via the cut surface and not via the epidermal surface. Sci. Hortic. 86:23-32.

Hol, T. and Van Der Linde, P., 1992.
Reduction of contamination in bulb-explant cultures of *Narcissus* by a hot-water treatment of parent bulbs. Plant Cell Tissue Organ Cult. 31:75-79.

Ishimori, T., Niimi, Y. and Han, D.S., 2007.
Benzyladenine and low temperature promote phase transition from juvenile to vegetative adult in bulblets of *Lilium* × *formolongi* 'White Aga' cultured in vitro. Plant Cell Tissue Organ Cult. 88:313-318.

Kim, S.K., Lee, J.S., Huang, K.H. and Ahn, B.J., 2003.
Utilization of embryogenic cell cultures for the mass production of bulblets in Oriental and Easter lilies. Acta Hortic. 625:253-259.
Kumar, S. and Choudhary, V., 2005.
Micropropagation from in vitro roots of lily oriental hybrid. Phytomorphology. 55:267-273.
Langens-Gerrits, M., Albers, M. and De Klerk, G.J., 1998.
Hot-water treatment before tissue culture reduces initial contamination in *Lilium* and *Acer*. Plant Cell Tissue Organ Cult. 52: 75-77.
Langens-Gerrits, M., De Klerk, G.J. and Croes, A., 2003.
Phase change in lily bulblets regenerated in vitro. Physiol. Plant. 119: 590-597.
Langens-Gerrits, M., Lilien-Kipnis, H., Croes, T., Miller, W., Kollfiffel, C. and De Klerk, G.J., 1997.
Bulb growth in lily regenerated in vitro. Acta Hortic. 430:267-273.
Langens-Gerrits, M.M., 2003.
Phase Change, Bulb Growth and Dormancy Development in Lily: Manipulation of the Propagation Cycle by in Vitro Culture. PhD thesis, Radboud University, The Nettherlands.
Leifert, C. and Waites, W.M., 1992.
Bacterial growth in plant tissue culture media. J. Appl. Bacteriol. 72:460-466.
Miyazaki, J., Tan, B.H. and Errington, S.G., 2010.
Eradication of endophytic bacteria via treatment for axillary buds of *Petunia hybrida* using Plant Preservative Mixture (PPM™). Plant Cell Tissue Organ Cult. 102:365-372.
Novak, F.J. and Petru, E., 1981.
Tissue-culture propagation of *Lilium* hybrids. Sci. Hortic. 14:191-199.
Paek, K.Y., Chakrabarty, D. and Hahn, E.J., 2005.
Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tissue Organ Cult. 81:287-300.
Raven, J.A., 1996.
Into the voids: The distribution, function, development and maintenance of gas spaces in plants. Ann. Bot. 78:137-142.
Robb, S.M., 1954.
Physiological Investigations on Regeneration from Bulb Scale Leaves of Lilium Speciosum Thun. PhD Thesis, Massey University, New Zealand
Robb, S.M., 1957.

The culture of excised tissue form bulb scales of *Lilium speciosum* Thun. J. Exp.Bot. 8:348-352.

Sheridan, W.F., 1968.
Tissue culture of monocot *Lilium*. Planta 82:189-192.

Smulders, M.J.M., Visser, E.J.W., Van Der Krieken, W.M., Croes, A.F. and Wullems, G.J., 1990.
Effects of the developmental state of the tissue on the competence for flower bud regeneration in pedicel explants of tobacco. Plant Physiol. 92:582-586.

Soliday, C.L., Kolattukudy, P.E. and Davis, R.W., 1979.
Chemical and ultrastructural evidence that waxes associated with the suberin polymer constitute the major diffusion barrier to water vapor in potato tuber, *Solanum tuberosum* L. Planta 146:607-614.

Staikidou, I., Selby, C. and Watson, S., 2011.
Efficient surface sterilization of snowdrop bulb explants with PPM. Acta Hortic. 886:259-266.

Stimart, D.P. and Ascher, P.D., 1978.
Tissue culture of bulb scale sections for asexual propagation of *Lilium longiflorum* Thunb. J. Am. Soc. Hortic. Sci. 103:182-184.

Stimart, D.P., Ascher, P.D. and Zagorski, J.S., 1980.
Plants from callus of the interspecific hybrid *Lilium* black beauty. HortScience 15:313-315.

Takayama, S. and Misawa, M., 1979.
Differentiation in *Lilium* bulb scales grown in vitro, effect of various cultural conditions. Physiol. Plant. 46:184-190.

Takayama, S. and Misawa, M., 1983.
A scheme for mass propagation of *Lilium* in vitro. Sci. Hortic. 18:353-362.

Teixeira, S.L., Ribeiro, J.M. and Teixeira, M.T., 2006.
Influence of NaClO on nutrient medium sterilization and on pineapple, *Ananas comosus* cv. Smooth cayenne behavior. Plant Cell Tissue Organ Cult. 86:375-378.

Thakur, R., Sood, A., Nagar, P., Pandey, S., Sobti, R.C. and Ahuja, P.S., 2006.
Regulation of growth of Lilium plantlets in liquid medium by application of paclobutrazol or ancymidol, for its amenability in a bioreactor system: growth parameters. Plant Cell Rep. 25:382-391.

Thomas, P., 2010.
Plant tissue cultures ubiquitously harbor endophytic microorganisms, Acta Hortic. 865:231-240.

Van Aartrijk, J., Blom-Barnhoorn, G.J. and Van der Linde, P.C.G., 1990. Lilies. In: Ammirato, P.V., Evans, D.R., Sharp, W.R.,, Bajaj, Y.P.S. , eds Handbook of plant cell culture Vol. 5, Ornamental species. McGraw-Hill Publishing Company, New York, pp. 535 - 576.

Van Den Dries, N., Giannì, S., Czerednik, A., Krens, F.A. and De Klerk, G.J.M., 2013. Flooding of the apoplast is a key factor in the development of hyperhydricity. J. Exp. Bot. (in press).

Van Le, B., Tân Nhut, D. and Tran Thanh Van, K., 1999. Plant production via shoot regeneration from thin cell layer pseudobulblet explants of Lilium longiflorum in vitro. C. R. Acad. Sci. Paris - Serie III 322:303-310.

Woolf, A.B., Combes, S., Petley, M., Olsson, S.R., Wohlers, M. and Jackman, R.C., 2012. Hot water treatments reduce leaf yellowing and extend vase life of Asiatic hybrid lilies. Postharvest Biol. Technol. 64:9-18.

Ziv, M. and Lilien-Kipnis, H., 2000. Bud regeneration from inflorescence explants for rapid propagation of geophytes in vitro. Plant Cell Rep. 19:845-850.

Avoidance of Cross-Contamination during the Initiation Step in Lily Tissue Culture

Naser Askari and Geert-Jan de Klerk

Wageningen UR Plant Breeding, The Netherlands

Introduction

Organs growing underground like bulbs are notorious for contamination. Acute contamination (caused by incomplete surface sterilization) and post-establishment contamination (caused by contaminants within the tissue or by inadequate manipulations of the operators during subculturing) are the main categories of contamination in plant tissue culture (Long et al., 1988). Several microorganisms (fungi, yeast, bacteria) have been identified as contaminants in plant tissue culture but bacterial contamination is probably the most common (Leifert and Cassells, 2001; Leifert et al., 1991).

Substantial cross-contamination (the spread of bacteria and other microorganisms from one explant to the other) may occur just after the transfer to the tissue culture environment during the first weeks of tissue culture and is avoided by culturing a single explant per container. Cross-contamination may also occur just after surface sterilization during rinsing of explants with sterile water. In this case, the possibility of cross-contamination is usually ignored because it is not feasible to process each explant individually and because it is believed that the period in which cross-contamination may occur is too short to cause serious problems.

Tissue culturalists rinse explants extensively after sterilization to remove all NaClO even though it has been found that NaClO is not toxic at low concentration. Some researchers even add low levels of NaClO during tissue culture to avoid flourishing of bacteria (Sawant and Tawar 2011; Teixeira et al. 2006; Yanagawa et al. 2007). The aim of the present study is to reduce cross-contamination in tissue culture of lily during rinsing by adding a low quantity of NaClO to sterile rinsing water.

Materials and Methods

Standard tissue culture conditions

Field-grown bulbs (circumference 18-20 cm) of *Lilium* cv. 'Santander' were harvested, cold-treated to break dormancy and stored at -1.0 °C until use. Scales were surface-sterilized for 30 min in 1% (w/v) NaOCl, rinsed for 1, 3 and 10 min with sterile water or with 0.03% NaOCl, and after that

stored until use for 1-2h in sterile water or 0.03% NaOCl, respectively. The rinsing and storage fluids were stored at 4 °C to examine bacterial incidence.

Explants of 7 x 7 mm were placed with the abaxial side on 15 ml medium in small plastic containers (3.5 cm diameter). The medium was composed of MS macro- and microelements (Murashige and Skoog 1962), 30 g l^{-1} sucrose, 7 g l^{-1} agar (Microagar) and 0.05 mgl^{-1} NAA (-naphthaleneacetic acid). The explants were cultured at 25°C and 30 $\mu E.m^{-2}.sec^{-1}$ (Philips TL 33) for 16h per day. After 11 weeks of culture, the bulblets were harvested and the parameters indicated in the graphs were determined.

Estimation of cross-contamination

Sixty outer scales and 30 inner scales were sterilized for 30 min in one beaker with 1% NaClO solution plus a few drops Tween 20. Then the scales were divided into two groups (30 outer scales and 15 inner scales), distributed over two beakers, rinsed three times (1, 3 and 10 min), the first group with sterile water and the second group with 0.03% NaClO, and then stored until use (1-2h) in water or 0.03% NaClO, respectively. The rinsing fluids were stored at 4 °C. We monitored contamination of the scales during 6 weeks of culture. The decrease of contamination by rinsing in 0.03% NaClO was taken as an estimation of cross-contamination using the following formula:

Determination of contamination in the rinsing fluids

The rinsing fluids (water and NaClO solutions) were inoculated on LB solid and 30 ml LB fluid medium. On the solid medium 25 µl was inoculated and on the fluid medium 30 ml. Bacterial growth was determined after 3 days in dark at 37°C.

Minimal concentration of NaClO for decontamination of fluids

To determine the minimal effective concentration, increasing quantities of NaClO were added to heavily contaminated storage water to obtain different concentrations (0, 0.01, 0.03, 0.06, 0.1 and 1.5%) and the solutions were stored for 24 hours at room temperature. After that 2 ml of LB fluid medium was added to 2 ml from each NaClO concentration and incubated at 37°C for 3 days. After that bacterial growth was determined.

Performance of scale explants

After 11 weeks of culture, bulblets regenerated on non-contaminated explants were separated from the scale explants and fresh weight of bulblets, fresh weight of leaves per explant, regeneration percentage (scale explants regenerating bulblets as a percent of the total noncontaminated scales) and bulblet number per explant were determined.

Results

Determination of the effective concentration of NaClO

As shown in Table 1, bacteria did only grow with 0% and 0.01% NaClO. The lowest NaClO concentration that fully inhibited bacterial growth was 0.03%. We used this concentration in the following experiments because a higher concentration might damage the scale tissue.

To determine contamination in the rinsing fluids, bacterial incidence was examined by inoculating on solid and liquid LB. Table 2 shows that there was no contamination in the rinsing NaClO-solutions but that contamination occurred in rinsing water. Bacterial colonies were present in the 3rd rinsing water and the storage water on both solid and liquid LB and the 2nd rinsing water on liquid LB only. The 1st rinsing water had no contamination probably because of carry-over of NaClO used for surface sterilization.

Table 1. Bacterial incidence after adding different quantities of NaClO (- not contaminated, ++ medium contaminated, +++ highly contaminated)

NaClO concentration (%)							
LB Liquid Medium		0	0.01	0.03	0.06	0.1	1.5
	1	+++	++	-	-	-	-
	2	+++	++	-	-	-	-
	3	+++	++	-	-	-	-

Table 2.Contamination of rinsing fluids as detected with LB solid (SM) and fluid (LM) medium

	Water	0.03% NaClO							
	1st rinse (1 min)	2nd rinse (3 min)	3rd rinse (10 min)	Storage (120 min)	1st rinse (1 min)	2nd rinse (3 min)	3rd rinse (10 min)	Storage (120 min)	
Test SM	1	-	-	+	++	-	-	-	-
	2	-	-	+	++	-	-	-	-
	3	-	-	+	++	-	-	-	-
Test LM	1	-	+	++	+++	-	-	-	-
	2	-	+	++	+++	-	-	-	-

Contamination of scale explants after rinsing with diluted NaClO (0.03%) and water.

After rinsing the scales, explants (7 x 7 mm, two per scale) were cut and transferred to standard lily medium. Contamination was monitored during 6 weeks. Inner scale explants showed lower contamination than outer ones: when rinsed with water 27% *vs.* 53% and when rinsed with 0.03% NaClO 3% *vs.* 37%. Contamination in outer scales is high because of damage of these scales and because they are much older. The contamination after surface sterilization may be attributed to endogenous contamination and cross-contamination during rinsing. We assumed that cross-contamination occurred from outer scale explants (highly endogenously contaminated) to inner scale explants (hardly endogenously contaminated), and also to noncontaminated outer scales. As in inner scales the percentage contamination decreased from 27% after rinsing with water to 3% after rinsing with NaClO, 24 % of the contamination in water-rinsed scales was due to cross-contamination during the rinsing (see formula in Material and Methods). Rinsing with 0.03% NaClO also reduced the contamination of outer scales and a similar calculation as done for inner scales showed that in this case cross-contamination was 25%.

Performance of scale explants after rinsing with water and 0.03% NaClO

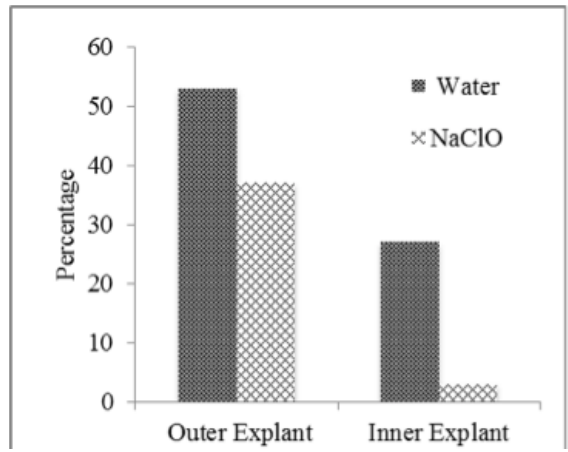

Figure 1. Contamination of explants cut from inner and outer scales after rinsing with water or 0.03% NaClO. Contamination was monitored for 6 weeks.

Discussion

When material from field-grown plants is surface-sterilized, a batch of a few to tens of explants is processed in one beaker because it is unfeasible to process the explants individually. We studied the occurrence of cross-contamination during this procedure in lily. We showed that the rinsing water used to remove the NaClO after surface sterilization became contaminated with bacteria. This resulted in considerable additional contamination of the explants. A simple way to reduce cross-contamination was rinsing with 0.03% NaClO instead of water. We had observed in a dose-response

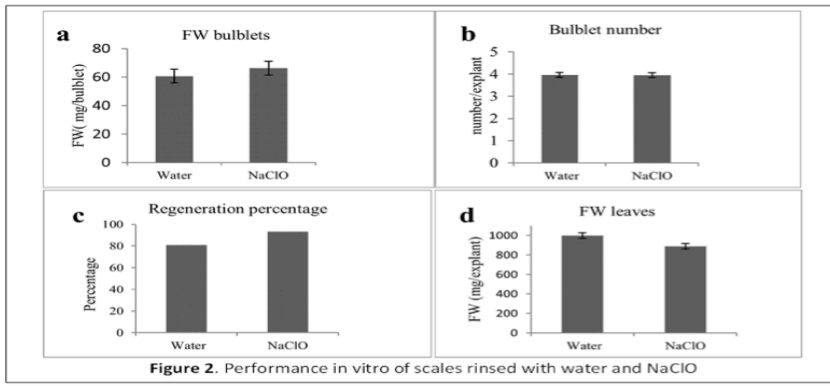

Figure 2. Performance in vitro of scales rinsed with water and NaClO

Figure 2. Performance in vitro of scales rinsed with water and NaClO

experiment that this was the lowest concentration at which bacterial growth was fully inhibited. After rinsing in NaClO, the performance of the scale explants was the same. The low toxicity (or the absence of toxicity) of a low concentration of NaClO agrees with studies in which tissue culture was performed in the presence of a low concentration of NaClO (Sawant and Tawar 2011; Teixeira et al. 2006; Yanagawa et al. 2007). Rinsing in diluted NaClO may also be considered for other crops.

Conclusion

In tissue culture of lily, substantial contamination may be caused by cross-contamination after surface sterilization when the excess of NaClO is removed by rinsing with "sterile" water. Cross-contamination is avoided by rinsing with a solution with a low concentration of NaClO. There was no impact on performance during tissue culture.

References

Leifert,, C. and Cassells, A.C., 2001. Microbial hazards in plant tissue and cell cultures. In Vitro Cell Dev.-Pl. 37: 133-138.

Leifert, C., Ritchie,J.Y. and Waites, W.M., 1991. Contaminants of plant-tissue and cell cultures. World J. Microbiol. Biotechn. 7: 452-469.

Long, R.D., Curtin, T.F. and Cassells, A.C., 1988. An investigation of the effects of bacterial contaminants on potato nodal cultures. Acta Hortic. 225: 83-92.

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant: 473-497.

Sawant, R.A. and Tawar, P.N., 2011. Use of sodium hypochlorite as

media sterilant in sugarcane micropropagation at commercial scale. Sugar Tech 13: 27-35.

Teixeira, S.L., Ribeiro, J.M., Teixeira, M.T., 2006. Influence of NaClO on nutrient medium sterilization and on pineapple (Ananas comosus cv Smooth cayenne) behavior. Plant Cell Tissue Organ Cult. 86: 375-378.

Yanagawa, T., Tanaka, R. and Funai, R., 2007. Simple micropropagation of ornamentals by direct application of NaClO disinfectants without equipment. Acta Hortic. 764: 289-298.

A Cytogenetics Lesson from Lilies

Rodrigo Barba-Gonzalez

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C.

I have been a member of the North American Lily Society for a few years now, and I always wondered about the possibility of writing in the Yearbook. This time I got an invitation from Dr. Jaap Van Tuyl, who was my mentor during my stay at Plant Research International (PRI), while I was studying at Wageningen University. Before, I had seen lilies in stores and I must confess that I did not know much about them. It was Jaap who shared his passion and taught me a lot about these magnificent flowers, and in no time they also became my passion. I will try to do my best to share in a few pages some years of research in what is for me one of the most exiting ornamental crops: The Lilies.

Without a doubt lilies are one of the most beautiful ornamental crops. They feature so many flower shapes, flower orientations, colors and fragrances that almost everybody who likes flowers may have the lily as a favorite. Such traits place the lilies as the fourth crop in the ornamental industry. The quest to combine traits from different species in order to create novel forms, disease resistance and color combinations has led breeders to apply different biotechnological tools to generate these coveted new hybrids. So, that was my job, to apply different biotechnological tools to obtain new lily combinations. For that purpose it was necessary to learn and understand different reproduction mechanisms that only recently had been discovered. During my stay at PRI I was lucky to work with some lily generations, so I have to go back in a little in history in order to depict the breeding program that was being developed.

A brief history of lily hybrids

Lilies have been cultured since ancient times (Woodcock and Stern, 1950). They belong to the genus *Lilium*; a monocotyledonous bulb crop of the Liliaceae family, the genus originated in the Himalayan region from where they have extended over the mountain areas in the Northern hemisphere, nowadays the genus includes over 80 species which have been classified into six sections (Comber, 1947; De Jong, 1974). From these sections, the following three have contributed to the creation of cut flowers (McRae, 1998): i) Section Leucolirion, the Longiflorum and trumpet hybrids; Trumpet-

shaped hybrids with white flowers and a distinctive fragrance. They can be forced year round.

ii) Section Sinomartagon, the Asiatic hybrids. They present a wide variation in colors from bright to soft and from white to red, including yellow. These hybrids are the most widely grown. Maybe, the major characteristic of these hybrids is their resistance to *Fusarium oxysporum* (Straathof and Van Tuyl, 1994) and to some viruses, resistances that are not present in hybrids from other sections.

iii) Section Archelirion, the Oriental lilies. These might be the most magnificent lilies; hybrids from this section have been used for breeding since the early ´50s, and in few years, the number of commercial varieties increased significantly. They have big and showy flowers with a sweet fragrance; a wide variety of colors within the whites, pink and yellows. Some of them are resistant to *Botrytis elliptica*.

Each of the different species of lilies has a special feature or trait such as small or large flowers, simple or fancy shapes, up or down facing flowers, colors, spots, leaves and many others. Breeders which are seduced by these attributes are always looking to include such characteristic in their hybrids to obtain novel and unique cultivars. A few decades ago it was only possible to hybridize lilies within a taxonomic section, however, interspecific hybrids between different taxonomic sections (intersectional hybrids) was not possible; the reason is that the pollen of a lily from a determined section cannot germinate in lilies from different sections. To overcome this pre-fertilization barrier Asano and Myodo (1977a) developed the intrastylar pollination technique and pollination between distantly related species became possible. However, just to a certain extent, because it is only possible to pollinate Longiflorum hybrids with pollen from Oriental and Asiatic hybrids; Oriental hybrids with pollen from Asiatic hybrids and not the other way around. Nevertheless pollination was possible, the intersectional embryos aborted due to incompatibility, lacking endosperm. To overcome this post-fertilization barrier Asano and Myodo (1977b) cultured the immature embryos *in vitro* (in this case embryosac culture) with success. With the application of such techniques it was possible to create intersectional hybrids. Later, at the end of the 80s, new techniques to obtain the desired intersectional hybrids were added to the existing ones. These techniques included mentor pollen, *in vitro* pollination and ovary- and ovule culture (Van Tuyl *et al.,* 1982; 1988; 1991). With all these techniques it has been possible to generate an enormous amount of hybrids, hybrids which combine all those desired traits and resulted in new groups of an almost endless collection.

The hybridization drawbacks

The problem with interspecific hybrids is that they tend to be sterile; this is also true for intersectional lily hybrids. By the time I arrived at PRI they had developed a number of intersectional hybrids, Longiflorum x Asiatic (LA); Oriental x Asiatic (OA); Oriental x Trumpets (OT) and so on. I focused in OA hybrids. Normally, the genome of a diploid organism is composed by chromosome pairs, these pairs are called homologous, one chromosome of each homologous pair comes from the mother and one

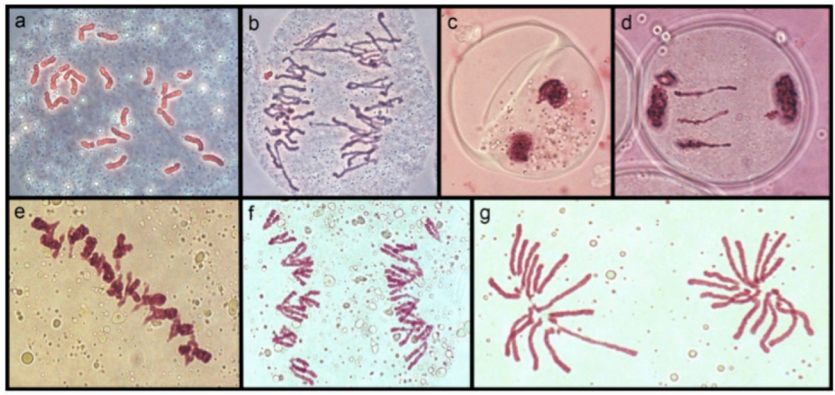

Figure 1. *Lilium* meiosis. a) Univalents at metaphase in the OA hybrid 951502-1; b) First Division Restitution in the OA hybrid 951502-1, the complete set of chromatids are segregated before the reductional division; c) Second Division Restitution in the OA hybrid 951502-1, the restituted nuclei before cytokinesis; d) Abnormal meiosis in the OA hybrid 951502-1; e) Bivalents at metaphase in the Asiatic hybrids "Pollyanna"; f) Anaphase I in the Asiatic hybrids "Pollyanna", the chromosomes segregate "reductionally"; g) Anaphase II in the Asiatic hybrids "Pollyanna".

comes from the father. During normal meiosis (cell division that produces reproductive cells, in the case of lilies the ovules and pollen) the homologous chromosomes pair and recombine during cross over, generating genetic variation. In interspecific hybrids, the chromosome pairs are not that similar anymore, because they come from different species and they are denominated homoeologous chromosomes. During meiosis the pairs of homoeologous chromosomes do not recognize each other and do not pair, being this and irregular chromosome segregation the main causes of sterility in lily intersectional hybrids. (Figure 1)

The traditional method to restore fertility in interspecific hybrids is doubling the chromosome number with certain chemicals such as colchicine

and oryzalin (Van Tuyl *et al.,* 1992). The results of such treatments are plants with double of chromosomes that they originally had. They are denominated polyploids, because they contain more than two sets of chromosomes.

In these hybrids, in a certain way, the meiotic division finds a balance, because each chromosome now has a "recognizable" pair and the main causes of sterility, (lack of chromosome pairing and irregular chromosome segregation) is overcome.

Well, now we had a solution, but there was a major drawback with these polyploids, even though the fertility is restored, these plants are called "permanent hybrids" because the chromosomes from the parental genomes do not recombine. This is because when the chromosomes are chemically "doubled" they will pair with its double, which is an exact copy of itself, so, it doesn't matter if there is pairing and recombination because the genetic information is the same and all the gametes (pollen and ovules) are identical (Figure 2), as an example I will refer to an OA hybrid,

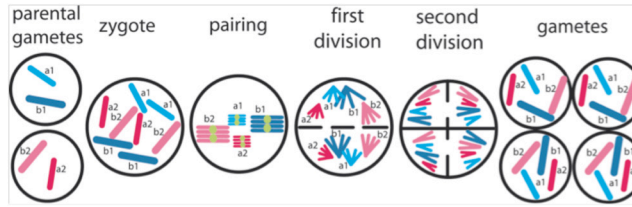

Figure 2. Schematic representation of chromosome segregation and recombination in a mitotically doubled hybrid.

Figure 3. Triploid population obtained from a tetraploid OA hybrid mitotically doubled.

let's think on it from the beginning and how we obtained it: if we cross an Oriental hybrid with an Asiatic hybrid (utilizing intrastylar pollination and embryo rescue) we obtain an OA hybrid, a sterile OA hybrid, now we can use oryzalin to double the chromosome number to obtain an OOAA hybrid (using the letters to represent the chromosome sets), during meiosis the O chromosomes will pair with their copies, the other O chromosomes and the A chromosomes will pair with the copies of the A chromosomes. So, there is no pairing and recombination between the O and A genomes and all the gametes (pollen and ovules) will be identical. Thus, if these hybrids are utilized to generate progeny, they will provide little or any genetic variation to the progeny (Figure 3).

A light in the darkness

Basically, the purpose of meiosis is to reduce the normal diploid cells (two copies of each chromosome / cell) to haploid cells (one copy of each chromosome / cell): the gametes. When the haploid gametes (ovule and pollen) join they produce a zygote with two copies of each chromosome (one copy from the ovule and one copy from the pollen). This being true, there are many polyploid species in nature. The question arises about their origin. One of the most accepted explanations is the "*2n*" or "unreduced" gametes, this kind of gametes occur in most of the angiosperm species and they might be the origin of polyploid species (Harlan and De Wet, 1975). Before the use of chemicals to restore fertility, the unreduced gametes were utilized to produce polyploids; nevertheless, their use was discarded because the breeders considered that the production of such gametes was only occasional. The advantage of the unreduced gametes is that there is a recombination between the parental

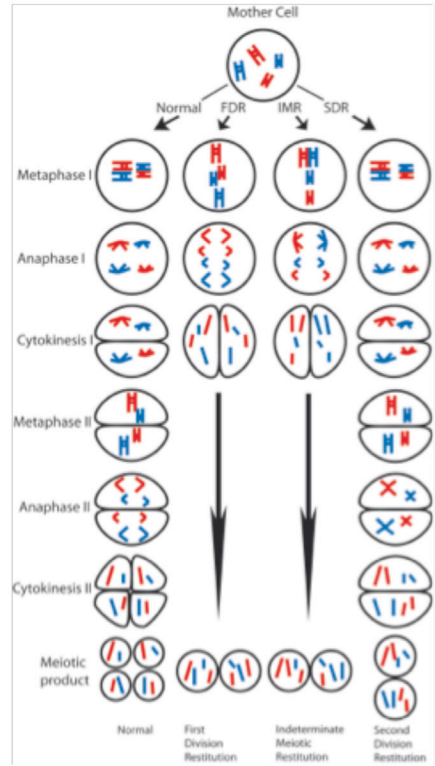

Figure 4. Schematic representation of the three different meiotic restitution mechanisms detected in lily hybrids

chromosomes and as a consequence the pollen grains and ovules provide genetic variation, making them more promising for breeding. What followed was obvious; we made a screening for OA hybrids that produced the *2n* gametes and among a number (400 or more!) and we found 12 of them (Barba-Gonzalez *et al*., 2005a). In a few of them we observed meiosis looking for the chromosomes recombining, for this we utilized Genomic *in situ* hybridization (GISH), a molecular cytogenetic technique that allows the identification of parental genomes by "painting" the chromosomes (Figure 5a). Furthermore, we wanted to know which mechanisms were originating them, so we looked deeply into meiotic configurations and we were able to identify two mechanisms: First Division Restitution (FDR) and Second Division Restitution (SDR). The importance of identifying the mechanisms that formed the *2n* gametes was that each mechanism has different genetic consequences. If FDR heterozygosity is maintained, keeping it simple, both chromosomes of the parental genomes (O+A) are transmitted to the progeny; if SDR chromosome assortment occurs, meaning that two copies of each of either the O or the A chromosome are transmitted to the progeny. There is another mechanism that occurs in lilies (Lim *et al.,* 2001); this is Indeterminate Meiotic Restitution (IMR) which is a mixture of the previous, where each independent chromosome might behave as in FDR or SDR (Figure 4). Once the OA hybrids and the mechanisms that produced the *2n* gametes were identified, we utilized those fertile hybrids in a number of crosses, obtaining hundreds of progeny plants (Barba-Gonzalez *et al.,* 2004; 2005b); many of them were screened by GISH to reveal the recombinant chromosomes and the composition of the new hybrids (Figure 5). As expected, most of the progeny was triploid, because the unreduced gametes

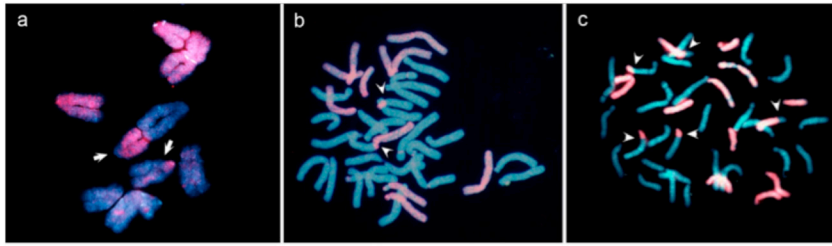

Figure 5. Genomic *in situ* hybridization of chromosomes of OA hybrids. The chromosomes in blue represent Asiatic chromosomes and the chromosomes in pink represent the Oriental chromosomes. a) Meiosis in the OA hybrid 951502-1, the arrows shows the product of recombination; b) Chromosome complement of the OA hybrid 012168-01; c) Chromosome complement of the OA hybrid 022604-05. Arrowheads show recombinant chromosomes.

Figure 6. Triploid population obtained from a *2n* gamete producer OA hybrid

contributed with two chromosome sets and the female progenitor only with one chromosome set and most important, there were recombinant chromosomes in almost all the triploid hybrids. When the time passed and we were able to take a look at the flowers of the triploid hybrids we observe a tremendous amount of variation among them, the variation that the unreduced gametes provided (Figure 6).

So far so good

Until now we had obtained hundreds (maybe thousands) of triploid hybrids, and the next question arose: are the triploid hybrids fertile? In many cultivars triploid hybrids are sterile and the breeding programs cannot further continue, but in the case of OA lily hybrids many of the triploid hybrids were a little fertile and again, hundreds of progeny plants were using embryo rescue obtained in backcrosses to Asiatic, OA hybrids and mitotically doubled OA hybrids (Barba-Gonzalez *et al.,* 2006). When we analyzed the chromosome configurations we found out that the progeny hybrids from the triploids were aneuploids, meaning that the chromosome sets were not complete or there were extra chromosomes, many of the progeny hybrids were nearly diploids plus some chromosomes. So we took a look at the meiosis of the triploid hybrids and we found that the Oriental chromosomes were not segregated properly and their presence in the hybrids could

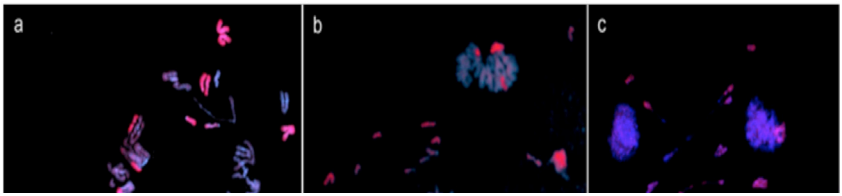

Figure 7. Genomic *in situ* hybridization at meiosis in the triploid AOA hybrid 002531-12. a) Anaphase, the Oriental chromosomes (pink) are segregated irregularly, while the Asiatic chromosomes (Blue) migrate to the poles; b) Early telophase, note all the Oriental chromosomes remaining in the middle of the cell; c) Telophase, note the Oriental chromosomes at the center of the cell that were not pulled to the poles

just by chance when the cells undergo cytokinesis (Figure 7). This gave us our last lesson, the triploid hybrids, which contained two sets of Asiatic chromosomes and one set of Oriental chromosomes, were not transmitting all the Oriental chromosomes and eventually they will be completely lost in further backcrosses to Asiatic hybrids. Anyway, the good news was that the recombinant chromosomes (those Asiatic chromosomes with segments of Oriental chromosomes) were maintained in the progeny plants and they will keep the Oriental genes by generations.

A final word

Through the years, the genus *Lilium* has not only been a beautiful flower and crop, but a model to genetics and cytogenetics, and we have learned many lessons from it. In this case, it has taught us a really important lesson. It has been possible to generate hybrids that were not possible before. From special pollination techniques, embryo- and ovule rescue to the elucidation of restitution mechanisms during meiosis, the use of unreduced gametes and triploid hybrids it has been possible to generate an enormous amount of intersectional lily hybrids, with a similar amount of genetic variation. The bases for a successful lily breeding program are clear now.

References

Asano, Y. and H. Myodo. 1977a. Studies on crosses between distantly related species of Lilies. I. For the intrastylar pollination technique. J Jpn Soc Hortic Sci 46: 59-65.

Asano, Y. and H. Myodo. 1977b. Studies on crosses between distantly related species of Lilies. II. The culture of immature hybrid embryos. J Jpn Soc Hortic Sci 46: 267-273.

Barba-Gonzalez, R., Lokker, A.C., Lim, K-B., Ramanna, M.S. and J.M.

Van Tuyl. 2004. Use of *2n* gametes for the production of sexual polyploids from sterile Oriental x Asiatic hybrids of lilies (*Lilium*). Theor Appl Genet 109: 1125-1132.

Barba-Gonzalez, R., Lim, K-B., Ramanna, M.S., Visser, R.G.F. and J.M. Van Tuyl. 2005a. Occurrence of 2n gametes in the F_1 hybrids of Oriental × Asiatic lilies (*Lilium*): Relevance to intergenomic recombination and backcrossing. Euphytica 143: 67-73.

Barba-Gonzalez, R., Ramanna, M.S., Visser, R.G.F. and J.M. Van Tuyl. 2005b. Intergenomic recombination in F_1 lily hybrids (*Lilium*) and its significance for genetic variation in the BC_1 progenies as revealed by GISH and FISH. Genome 48: 884-894.

Barba-Gonzalez, R., Van Silfhout, A.A., Visser, R.G.F., Ramanna, M.S. and J.M. Van Tuyl. 2006. Progenies of allotriploids of Oriental x Asiatic lilies (*Lilium*) examined by GISH analysis. Euphytica 151: 243-250.

Comber H.F. 1947. A new classification of the Lilium. Lily Yearbook, Royal Hortic Soc 15: 86-105.

De Jong P.C. 1974. Some notes on the evolution of lilies. Lily Yearbook North Am Lily Soc 27:23-28.

Harlan, J.R. and J.M.J De Wet. 1975. On Ö. Winge and a prayer: The Origins of Polyploidy. Bot Rev 41:361-390.

Lim, K.B., Ramanna, M.S., De Jong, J.H., Jacobsen, E. and J.M. Van Tuyl. 2001a. Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. Theor Appl Genet 103: 219–230.

McRae E.A. 1998. Lilies: a guide for growers and collectors. Timber press. Portland, Oregon. pp 239-257.

Straathof, T.P. and J.M. Van Tuyl. 1994. Genetic variation in resistance to *Fusarium oxysporum* f. sp *lilii* in the genus *Lilium*. Ann Appl Biol 125: 61-72.

Van Tuyl, J.M., Marcucci, M.C. and T. Visser. 1982. Pollen and pollination experiments. VII. The effect of pollen treatment and application method on incompatibility and incongruity in *Lilium*. Euphytica 31: 613-619.

Van Tuyl, J.M., Keijzer, C.J., Wilms, H.J. and A.A.M. Kwakkenbos. 1988. Interspecific hybridization between *Lilium longiflorum* and the white Asiatic hybrid 'Mont Blanc'. Lily Yearbook North Am Lily Soc 41: 103-111.

Van Tuyl, J.M., Van Diën, M.P., Van Creij, M.G.M., Van Kleinwee, T.C.M., Franken, J. and R.J. Bino. 1991. Application of in vitro

pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci 74: 115-126.

Van Tuyl, J.M., Van Creij, M.G.M. and Van Dien, M.P. 1992. In vitro pollination and ovary culture as a breeding tool in wide hybridization of *Lilium* and *Nerine*. Acta Hort 325:461-466.

Woodcock, H.B.D. and W.T. Stearn. 1950. Lilies of the world. Their cultivation & classification. Country life limited. London. pp 15-22.Figures

How To Obtain Unreduced Gametes

Rodrigo Barba-Gonzalez

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C.

Interspecific hybridization is one of the most important ways to obtain genetic variability. In the case of lilies the interspecific hybridization has been accomplished not only between taxonomically related species, but with distantly related species from other taxonomical sections with the aim of special pollination techniques and embryo- and ovule-culture. New groups have been created from these crosses; however, further hybridization is hampered by the sterility of these hybrids. The traditional method to restore fertility is to double the chromosomes with some chemicals such as oryzalin and colchicine. The major drawback of these methods is that autosyndetic paring is promoted, converting the new allopolyploids in permanent hybrids. An alternative to the use of chemicals to double the chromosome numbers is the use of the naturally occurring "*2n*" or "unreduced" gametes, which are preferred over the synthetic polyploids because there is recombination among the parental genomes and thus genetic variability.

There is still a problem with the unreduced gametes; this is related to its frequency and the ways to obtain them. It is known that they occur in most of the angiosperm species and they might be the origin of polyploid species (Harlan and De Wet, 1975), but there is still no certainty about when will they occur. It seems that the production of such gametes is both environmental and genetically controlled. In the work of Lokker *et al.* (2005) they made a huge screening among Oriental x Asiatic (OA) hybrids to induce the formation of *2n* gametes. They placed many OA hybrids in different greenhouses, heated .and non heated and they even used a phytotron, were they exposed the lilies to drastic environmental changes for weeks, at the end they succeeded in generating the unreduced gametes, but even between clones they got different frequencies, suggesting that not the same environmental conditions will activate the genes to produce the *2n* gametes. I remember the summer of 2004 in the Netherlands, I was working at Plant Research International with some OA hybrids that regularly produced unreduced gametes, specially the genotype 951502-1 was very fertile and it was used in many combinations, There was also the genotype 952400-1, that in other years produced considerable amounts of *2n* gametes and some progeny plants were obtained. The plants were placed in a plastic greenhouse and that special summer was really hot and the temperatures raised at noon to

several degrees. Both plants had their flower buds in several stages, some of them were about a centimeter (the size of the buds where meiosis takes place in those hybrids) and were exposed to high temperatures during those hot days. Then the temperatures would drop for a few days and when those flowers opened I observed the pollen and tested its germination, that of the most fertile hybrid (951502-1) barely germinated, but the one of 952400-1 had a high germination frequency. The days passed and the temperature continued to drop, in those days more flowers of both hybrids whose buds were not exposed to high temperatures opened and the pollen germination frequencies came back to normality, 951502-1 had a high frequency of germination and 952400-1 was producing just a few fertile pollen grains again. This suggests as the experiment of Lokker *et al.* that different environmental conditions might activate the productions of the *2n* gametes depending on the genotype of the hybrid.

Other attempt to produce the unreduced gametes was made by Lim *et al.* (2005). He injected caffeine in small flower buds of different known sterile OA hybrids just before the first cytokinesis took place during meiosis. Caffeine inhibits the cytokinesis so he expected that the chemical compound could induce the formation of unreduced gametes. When the flowers opened he utilized them as both, male and female parents in different crosses. Indeed the caffeine succeeded and he obtained 279 triploid progeny plants. However, only a few number had recombinant chromosomes and just when the flowers were used as the female progenitor.

Figure 1. Gas chamber

There is a third compound that has been utilized to induce the formation of *2n* gametes; this is the nitrous oxide (N_2O) (see also the article of Jianrang Luo in this NALS volume; Luo *et al*. 2012). This chemical acts as a "spindle poison", this means that during meiosis it inhibits the

chromosome segregation and as a result the nucleus restitutes forming an unreduced gamete. The advantage of this chemical over the others is that it is a gas, and it can be directly applied under pressure to the tissues, and the effects are mitigated just by simply removing the tissue from the chamber. We utilized the N_2O to treat eight different OA hybrids (Barba-Gonzalez *et al.,* 2006); five of them were known to be completely sterile, while the three remaining produced unreduced gametes in a regular way. The hybrid plants with flower buds that ranged from 0.5 to 1 cm were placed in a gas chamber (Figure 1) and were treated at a pressure of 6 bars with the N_2O during 0 h (untreated control), 24h and 48h. After the treatment and when the flowers opened, we observed the pollen grains; the unreduced gametes are around twice the size a normal pollen grain (Figure 2) and registered the germination. Of the eight OA hybrids, six of them (three of the completely sterile) contained fertile pollen, unreduced pollen. In most of the cases, the more the exposure lasted, the higher germination percentage was obtained (Figure 3). The treated hybrids were utilized both as female and male progenitors in different crosses and progeny was obtained in both cases for five of the hybrids. Of these five, three had never had offspring. We analyzed the ploidy and the chromosome constitution of 12 of the progeny plants; eight of them were triploids, while the other four were tetraploid. The chromosome constitution showed that in most cases the unreduced gametes formed through first division restitution, and in a few cases through indeterminate meiotic restitution and also important, recombination among the parental genomes was present in some of the chromosomes.

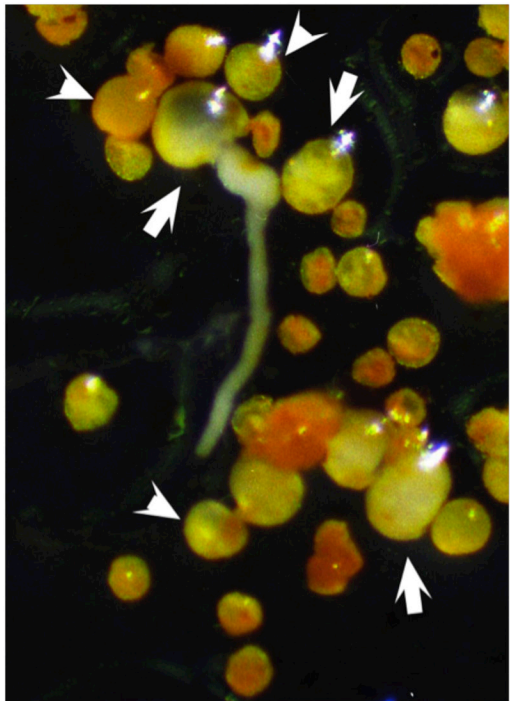

Figure 2. Pollen grains of the OA lily hybrid 951502. Arrows show the unreduced pollen and the arrowheads the normal pollen. In the center unreduced pollen is germinating.

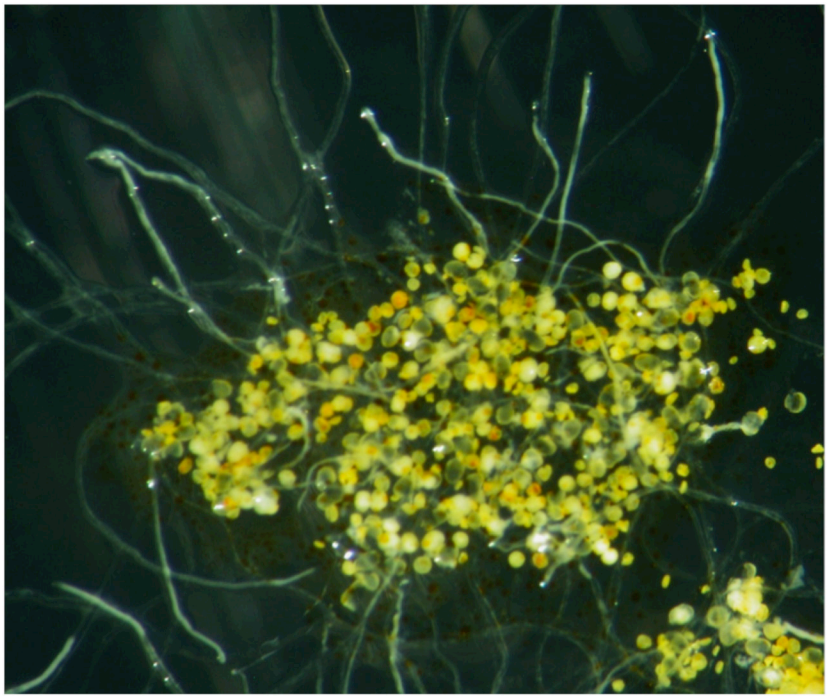

Figure 3. Unreduced pollen germinating after N_2O treatments.

This proved that the nitrous oxide under pressure can induce the formation of unreduced gametes in completely sterile lily hybrids. However, to obtain a gas chamber is not always that easy, but the observation of the pollen and the identification of the unreduced gametes by its size could be enough to discover those naturally occurring *2n* gametes, especially when there are unusual climate changes.

References

Barba-Gonzalez, R., Miller, C.T., Ramanna, M.S. and J.M. Van Tuyl. 2006.Nitrous oxide (N_2O) induces *2n* gametes in sterile F_1 hybrids between Oriental x Asiatic (*Lilium*) hybrids and leads to intergenomic recombination. Euphytica 148: 303-309.

Harlan, J.R. and J.M.J De Wet. 1975. On Ö. Winge and a prayer: The Origins of Polyploidy. Bot Rev 41:361-390.

Lim, K-B., Barba-Gonzalez, Zhou, Z., R., Ramanna, M.S. and J.M. Van Tuyl. 2005. Meiotic polyploidization with homoeologous recombination induced by caffeine treatment in interspecific lily hybrids. Korean J Genetic 27: 219-226.

Looker, A.C., Barba-Gonzalez, R., Lim, K-B., Ramanna, M.S. and J.M. Van Tuyl. 2005. Genotypic and environmental variation on production of *2n*-gametes of Oriental x Asiatic lily hybrids. Acta Hort (ISHS) 673: 453-456.

Luo, J.R., Ramanna, M.S., Arens, P., Niu L.X. and J.M. Van Tuyl. 2012. GISH analyses of backcross progenies of two Lilium species hybrids and their relevance to breeding. J Hortic Sci Biotech 87: 654-660.

Fertility Recovery and Polyploidization of Interspecific Hybrids

Lim, Ki-Byung[1] & Jaap M. van Tuyl[2]

Department of Horticultural Science,

Kyungpook National University, Daegu 702-701, Korea

Department of Biodiversity and Breeding, Wageningen

UR, The Netherlands

Longiflorum and Asiatic (LA) hybrids

The species of section Sinomartagon have a close genetic relationship. About 55 species of Lilium inhabitat E. Asia, among them, nearly 35 are endemic. The major Asiatic species are in China, Korea and Japan. Asiatic hybrids were made by many anonymous breeders long ago in Asia. Asiatic hybrids have developed rapidly in the USA and Western countries since the 1900s. The current Asiatic cultivars developed by commercial breeders are regarded as mixed genetic background within section Sinomartagon. Therefore F1 hybrids between Asiatic cultivars show, in general, fertility in both female and male gametes.

When we make crossing between Lilium longiflorum as female and Asiatic hybrid as male, the fertility is dramatically decreased. They often show no fertility or very low fertility. Normally the female organ shows higher fertility than the male organ of the F1 interspecific LA plant.

Pollen fertility is highly related to the meiosis. Meiosis in a plant is a critical process of chromosome rearrangement. The chromosomes are very concise elements of DNA threads of genetic information which are transmitted into the next generation. Meiotic processes are controlled by genes. Interspecific hybrid possesses two different sources of chromosomes (DNA) from both parental species (genome).

The key point of an interspecific hybrid about pollen fertility is highly related to the pairing of chromosomes during meiosis I stage. If two parental species are relatively closely related , there is a higher frequency of bivalent formation in meiosis I. Then paired chromosomes behave more regularly or stably during the rest of meiosis. Therefore higher bivalent formation may show higher fertility. If individual chromosomes among the chromosome set of parental genomes paired perfectly 100% during meiosis I stage, most of pollen may show highest pollen fertility. The meiosis of those cells is similar to what the normal plant does. When we look at the meiotic cells of sterile

interspecific hybrids, individual chromosome behavior of meiosis is unstable, irregular and not uniform. The genes of each species controlling meiosis are also different to each other. For example, function of mode, activation time of certain step(s) during meiosis and so on. Therefore abnormal division of meiosis leads to the incomplete meiosis resulting in a skip of first or second division of the meiosis. These abnormal meiosis may result in duplicated chromosome sets instead of monoploid (one set of chromosomes). The composition of completely duplicated chromosome sets is the same chromosome number of diploid parental plant, resulting in 2n gametes.

There are three types of 2n pollen produced by different mechanism in F1 LA hybrids. The most abundant type is FDR (First Division Restitution). FDR is a mechanism by omission of some meiosis division from anaphase I till prophase II. The chromosomes at meiosis metaphase I lie on the equatorial plate (at this stage, some chromosomes are paired and the rest of chromosomes not). We call the paired chromosomes bivalent, and not paired chromosomes are univalent. The paired chromosomes appear more thick than univalent (not paired one under the microscope observation). After meiosis I, chromosomes both once paired (we call it as half-bivalent) and not paired (univalent) stay at the equatorial plate and divided into two chromatids(univalent is composted with two sister chromatids), move to opposite poles, and finally cytokinesis formed.

Second meiotic restitution (SDR) is a mechanism by omission of some meiotic stages. 2n gametes from SDR are mainly due to independent assortment of bivalents and univalents. At anaphase I, both bivalents and univalents move into both poles and then sister chromatids disjoin (separate) each other followed by cytokinesis. In this case, two hypotheses would be considered. One would be high homozygosity; homoeologous chromosomes may have a chance at the same daughter cell and the other counterpart stays with the other cell. For example in F1 interspecific LA hybrid, two chromosome number 1 of L genome(L. longiflorum species) in one daughter cell, but the other counterpart cell only contains two chromosome number 1 of A genome(Asiatic hybrid). As a result, 2n gametes derived from SDR mechanism show a lower viability and fertility rather than those derived from FDR gametes. Therefore SDR 2n gametes has shown more frequently in a hybrid between closely related species and on the other hand, FDR 2n gametes have shown more in relatively unrelated species such as LA hybrids. We would expect that there are some viable n gametes also possible in very closely related hybrids such as hybrids between Asiatic species. We have confirmed this mechanism by analysis of chromosome techniques such as GISH (Genomic in situ Hybridization) method. GISH techniques enable us

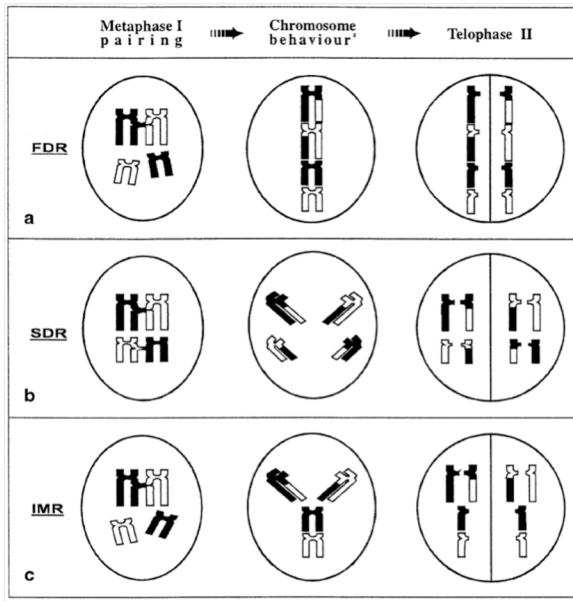

Fig. 1a–c A schematic representation of three possible types of meiotic nuclear restitution in a diploid interspecific hybrid in the case of 2n=2x=4. The homoeologous pairs of chromosomes are shown as black and white chromosomes. a; First division restitution (FDR) with recombination. At metaphase I, one bivalent and two univalents are formed. In the subsequent stage two half-bivalents and two univalents align on the equatorial plate and divide equationally. The result is that the homoeologous chromosomes do not assort independently and that the centromeres of both genomes are intact in the 2n-gametes. b; Second division restitution (SDR) with recombination showing independent assortment of homoeologous pairs of chromosomes. In this case both pairs of homoeologous chromosomes disjoin at anaphase I but restitute subsequently, i.e. without the second division. The notable features of SDR are that the homoeologous pairs assort independently of each other and that the number of centromeres of the parental genomes are not preserved intact in the resulting 2n-gametes. Moreover, each centromere is always represented in pairs. c; Indeterminate meiotic restitution (IMR) showing unequal distribution of the centromeres of the parental genomes. At metaphase I a bivalent and two univalents are formed. The bivalent disjoins normally as in the anaphase I, whereas the two univalents divide equationally. Consequently, the chromosome constitution of the parental genomes is not preserved in the 2n-gametes and, furthermore, the centromeres of each of the parental genomes are present in odd numbers. In all three cases (a–c), meiosis is incomplete. Because of this, the different stages of meiosis cannot be strictly defined.

to discriminate the parental chromosomes (in case of LA hybrid, Longiflorum and Asiatic chromosomes discriminated by color) (Lim, 2000; Lim et al. 2001). Indeterminate meiotic restitution (IMR) is a mixed mechanism between FDR and SDR. The results of IMR depend on the bivalent formation frequency. More bivalent results in higher homozygosity, on the other hand, more univalent formation would result in higher heterozygosity (Figure 1).

How to select 2n pollen producing plants?

There is one simple way to discriminate 2n pollen producing plants. The method is a pollen germination test on an artificial medium. Lily pollen grains germinate on the artificial media containing 200mg/L H_3BO_3, 6% Sucrose, 8g/L Agar. Put some pollen grains on the media and then incubate at 25C for about 6 hours. Germination of pollen can be checked under the stereo microscope. In case of 2n pollen producing LA, pollen germination may be about 1% as minimum to 15% as maximum depending on the crossing combinations. Normally 4-5% germination rate is enough to make crossing for the production of next generation. If you find one good F_1 interspecific hybrid which shows pollen germination by 2n gametes formation, you can make different off-springs by changing female partner.

2n gametes increase ploidy level in next generation

In lily, the chromosome number in the somatic cell of a diploid lily is 2x=24. Diploid plant (2x) produces haploid (n) gamete. The chromosome number of a haploid (n) gamete is 12. It is normal that the crossing between diploid female and male produces diploid offspring. However, sometimes the ploidy level of offspring is various including diploids, triploids and tetraploids. How do you explain these results? As based on chromosome analysis, triploid offsprings are formed by n-gametes from one parent and 2n-gamete from the other parent. And further, tetraploids are formed by 2n-gametesfrom both parents. Therefore 2n-gametes producing diploid plants increase the ploidy level of its off-spring. Increasing ploidy level may decrease the fertility in subsequent generation. Comparing the fertility between diploid and triploid, and diploid and tetraploids, diploid plants have higher fertility than triploid. Tetraploids may show higher fertility than triploids. Higher chromosome pairing at meiosis metaphase I is a critical point in relation to the fertility. Therefore metaphase I during meiosis is very important among any stages of gamete formation. The higher ploidy level ihas a lower fertility than diploid. Diploid is the most stable level, because of a higher chance of chromosome pairing at meiotic metaphase I. The odd ploidy level (triploid, pentaploid) may show lower fertility than even ploidy level (diploid, tetraploids). An

aneuploidy plant may have lower fertility than euploidy. Aneuploidy is a plant genome possessing an incomplete chromosome set. Increasing ploidy level by crossing methods is resulting in a lower fertility; the plant will have no fertility at the end of the crossing. Therefore increasing the ploidy level by crossing is not actually a preferable way of breeding. Pairing between homoeologous chromosomes during meiosis metaphase I stage is a key point for the pollen fertility. Introgression between two different genomes is another important point. It is highly related to the frequency of the homoeologous chromosome pairing. Incredibly diverse genetic phenomenon between different crossings happens in nature which we do not yet explain scientifically.

Table 1. Theoretical descriptions on ploidy level between different crosses and gamete formations.

Crossing				Expected off-spring ploidy level	Theoretical description
Female		Male			
Ploidy level of somatic cell	Gamete pro-duction	Ploidy level of somatic cell	Gamete pro-duction		
2x	N	2x	n	2x	Between normal diploid plants crosses, most common, stable and normal seed mature
	2n			3x	One parent produces 2n-gamete and other parent produces normal n-gamete
3x	n	2x	n	2x	Triploid female produces very low fequency of viable n-gamete
	2n			3x	Triploid female produces very low frequency of viable 2n-gamete
	n	2x	2n	3x	In case of 2n-pollen from diploid male parents
	2n			4x	A very low frequency 2n-gametes from triploid female and male parents

	n+	2x	n	2x+	A very low frequency of viable gamete with n(12 chromosomes plus few chromosomes from triploid female, and further 2n-gamete from male parent
			2n	3x+	A very low frequency of viable gamete with n(12 chromosomes) plus few chromosomes from triploid female, and further 2n-gamete from male parent
	2n+	2x	n	3x+	A very low frequency of viable gamete with 2n(24 chromosomes) plus few chromosomes from triploid female
			2n	4x+	A very low frequency of viable gamete with 2n(24 chromosomes) plus few chromosomes from triploid female, and further 2n-gamete from male parent
4x	2n	2x	n	3x	More common in nature, but embryo rescue needed.
			2n	4x	More stable embryo rescue may not needed, normal seed mature
		3x	n	3x	A very low frequency of formation
			n+	3x+	Even lower frequency of formation
			2n	4x	A very low frequency of formation
			2n+	4x+	Even lower frequency of formation
		4x	2n	4x	Mostly normal seed mature

Polyploidy by somatic chromosome doubling reduces genetic variation

Most of the interspecific hybrids show both sterile male and female gametes. Therefore, people often use somatic chromosome doubling using chemical treatment such as colchicine. Colchicine is the most commonly used chemical for chromosome doubling in plants. It is performed by dipping scales in a colchicine solution (or using the tissue culture technique). The first mitotic chromosome doubling technique and tetraploid(amphidiploid) of 'Black Beauty' were developed by Emsweller (Emsweller and Brierley, 1940; Emsweller and Uhring, 1966). Recently oryzalin was used for the chromosome doubling by tissue culture and culture about three months for the new bulbs formation. The new bulbs are checked by a ploidy analyzer in comparison with reference such as original diploid material. The small tetraploid bulbs are then grown in the greenhouse to flowering size. Flowers from tetraploids produce 2x-gametes instead of n-gametes. We indicate that n-gamete is a haploid chromosome set often produced in a diploid plant, but tetraploid plants produce a doubled chromosome number (diploid chromosome sets) in gamete instead of haploid chromosome set n=12. For example, LR (L. longiflorum x L. rubellum) interspecific hybrid (2x=12L+12R=24) produces sterile pollen due to mismatches of homoeologous chromosomes. The metaphase I stage of this LR interspecific hybrid shows it not fully paired (Lim et al. 2000). Normal diploid hybrid produces viable pollen by perfectly paired (12 bivalents at metaphase I). Tetraploid (4x=12L+12L+12R+12R=48) LR hybrid by chromosome doubling shows 24 bivalents at meiosis metaphase I. In this case, 12L (chromosome number 1, 2, 3, 4, 5, …… 12) paired another 12L (chromosome number 1, 2, 3, …. 12). More precisely speaking, L. longiflorum chromosome number 1 paired another L. longiflorum chromosome number 1, chromosome number 2 paired chromosome number 2, etc.. The L. rubellum chromosomes are also paired the same way of L. longiflorum chromosomes. Therefore, meiosis metaphase I stage shows 24 bivalents in tetraploids LR hybrid lily. The subsequent meiosis will be preceded without any problems. The final product of meiosis in this case is four pollen. Each of which contains 2x gamete (12L+12R). There is one problem occurring in tetraploids which are produced by mitotic polyploidisation. Due to perfect pairing between 12L+12L and 12A+12A, there is no introgression between L (longiflorum) and A (Asiatic) chromosomes. Pollen of tetraploids LR hybrid deliver simply 12L and 12R chromosomes for the next generation. Introgression by homoeologous recombination (recombination between L and R chromosomes) have a more positive effect for making variation in a subsequent generation. Variation is always necessary for enhancing selec-

tion efficiency. However the mitotic polyploidization method hardly shows homoeologous recombination between L and R genomes (Figure 2). The results from mitotic chromosome doubling would be the same as in LA, LO, OA and any other interspecific hybrids.

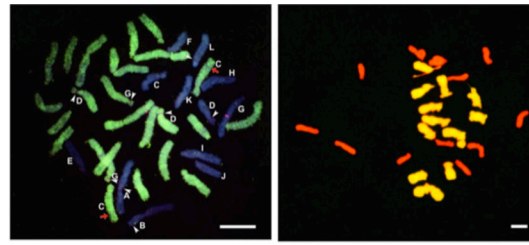

Fig. 2. GISH analysis of triploid LLR hybrid (left) indicates that two sets of L. longiflorum (24 chromosomes, green color) and one set of L. rubellum (12 chromosomes, blue) by crossing between diploid L. longiflorum and chromosome doubled tetraploid LR(LLRR, L=L. longiflorum, R=L. rubellum). Meiosis metaphase I stage of LLR hybrid shows that 12 bivalents (paired chromosome, yellow=L. longiflorum) and 12 univalents (red=L. rubellum), respectively. There is no homoeologous recombination between L. longiflorum and L. rubellum chromosomes at metaphase I.

Reference

Emsweller SL, Brierley P (1940) Colchicine-induced tetraploidy in Lilium. J Hered 31: 223 - 230

Emsweller SL, Uhring J (1966) Lilium x 'Black beauty' - Diploid and amphidiploid. Royal Hort Soc Lily YB. 29: 45 - 47

Lim KB, Chung JD, Van Kronenburg BCE, Ramanna MS, De Jong JH, Van Tuyl JM (2000) Introgression of Lilium rubellum Baker chromosomes into L. longiflorum Thunb.: a genome painting study of the F1 hybrid, BC_1 and BC_2 progenies. Chromosome Res 8: 119 - 125

Lim KB (2000) Introgression breeding through interspecific polyploidisation in lily: a molecular cytogenetic study. Ph D Thesis, Wageningen University, The Netherlands. ISBN:90-5808-311-X

Lim KB, Ramanna MS, De Jong JH, Jacobsen E, Van Tuyl JM (2001) Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. TAG 103: 219 – 230.

of this, the different stages of meiosis cannot be strictly defined.

Overcoming Crossing Barriers in Hybridisation with OT-hybrids

Jianrang Luo, Paul Arens & Jaap M. van Tuyl.

College of Forestry, Northwest Agricultural and Forestry University, P. R. China

Wageningen University (WUR), Plant Breeding, The Netherlands

Introduction

Lily is one of the most important cut-flower crops in the world. Approximately 100 species are distributed throughout the northern hemisphere (McRae, 1998). It can be classified into seven sections (Comber, 1949; De Jong, 1974). Each section has its own unique set of horticultural traits. It is desirable to combine valuable horticultural traits from the different lily sections into new cultivars through breeding. In order to do this, interspecific hybridization is the most important tool. During interspecific hybridization of distantly related *Lilium* species, multiple crossing barriers occur. In brief, there are three barriers (pre-fertilization barriers, post-fertilization barriers and sterility of interspecific hybrids). Pre-fertilization barriers that occur before fertilization can be overcome by using special pollination techniques, such as the cut style method, the grafted-style method and the *in vitro* isolated ovule pollination method (Asano and Myodo, 1977a,b; Van Tuyl et al., 1991; Van Creij et al., 2000; Chi, 2000). Post-fertilization barriers that occur during the development of the hybrid embryo can be overcome by using embryo rescue, ovule culture or ovary-slice culture (Asano, 1980; Van Tuyl et al., 1991; Okazaki et al., 1994; Wang et al., 2009). The third barrier is sterility of interspecific hybrids, which means that gametes of F_1 hybrids are usually sterile, so they cannot be used for further crossing in lily breeding. However, we found few OT (Oriental × Trumpet) lily cultivars which produced fertile gametes (most of them were female gametes). Some progenies from these OT lily cultivars were produced and their genome compositions were analysed with GISH (Genomic *in situ* hybridization). In addition, we also induced some fertile 2n pollen from highly sterile OT lily cultivars by N_2O treatment.

Materials And Methods

Plant material

Four diploid OT lily cultivars, four triploid OOT cultivars, seven dip-

loid Oriental cultivars (OO), and one diploid Oriental breeding line (OO) were used as parents (Table I; Table II). Most of the BC_1 progenies of the OT hybrids were produced by crossing diploid F_1 OT cultivars (female) × diploid Oriental cultivars (male).The two BC_1 progenies (109102-1and 109389-1) arose from diploid Oriental cultivars (female) × diploid F_1 OT cultivars (male). The BC_2 progenies were produced by crossing triploid OOT cultivars (female) × diploid Oriental cultivars (male). In this experiment, normal stigma pollination was used.

In the induction of 2n pollen, four sterile diploid OT lily cultivars ('Nymph', 'Gluhwein', 'Yelloween' and 'Shocking') obtained from the lily breeding company Word Breeding were used. A single bulb (16-18 cm) of each cultivar was planted in 17 cm pots in a heated greenhouse (25-30°C/18°C). For each of the treatments, 20-25 plants whose flower buds are in an early meiotic stage were used. For the N_2O treatment, plants were placed in a gas chamber (Barba-Gonzalez et al., 2006a) and treated with N_2O at a pressure of six bars during 24 h, 48 h and 72 h, untreated plants are used as control.

All the plant materials were maintained at Wageningen UR Plant Breeding, Wageningen, The Netherlands.

Verification of meiotic stage

Because 2*n* pollen production only occurs in a particular meiotic stage, it is only possible to induce 2*n* pollen from flower buds which contain pollen mother cells in that meiotic stage. In order to efficiently induce 2*n* pollen, we need to know the development of the meiotic stages of flower buds to pinpoint the optimal developmental stage for treatment. In *Lilium*, bud length is highly correlated with the stages of meiotic development (Bennett and Stern, 1975). Therefore, we can get information about the meiotic stage of flower buds once the correlation between bud length and meiotic stage in pollen mother cells has been established. For this, more than 180 different sized flower buds in each cultivar were used to check the meiotic stage of their pollen mother cells.

Checking 2n pollen viability

Two criteria were used to determine the production of viable 2*n* pollen. (1) Pollen grain size, at flowering time, a small amount of pollen was collected from one to three anthers of each bud, mixed and stained with aceto-carmine. For each bud, over 200 pollen grains were checked under a microscope, only big and stained pollen grains were considered to be viable 2*n* pollen.(2) *In vitro* pollen germination was carried out in artificial medium(100g sugar, 5g bacteriological agar,200mg calcium nitrate and 20mg

boric acid per litre) overnight at 25°C. After 24 h, the pollen germination percentage was observed using a light microscope.

Results

Chromosome composition of the BC progenies from the OT hybrids

The chromosome compositions of all BC progenies from the OT hybrids were summarized in Table I and Table II. Representative GISH images of chromosomes of BC progenies from the OT hybrids were shown in Figure 1. From Table I, we checked 21 BC_1 OT lily progenies in total. Among them, there were 15 euploids and six aneuploids. Many chromosome recombinations occurred in these progenies, which means that some horticultural traits may be combined together in these progenies. It also implies that we can select some good cultivars from these OT progenies. From Table II, we know that all checked BC_2 progenies of the OOT cultivars were aneuploid . Similar phenomena were also found in the BC_2 progenies of OA hybrids (Barba-Gonzalez *et al.*, 2006b). In one genotype (109439-1), a small Trumpet chromosome was observed that may be a putative B chromosome (Figure 1C).

Table I. *Chromosome composition of BC_1 progenies from Oriental × Trumpet hybrid lilies*

Genotype (code)	Female Parent	Male Parent	Cross combination*	No. of chromosome
109057-2,3,7,9	'Gluhwein'	'Montezuma'	OT×OO	36
109102-1	'Kordesa'	'Invasion'	OO×OT	24
109294-1,2,3,6,8,9	'Gluhwein'	'Lake Carey'	OT×OO	36
109365-1,3,7	'Gluhwein'	'Lake Carey'	OT×OO	36
109374-1,2	'Invasion'	'Invasion'	OT×OO	25
109389-1	'Curie'	'Curie'	OO×OT	25
109307-1,2,3	'Nymph'	'Nymph'	OT×OO	35

*OT means diploid F_1 cultivars from Oriental × Trumpet lily hybrid; OO means diploid Oriental lily.

Table II. *Chromosome composition of BC_2 progenies from Oriental × Trumpet hybrid lilies*

Genotype (code)	Female Parent	Male Parent	Cross Combination*	No. of chromosome
109095-1	'Catina'	'Montezuma'	OOT×OO	25
109439-1	'Robina'	'Sorbonne'	OOT×OO	28
109136-2	'Robina'	'Sorbonne'	OOT×OO	25
109136-5	'Robina'	'Sorbonne'	OOT×OO	27
106950-1	'Cocossa'	'Cherbourg'	OOT×OO	25
106950-2	'Cocossa'	'Cherbourg'	OOT×OO	27
106950-4	'Cocossa'	'Cherbourg'	OOT×OO	29
109180-1	'Concador'	'Cobra'	OOT×OO	26
109180-4	'Concador'	'Cobra'	OOT×OO	28

*OOT means triploid BC_1 cultivars from Oriental × Trumpet lily hybrid; OO means diploid Oriental lily.

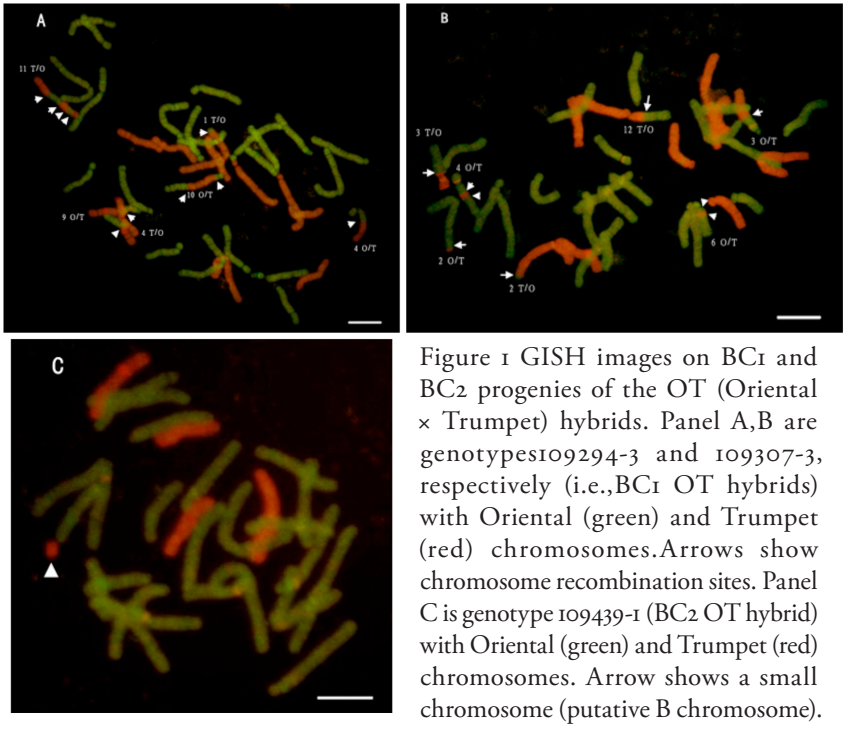

Figure 1 GISH images on BC1 and BC2 progenies of the OT (Oriental × Trumpet) hybrids. Panel A,B are genotypes109294-3 and 109307-3, respectively (i.e.,BC1 OT hybrids) with Oriental (green) and Trumpet (red) chromosomes.Arrows show chromosome recombination sites. Panel C is genotype 109439-1 (BC2 OT hybrid) with Oriental (green) and Trumpet (red) chromosomes. Arrow shows a small chromosome (putative B chromosome).

Meiotic stage of pollen mother cells in different sizes buds

In most cases (70%), there were similar meiotic stages in one flower bud. In others (30%), mixed meiotic stages were also observed in the same flower bud (Figure 2B). In this case, the predominant meiotic phase was taken as the meiotic phase and used to establish the correlation with bud length (Table III). In the case of 'Gluhwein' and 'Shocking', meiosis did not start in buds whose bud length was less than 23 mm. Buds ranging from 23 to 29 mm exhibited predominantly prophase I whereas buds ranging from 30 to 33 mm exhibited metaphase I-telophase II. Most pollen mother cells completed meiosis and become microspore when bud length was over 36 mm. In the case of 'Nymph' and 'Yelloween', meiosis did not start before buds were 26 mm long. Prophase I was observed in buds ranging from 26 to 32 mm in length and metaphase I-telophase II stages were observed in buds ranging from 33 to 36 mm. When bud length was over 40 mm, almost all pollen mother cells finished their meiosis and become microspore.

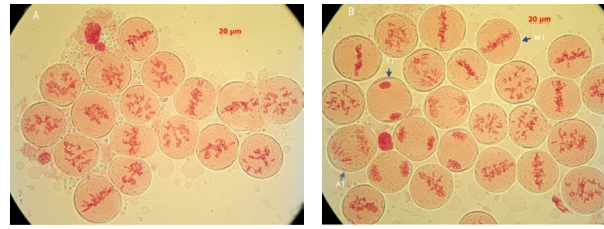

Figure 2 Meiotic stages in pollen mother cell of 'Gluhwein'. A shows similar meiotic stages (metaphase I) in one flower bud (bud length 30mm); B shows mixture meiotic stages in one flower bud (bud length 31mm). M I means metaphase I, A I means anaphase I and T I means telophase I.

Table III. *Correlation between bud size and meiotic stage in pollen mother cells in Lilium*

Genotypes	Bud length	Meiotic stage
'Gluhwein' & 'Shocking'	<23	Interphase
	23-29	Prophase I
	30-33	Metaphase I-Telophase II
	>36	Microspore
'Nymph' & 'Yelloween'	<26	Interphase
	26-32	Prophase I
	33-36	Metaphase I-Telophase II
	>40	Microspore

Table IV. *Effect of different inducing methods on pollen fertility in Lilium*

Inducing methods	Genotypes	Treating time	No. of treated flowers	No. and % of fertile flower	Germination (%)
N2O treatment	'Gluhwein'	24 h	45	5(11.1%)	5-90%(32.0%)
		48 h	41	6(14.6%)	45-95%(72.5%)
		72 h*	31	0	0
	'Nymph'	24 h	74	4(5.4%)	5-50%(18.8%)
		48 h	50	8(16.0%)	5-90%(32.5%)
		72 h*	31	0	0
	'Yelloween'	24 h	60	0	0
		48 h	46	2(4.3%)	10-90%(50%)
		72 h*	30	0	0
	'Shocking'	24 h	49	0	0
		48 h	48	0	0
		72 h*	25	0	0
Control (green-house)	'Gluhwein'	-	60	0	0
	'Nymph'	-	63	0	0
	'Yelloween'	-	65	0	0
	'Shocking'	-	60	0	0

* Most flower buds were damaged in N_2O treatment

Effect of N2O treatment on pollen fertility

From our previous studies on other genotypes of lily, it was established that only giant, well filled and stainable pollen grains are viable 2*n* pollen. However, a more reliable criterion to detect viable 2*n* pollen is pollen germination. For these four OT cultivars, there was no pollen germination in the control (Table IV, Figure 3, 4). After N_2O treatment, three OT hybrids ('Nymph', 'Gluhwein' and 'Yelloween') produced some pollen which can germinate. In addition, both fertile flower percentage and pollen germination were all higher in the 48 h treatment than that in the 24 h treatment. In the case of 72 h treatment, most flower buds or plants were damaged whereas other undamaged flower showed aberrant anthers at flowering time. This indicated that 48 h N_2O treatment was optimal in OT lily

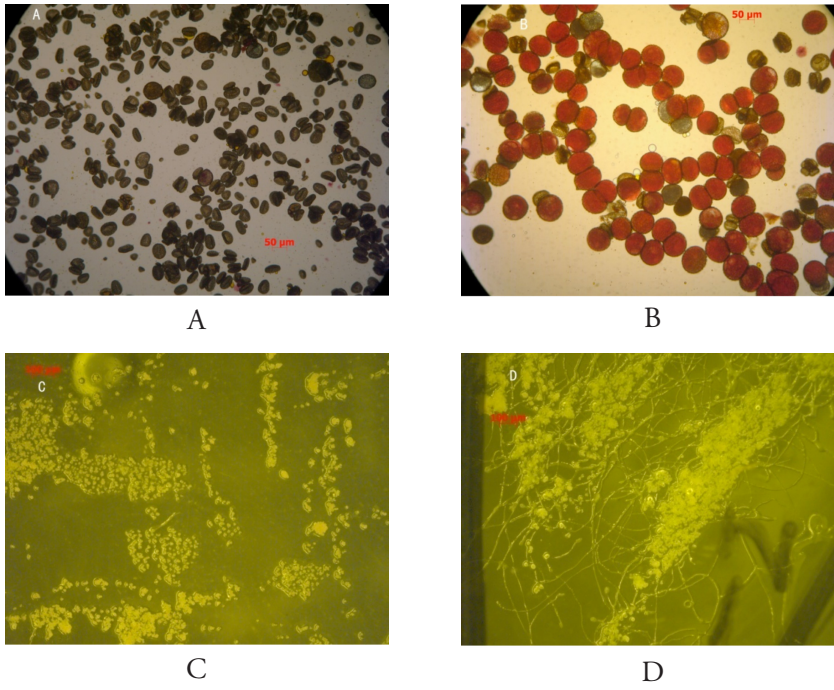

Figure 3 Comparion of sterile pollen and fertile 2*n* pollen in 'Gluhwein'. **A** shows sterile pollen which was small and shrinked and cannot be stained by aceto-carmine; **B** shows fertile 2*n* pollen which was big and round and can be stained by aceto-carmine; **C** shows sterile pollen which does not germinate on medium; **D** shows fertile 2*n* pollen which germinated on medium.

Conclusions

Gametes of OT lily hybrids are usually sterile, but a few gametes of some OT lily cultivars were fertile and can produce viable 2n female gametes.

N_2O can induce highly sterile OT lily cultivars to produce viable 2n pollen. 48 h N_2O treatment was optimal in OT lily hybrids.

Figure 4 Control flower (sterile) and N_2O treatmented flower (fertile) a: Control flower (Gluhwein); b: N_2O treatmented flower (Gluhwein); c: Control flower (Nymph); d: N_2O treatmented flower (Nymph); e: control flower (Yelloween); f: N_2O treatmented flower (Yelloween)

References

ASANO, Y. and MYODO, H. (1977a). Studies on crosses between distantly related species of Lilies. I. For the intrastylar pollination technique. *Journal of the Japanese Society for Horticultural Science*, **46,** 59-65.

ASANO, Y. and MYODO, H. (1977b). Studies on crosses between distantly related species of lilies. II. The culture of immature hybrid embryos. *Journal of the Japanese Society for Horticultural Science*, **46**, 267-273.

ASANO, Y. (1980). Studies on crosses between distantly related species of lilies. IV. The culture of immature hybrid embryos 0.3-0.4 mm long. *Journal of the Japanese Society for Horticultural Science,* **49**, 114-118.

BARBA-GONZALEZ, R., MILLER, C.T., RAMANNA, M.S. and VAN TUYL, J.M. (2006a). Nitrous oxide (N_2O) induces 2*n* gametes in sterile F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids and leads to intergenomic recombination. *Euphytica*, **148**, 303-309.

BARBA-GONZALEZ, R., SILFHOUT, A.A., VISSER, R.G.F., RAMANNA, M.S. and VAN TUYL, J.M. (2006b). Progenies of allotriploids of Oriental ×Asiatic lilies examined by GISH analysis. *Euphytica*, **151**, 243-250.

BENNETT, M.D. and STERN, H. (1975).The Time and Duration of Female Meiosis in *Lilium*. *Proceedings of the Royal Society of London. Series B, Biological Sciences,***188**,459-475.

CHI H.S. (2000). Interspecific crosses of lily by in vitro pollinated ovules. *Botanical Bulletin of Academia Sinica*, **41**,143-149.

COMBER, H.F. (1949). A new classification of the genus Lilium. In: Lily Yearbook, Royal Horticulture Society, **13**, 86-105.

DE JONG, P.C. (1974). Some notes on the evolution of lilies. In: Lily yearbook, North American Lily Society, **27**, 23-28.

MCRAE, E.A. (1998). Lilies: a guide for growers and collectors. Timber Press, Portland, OR, USA.381pp.

OKAZAKI, K., ASANO, Y. and OOSAWA, K. (1994). Interspecific hybrids between *Lilium* 'Oriental' hybrid and *L.*'Asiatic' hybrid produced by embryo culture with revised media. *Breeding Science*, **44**, 59-64.

VAN CREIJ M.G.M., KERCKHOFFS D.M.F.J., VAN TUYL J.M. (2000). Application of four pollination techniques and of hormone treatment for bypassing interspecific crossing barriers in

Lilium. *Acta Horticulturae*, **508**, 267–276.

VAN TUYL, J.M., VAN DIËN, M.P., VAN CREIJ, M.G.M., VAN KLEINWEE, T.C.M., FRANKEN, J. and BINO, R.J. (1991). Application of *in vitro* pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. *Plant Science*, **74**, 115-126.

WANG J., HUANG L., BAO M.Z., LIU G. F. (2009).Production of interspecific hybrids between *Lilium longiflorum* and *L. lophophorum* var. *linearifolium* via ovule culture at early stage. *Euphytica*, **167**:45–55.

Cytological Maps Based on Recombination Sites Detected by GISH in Interspecific Lily Hybrids

Nadeem Khan, Agnieszka Marasek-Ciolakowska, Munikote Ramanna, Paul Arens, Alex van Silfhout, Jaap M Van Tuyl

Introduction

Cytological maps are constructed by localizing DNA markers along the chromosomes in relation to chromosomal structures such as centromeres, telomeres and secondary constrictions (if any). Such maps are created by microscopic determination of the position of visible structures or markers in fixed and stained chromosomes. These cytological maps are also called chromosome maps. These maps are used to relate genetic data based on molecular markers or DNA sequences to morphological features of chromosomes (Cheng et al. 2001). Such cytological maps were most useful for assigning the recombination sites and their physical distance on individual chromosomes in some crops (Singh et al. 1996). Differential staining techniques, such as Giemsa C-and Q-banding were used extensively as chromosome markers in lilies (Holm 1976). In these techniques each chromosome shows a distinct pattern of banding. Beside this the chromosome deletions and translocations which can be visualised cytologically were also used for mapping genes in some plants (Howell et al. 2002; Bhat et al. 2007).

Discovery of Fluorescent *in situ* Hybridization (FISH) has opened the possibilities for localizing large numbers of cloned DNA sequences directly on chromosomes for mapping purposes. This technique has been used to construct chromosome maps or, the so-called, cytomolecular maps in different plant species (Islam-Faridi et al. 2007; Sun et al. 2013). Since the cloned DNA sequences can be directly localized on chromosomes, this method is becoming increasingly important in plant molecular cytogenetics. However, the plants with large chromosomes possessing huge amounts of dispersed repetitive DNA sequences, such as *Lilium* the hybridization of DNA probes for FISH is not so specific. Furthermore, it is less suitable to unravel the process of crossing over event.

Genomic DNA *in situ* hybridization (GISH) where whole nuclear DNA is used as probe in hybridization experiments can be used successfully for analysing the process of inter-genomic recombination as well as for the elucidation of chromosome organization. But GISH can only be applied in case of distant hybrids (hybrids obtained from the parents with quite dis-

tant or variable DNA). Because when the parental genomes are sufficiently differentiated, as is the case of lily interspecific hybrids, GISH can be most effective for accurately distinguishing the parental genomes in the hybrids and estimate intergenomic recombination in their progenies.

GISH and Cytogenetic mapping in interspecific lily hybrids

The lily species genomes are completely differentiated and amenable for GISH analysis (Lim et al. 2003; Barba-Gonzalez et al. 2004). GISH has been used extensively for cytological analysis to study the mechanism of 2*n* gamete formation in interspecific lily hybrids (Lim et al. 2001a). Intergenomic recombination was monitored in backcross progenies of Longiflorum × Asiatic and Oriental × Asiatic interspecific hybrids using GISH (Lim et al. 2003; Barba-Gonzalez et al. 2004; Khan et al. 2010). Cytological maps of three genomes of lilies were constructed based on the recombination sites identified through GISH in the backcross progenies of two interspecific lily hybrids. Khan et al. (2009) investigated that manipulation of genomes in lily could be facilitated by molecular cytogenetic techniques of individual chromosomes. The considerable frequency of homoeologous recombination and easy discrimination of parental genomes in hybrids by GISH created a new strategy for chromosomes mapping in *Lilium.* The availability of crossing over points permits comprehensive studies of the chromosomal recombination and the localization of the introgressed segments in different backgrounds of interspecific lily hybrids. In this study mostly triploid progenies derived from functional 2*n* gametes with recombinant chromosomes were mapped. These involve hybrids between three main groups of diploid (2n = 2x = 24) cultivars, i.e., Asiatic (A), Longiflorum (L) and Oriental (O) which belong to three different taxonomic (Figure 1) sections of lilies. These cultivars were used to produce F1 hybrids when crossed with the species from other sections. For example, a Longiflorum cultivar was crossed with an Asiatic and Oriental cultivar was used to cross with Asiatic cultivars. These crosses resulted in to the formation of two types of hybrids: Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA). For backcrossing, the LA hybrids were used either as female or male parents and crossed with Asiatic parents to get (LA × A or A × LA) BC1 progenies. Similarly OA hybrids were used as male parents for backcrossing with the Asiatic cultivars (i.e., A × OA).

Mitotic chromosomes where prepared from root tips of interspecific lily hybrids to carry out GISH analysis. For GISH the genomic DNA of Longiflorum cultivar 'White Fox' and Oriental cultivar 'Sorbonne' were used as probe DNA and labeled with either Digoxigenin-11-dUTP or Biotin-16-dUTP by a standard nick translation protocol (Roche Diagnostics

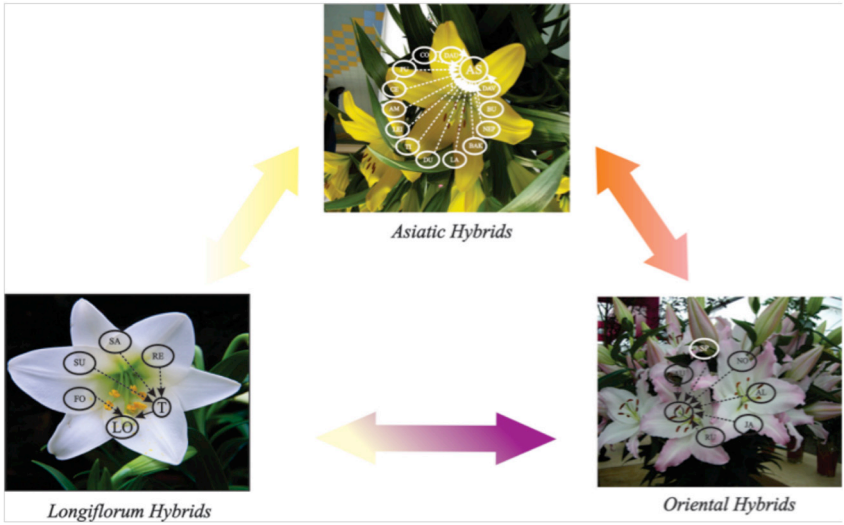

Fig. 1 Simplified crossing polygon amongst the species from Asiatic, Longiflorum and Oriental hybrids used to construct chromosomal map based on GISH. The ellipses show the species with in respective sections.

GmbH, Mannheim, Germany). The genomic DNA from Asiatic cultivar 'Connecticut King' was used as blocking DNA. After hybridization the preparations were analysed using an epifluorescence microscope and photographed for the determination of total number of chromosome and the number of recombination sites.

In all the three genomes the chromosomes are arranged in sequence of decreased short arm length according to Stewart (1947) taking into account the position of 45S rDNA hybridization signals in LL and OA hybrids (Lim et al. 2001b) . Images of mitotic metaphase chromosomes from each genotype were measured in micrometers using the computer program MicroMeasure (Reeves & Tears 2000). For mapping purposes the centromere of each chromosome was taken as the starting point and recombinant point found by GISH was used as markers for recombination mapping. Recombination sites were identified and measured as a percentage of the arm length from the centromere (both short and long arm) as shown in Fig. 2. After compiling the recombination data, the recombination distribution was determined on each chromosome based on its length in relation to the size of whole genome in micrometers. The calculated expected values were compared with the observed ones (the visible cytological markers found on each chromosome based on GISH analysis) to measure the frequency of recombination distribution in each chromosome in three different genomes of lily.

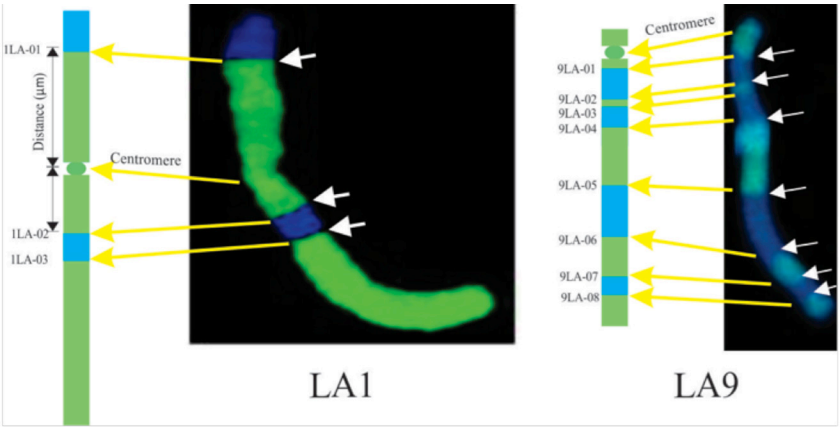

Fig. 2 Identification of individual chromosomes and mapping of recombination sites in the respective chromosomes. Green colour represents the Longiflorum genome while blue colour represents the Asiatic genome. LA1 represents the introgression of Asiatic segments in the chromosomes 1 of Longiflorum genome while LA9 is the introgression of Asiatic segments in the chromosomes 9 of Longiflorum genome. The arrow head show the site of recombination in different Longiflorum chromosomes.

Genome contribution and recombination sites

In order to construct cytological maps, the genome composition and recombinant chromosomes were identified in 71 backcross progenies (BC) of LA hybrids and 41 BC progenies of OA hybrids. Based on this data, the frequencies and distribution of recombination sites in different chromosomes of four genomes were determined.

With GISH the recombinant chromosomes could be clearly distinguished from the non-recombinant chromosomes and there were two distinct types (Figure 3A and 3B). A chromosome with Longiflorum centromere possessing Asiatic recombinant segment is indicated as L/A and Asiatic centromere possessing Longiflorum recombinant segment as A/L. Similarly in case of OA hybrids a chromosome of Oriental with a recombinant segment of Asiatic is indicated as O/A and *vice versa,* A/O (Fig 3C). The number of these four types of recombinant chromosomes (L/A. A/L, O/A and A/O) varied in different BC1 genotypes. This variation was expected to occur in view of the disturbed homoeologous chromosome pairing during meiosis in these interspecific hybrids. The identification of two types of recombinant chromosomes and the recombination sites in the progenies of each of the hybrids, i.e., L/A, A/L (in LAA or ALA), and O/A, A/O (in AOA) progenies, enabled to map recombination sites on all the 12 individual chromosomes of the con-

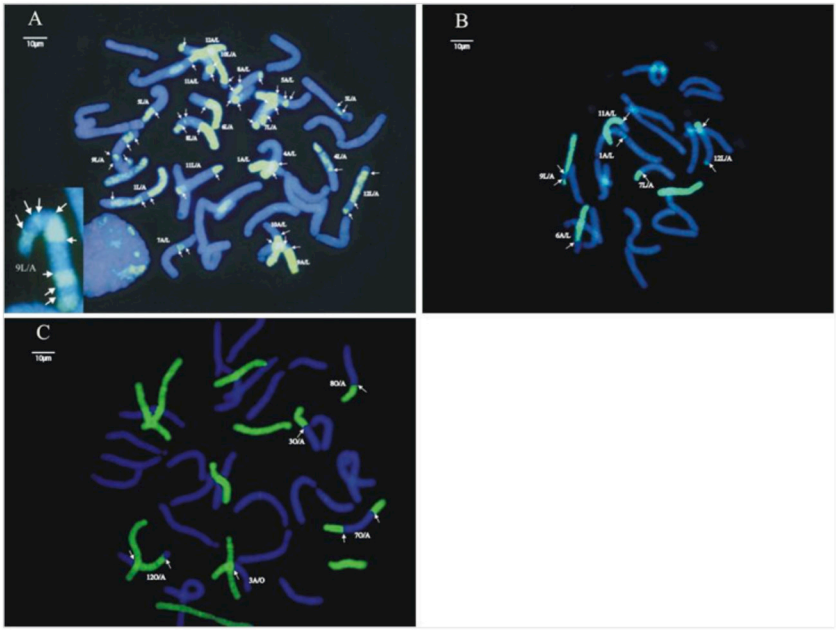

Fig. 3 (A-C). Somatic metaphase chromosomes of BC1 progenies of LA and OA hybrids showing recombination sites on different chromosomes after GISH (arrows). **A**. A triploid ($2n = 3x = 36$) BC_1 progeny of LA hybrid (LAA, 066994-3) with 49 recombination sites (arrows). Inset: a recombinant chromosome showing 8 recombination sites in BC1 LA hybrid (062071-2). **B**. A diploid ($2n = 2x = 24$) BC_1 progeny of LA hybrid (LAA, 066828-5) with 8 recombination sites (arrows). **C**. A triploid ($2n = 3x = 36$) BC_1 progeny of OA hybrid (AOA, 022605-24) with 7 recombination sites (arrows). Blue colour represents the Asiatic genome while green colour indicates the Longiflorum genomes. (Khan *et al*. 2009)

stituent genomes of both LA and OA hybrids. A total of 248 recombination sites were mapped to L and A genomes and a total of 122 recombination sites were mapped on O and A genomes. The four genomes were mapped based on GISH are: i) Asiatic (L) indicating the chromosomes of A with recombinant segments of Longiflorum, ii) Longiflorum (A) representing the chromosomes of L with recombinant segments of Asiatic. Similarly, the same pattern was followed for iii) Asiatic (O) and iv) Oriental (A). The recombination sites were most unevenly distributed on different chromosomes in all the four genomes (Figure 4A, B, C and D).

The cytological maps constructed in the present investigation prove that the entire genomes of lilies can be mapped through GISH. No doubt that the BC progenies from distant hybrids only could be used for mapping

– without which the constituent genomes and the recombinant sites could not be distinguished through GISH. These maps based on recombination sites identified by GISH have three important advantages; i) They serve as permanent cytological land marks on chromosomes that can be used for mapping molecular markers or genes of interest, ii) they provide information on the phenomenon of crossing-over event i.e., the number, position and distribution of recombination site on chromosomes and in the genomes and iii) these maps give a clear picture of whole genomic structure rather than concentrating on only one or two chromosomes.

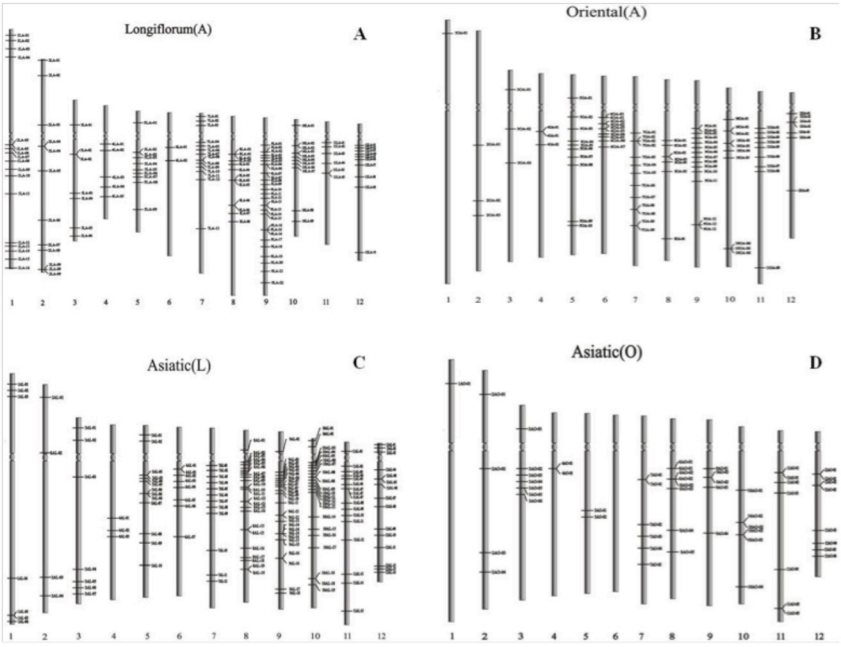

Fig. 4 (A-D). Four chromosomal recombination maps resulting from the analysis of BC progenies of LA and OA hybrids. A. Longiflorum (A); B. Oriental (A) C. Asiatic (L) and D. Asiatic (O)- the recombination partner in each is given in parenthesis. (Khan et al. 2009)

References

Barba-Gonzalez R, Lokker BH, Lim K-B, Ramanna MS, Van Tuyl JM (2004) Use of 2*n* gametes for the production of sexual polyploids from sterile Oriental x Asiatic hybrids of lilies (*Lilium*). Theor Appl Genet 109: 1125-1132

Bhat PR, Lukaszewski A, Cui A, Xu J, Svensson JT, Wanamaker S,

Waines JG, Close TJ (2007) Mapping translocation breakpoints using a wheat microarray. Nucleic Acid Res 35: 2936-2943

Cheng Z, Buell CR, Wing RA, Gu M, Jiang J (2001) Toward a cytological characterization of the rice genome. Genome Res 11: 2133-2141

Holm PB (1976) The C and Q banding patterns of the chromosomes of *Lilium longiflorum* (thunb.). Carl Res Comm 41: 217-224

Howell EC, Barker GC, Jones GH, Kearsey MJ, King GJ (2002) Integration of the cytogenetic and genetic linkage maps of *Brassica oleracea*. Genetics 161: 1225-1234

Islam-Faridi MN, Nelson CD, Kubisiak TL (2007) Reference karyotype and cytomolecular map for loblolly pine (*Pinus taeda* L.). Genome 50: 241-251

Khan N, Barba-Gonzalez R, Ramanna MS, Arens A, Visser RGF, Van Tuyl JM (2010) Relevance of unilateral and bilateral sexual polyploidization in relation to intergenomic recombination and introgression in *Lilium* species hybrids. Euphytica 171: 157-173

Khan N, Barba-Gonzalez R, Ramanna MS, Visser RG, Van Tuyl JM (2009) Construction of chromosomal recombination maps of three genomes of lilies (*Lilium*) based on GISH analysis. Genome 52: 238-351

Lim K-B, Ramanna MS, De Jong JH, Jacobsen E, Van Tuyl JM (2001a) Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. Theor Appl Genet 103: 219-230

Lim K-B, Ramanna MS, Jacobsen E, Van Tuyl JM (2003) Evaluation of BC2 progenies derived from 3x - 2x and 3x - 4x crosses of *Lilium* hybrids: a GISH analysis. Theor Appl Genet 106: 568-574

Lim K-B, Wennekes J, de Jong JH, Jacobsen E, Van Tuyl JM (2001b) Karyotype analysis of *Lilium longiflorum* and *Lilium rubellum* by chromosome banding and fluorescence in situ hybridization. Genome 44: 911–918

Reeves A, Tear J (2000) MicroMeasure for Windows, version 3.3. Free program distributed by the authors over the Internet from http://www.colostate.edu/Depts/Biology/MicroMeasure

Singh K, Ishii T, Parco A, Huang N, Brar DS, Khush GS (1996) Centromere mapping and orientation of the molecular l**in**kage map of **rice** (*Oryza sativa* L.) Proc Natl Acad Sci 93: 6163–6168

Stewart RN (1947) The morphology of somatic chromosomes in *Lilium*. Amer J Bot 34: 9-26

Sun J, Zhang Z, Zong X, Huang S, Li Z, Han Y (2013) A high-resolution cucumber cytogenetic map integrated with the genome assembly. BMC Genomics 14: 461

Zhou S, Ramanna MS, Visser RGF, Van Tuyl JM (2008) Genome composition of triploid lily cultivars derived from sexual polyploidization of Longiflorum × Asiatic hybrids (*Lilium*). Euphytica 160: 207-215

The Use of Chromosomal Markers for Interspecific Hybrids Verification in *Lilium*

Agnieszka Marasek-Ciolakowska, Teresa Orlikowska,

Research Institute of Horticulture, Skierniewice, Poland

A hybridization of taxonomically distant genotypes (wide or interspecific crosses) is one of the most important methods of lily breeding enabling the introduction of the desired traits. However, not all seedlings obtained as a result of distant crosses are hybrids. Apomixis (the process of embryo development from unfertilised maternal cell), which was noticed in genus *Lilium* e.g., in *Lilium regale* (North and Wills, 1969), *L. speciosum*, *L. canadense*, *L. szovitisianum*, *L. longiflorum*, *L. superbum* and *L. pumilum* (Georgi 1985) or the mistaken pollination cause the necessity to confirm whether seedlings obtained from distant crosses are indeed real hybrids (Figure 1). The hybrid status of lily seedlings from distant hybridisation can be confirmed based on the morphological traits, especially flowers, which takes 2-3 years (Obata at al., 2000). This is why methods enabling earlier verification of hybrids are necessary. In this study chromosomal markers for parental forms were established on the basis of the chromosome morphology, silver staining of nucleolar organizing regions (NORs), fluorescent (CMA_3/DA/DAPI) and Giemsa staining and they were subsequently used for verification of hybrid status of F_1 lily plants obtained from crosses Oriental hybrid (OR) 'Marco Polo' × *Lilium henryi*, *L. henryi* × OR 'Marco Polo', OR 'Expression' × *L. henryi,* OR 'Alma Ata' × *L. pumilum* and OR 'Muscadet' × *L.* × *formolongi*.

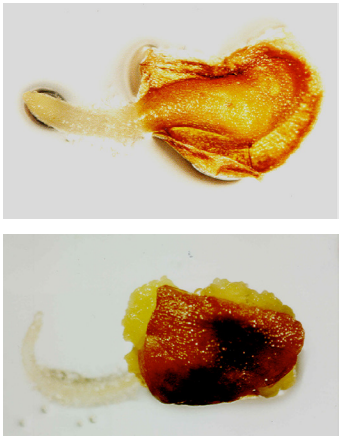

Figure 1.Normal seed germination; b. Regeneration from callus (interspecific hybrid).

Lily chromosomes are convenient for cytological analyses due to their large size. However, morphology of chromosomes turned out rather conserved within and between species and only secondary constrictions could be used as markers for both identification of individual chromosomes and hybrid status verification. In genotypes analysed in this study the number of chromosomes having secondary constrictions that could serve for markers varied from 4 to 10. Standard staining of chromosomes using e.g. acetocar-

mine or Schiff's reagent does not always reveal all secondary constrictions. They can be invisible especially at high chromosomes condensation (Figure 2). This problem also appeared in our study concerning D chromosomes of 'Expression' and 'Marco Polo' on which the presence of secondary constrictions were not seen after Feulgen staining (Marasek and Orlikowska, 2003) and was revealed only when silver staining was used.

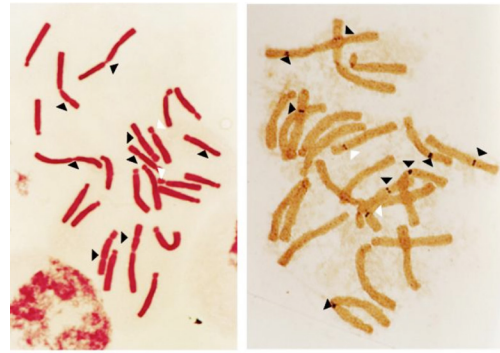

Figure 2. Metaphase chromosomes of *L. pumilum* a. Feulgen-stained; b. Silver stained nucleolar organizing regions (NORs). Secondary constrictions marked by arrowheads. White arrowheads indicate secondary constrictions revealed after silver staining.

Silver staining of nucleolar organizing regions (NORs)

Silver bands are important for idiograms construction, especially when primary and secondary constrictions are localised close to each other (Marasek and Orlikowska, 2003). Silver staining helps in construction of idiograms enabling the differentiation between primary and secondary constrictions. In genotypes analysed the following numbers of NORs were found: 2 pairs *(L. henryi)*, 3 pairs (*L.* × *formolongi*), 5 pairs (*L. pumilum*), 5 NORs (2II + 1I) (*L. candidum*, 'Expression' and 'Muscadet'), 6 NORs (2II + 2I) ('Marco Polo'), 7 NORs (3II + 1I) ('Alma Ata').

Fluorescence staining

The double fluorescence banding obtained using CMA_3/DA/DAPI, reveals only CMA_3 bands that are easily recognizable on chromosomes, co-localizing in the positions of secondary constrictions or were situated intercalary on chromosomes. In genotypes analysed the following numbers of CMA_3 bands were found: 2 pairs (*L. henryi*), 3 pairs (*L.* × *formolongi*), 5 pairs (*L. pumilum*) and 7 CMA_3 bands (2II + 3I) (*L. candidum*). In cultivars 10 CMA_3 bands in 'Muscadet' (5II) and in 'Alma Ata' (4II + 2I), 9 bands in 'Marco Polo' (3II + 3I) and 7 bands in 'Expression' (3II + 1I).

Giemsa staining

Most C bands were located near primary and secondary constrictions. They were also observed in intercalary positions both, on the short and the long arms of chromosomes. The number of C-bands obtained was often

unrepeatable, therefore only these seen in all metaphase plates were considered. In genotypes analysed the following numbers of C bands were observed: 23 in *L. candidum* (10II + 3I), 12 in *L.* × *formolongi* (6II), 18 in *L. henryi* (9II) and 13 in *L. pumilum* (6II + 1I), 12 in 'Alma Ata' (5II + 2I), 23 in 'Expression' (9II + 5I), 13 in 'Marco Polo' (4II + 5I) and 12 in 'Muscadet' (10II + 2I). In all genotypes tested the polymorphism between homologous chromosomes was observed in the number and the size of bands. In our investigation, C banding has provided the most markers for identification of individual chromosomes. Nevertheless, standardisation of this method was not easy and results obtained were often unrepeatable. In genotypes analysed C bands were found in different locations on chromosomes: close to primary and secondary constrictions or in pericentromerical position.

Marker chromosomes and hybrids verification

The first step in hybrid verification relies on the choosing of marker chromosomes for parental genotypes. Only chromosomes having characteristic bands on both homologous chromosomes could be accounted for markers. It was assumed that the presence of marker chromosomes of paternal genotype was the confirmation of hybridity. The list of marker chromosomes enabling verification of hybrids of all possible combinations between species and cultivars is presented in Table 1. For example, hybrids of 'Expression' × *L. henryi* can be verified easily by the presence of paternal F chromosome (staining Ag-NOR and CMA_3) or A, C, D, F, H and L (paternal chromosomes stained by Giemsa) (Figure 3).

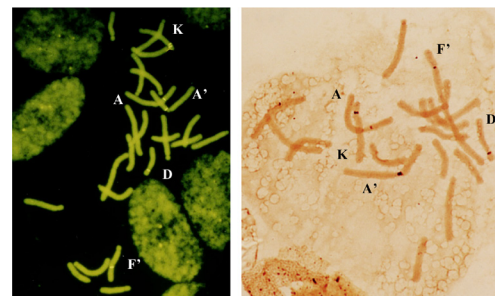

Figure 3. Metaphase chromosomes of Oriental hybrid 'Expression' × *L. henryi.* a. staining; b. silver staining. Chromosomes A, D, K are characteristic for 'Expression' and A', F' for *L. henryi*

All the plants obtained from crosses 'Expression' × *L. henryi* and 'Marco Polo' × *L. henryi* represented markers of paternal form. In *L. henryi* × 'Marco Polo', only in one out of 4 putative hybrids chromosomes characteristic for paternal form were found. Hybrids were not found in the progeny of combinations 'Alma Ata' × *L. pumilum* and 'Muscadet' × *L.* × *formolongi.*

Cultivar	Staining	Species			
		L. candidum	*L.* × *formolongi*	*L. henryi*	*L. pumilum*
'Alma Ata'	Ag-NOR3	D, F	C, D, G	A, F	A, B, C, D, F
	CMA3	F, I	C, D, G	A, F	A, B, C, D, F
	C-bands	D, F, H, I, J, K	C, D, G, I	C, F, H, L	B, C, F
	Ag-NOR	K	K	K	K
	CMA3	A, C, K	A, C, K	A, C, K	A, C, K
	C-bands	D, K	C, K	C, K	C, K
'Expression'	Ag-NOR3	D	C, G	F	A, B, C, F
	CMA3	F, I	C, G	F	A, B, C, F
	C-bands	D, F	C, G, I	A, C, D, F, H, L	B, C, F
	Ag-NOR	D, K	K	D, K	K
	CMA3	D, K	K	D, K	K
	C-bands	A, D, E, K	A, C, E, F, J, K	A, C, D, E, J, K	C, J, K
'Marco Polo'	Ag-NOR3	F	C, G	F	C, F
	CMA3	F, I	C, G	F	C, F
	C-bands	D, F, H, J, K	C, G, I	C, F, G, H, L	C, F
	Ag-NOR	K	K	K	K
	CMA3	K	K	D, K	K
	C-bands	D, K	K	C, K	K
'Muscadet'	Ag-NOR3	F	C, G	A, F	A, B, C, F
	CMA3	F, I	C, G	A, F	A, B, C, F
	C-bands	D, F, H. I, J, K	C, G, I	C, F, G, H, L	A, C, F
	Ag-NOR	K	K	D, K	K
	CMA3	A, K	A, C, K	A, C, D, K	C, K
	C-bands	A, D, B, F	A, B, C, E, F, K	B, K	A, K

Table 1. Marker chromosomes chosen for distant lily hybrid between species and cultivars verification obtained after silver staining, fluorescent banding and C banding. Chromosomes characteristic for species are shadowed

Conclusions

Each of the three chromosome banding methods can be used for verification of lily distant hybrids. The largest polymorphism was observed in Giemsa treated chromosomes but this method was the most troublesome due to standardization and reproducibility. The silver staining is the most easy and therefore most helpful in construction of idiograms. Fluorescence staining is also easy, informative and reproducible but needs a fluorescence microscope.

References

Georgi, H., 1985. The significance of apogamy and apomictic phenomena in lily hybridization. The Lily Yearbook NALS 85: 45 – 46

Marasek A., Orlikowska T. 2003: Karyology of nine lily genotypes. Acta Biologica Cracoviensia, Seria Botanica 45/2:165-174

North, C. & A.B. Wills, 1969. Inter-specific hybrids of *Lilium lankongense* Franchet produced by embryo-culture. Euphytica 18: 430-434

Stewart RN. 1947. The morphology of somatic chromosomes in *Lilium*. American Journal of Botany 34: 9–26

Meiosis in Interspecific Lily Hybrids

Songlin Xie

Group of bulbous flower breeding, Sino-Europe Agricultural Development Centre. Zhangzhou, Fujian 363105, P.R. China

Nine years ago, I started working on lily as a MSc student. At that time, I was investigating and collecting the wild lily species in Qinling Mountains in central China. After two years, using these collected species, I studied the genetic diversity on morphology, palynology, micro-morphology and chromosome level. At the end of 2007, I went to Wageningen University for learning lily breeding and cytogenetics under supervision of Dr. Jaap van Tuyl. In 2012, I went back to China and started working as a group leader of bulbous flower breeding in Sino-Europe Agricultural Development Centre (SEADC).

The significance of interspecific hybridization has been realized by lily breeders for a long time. In order to combine desirable traits from different lily species, crosses were made and hybrid groups like Asiatic (A), Oriental (O) and Longiflorum (L) were bred in the last century (McRae, 1998). These crosses were only successful within botanical sections of genus *Lilium* and intersectional crosses were difficult to get, while hybrids and seeds cannot be expected. With the application of some specific techniques like cut-style pollication and embryo rescue, more and more hybrids from crossing between distant parent were obtained, a few new groups like LA, OT, OA and LO were created (Van Tuyl and Arens, 2010; Van Tuyl et al., 1991).

The sterility of interspecific lily hybrids is still a bottleneck for lily breeding. The hybrids crossed from distant parents are generally sterile and cannot be directly used in further crosses (Asano, 1982). Although fertility can be restored by chromosome doubling or unreduced (2n) gametes, the ploidy level of resulted progenies is also improved to 3x or 4x. So questions arise: what determines the fertility of the hybrids and is it possible to use the hybrids directly for crossing and can the ploidy level be maintained in lily breeding? Before answering these questions, it is necessary to know that lily is quite a good material for cytogenetic analysis: 1. Lily has a large chromosome size and a large genome; 2. Genomes from different hybrid groups could be discriminated by genomic *in situ* hybridization (GISH). With the help of cytogenetic methods, the process of gametes production (meiosis) in interspecific lily hybrids could be clearly and systematically analyzed.

First, we need to have a look at the normal meiosis which gives rise to fertile gametes. Meiosis is a special type of nuclear division which segre-

gates one copy of each homologous chromosome into each new gamete. Different with mitosis, homologous chromosomes pair together before divisions (association) and all of the homologous chromosomes pairs as bivalents. After association there are two divisions, one is called reduced division in which two associated homologous chromosomes segregate, and the other one is called equal division in which sister-chromatids segregate. After these two divisions, haploid gametes are formed.

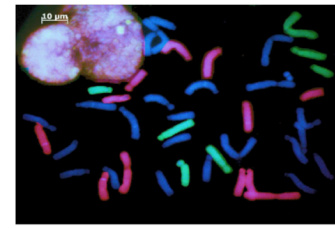

Figure 1. Chromosome painting of an aneuploid progeny from a cross between a triploid LLO and a tetraploid LLTT. Blue represents chromosomes from L genome, green represents chromosomes from O genome and red represents chromosome from T genome.

Crossover, which happens between homologous/homoeologous chromosomes, is a unique feature that differs with mitosis. In distant hybrids like LA, OT, OA, genomes from each group could be distinguished by the application of genomic *in situ* hybridization (GISH) (Figure 1), and different types of crossovers between different genomes could be detected from analyzing the segregation of anaphase I stage (Khan et al., 2009a; Xie et al., 2010). Statistics showed that majority of the crossovers is single crossover, and multiple crossover also happens (Figure 2).

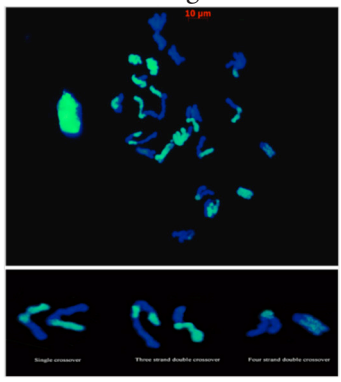

Figure 2. Different crossovers at Anaphase I stage during meiosis of an interspecific LA hybrids. Blue represents chromosomes from A genome, green represents chromosomes from L genome. In this cell, single crossover, three strand double crossover as well as four strand double crossover were identified.

In interspecific lily hybrids, the failure of association is the main reason which determines the loss of fertility. In *Lilium* hybrids, bivalents as well as univalents have been found during meiosis (Figure 3) and hybrids with high frequency of univalents are generally sterile, and only those which have higher frequency of bivalents show a low level of fertility (Xie, 2012). These elite genotypes can produce few haploid gametes and resulted to diploid progenies in further crosses. Diploid LA and OT cultivars are examples for such phenomenon (Khan et al., 2009b).

Some other abnormalities could also be found during meiosis of some interspecific lily hybrids. Except bivalents and univa-

lents, multivalents which involves more than two chromosomes could also be found at metaphase I in some interspecific lily hybrids (Figure 4). In addition, chromosome breakage and fusion could also be happen in the hybrids, and lead to the production of isochromosomes (Figure 5). These isochromosomes are the fusion of two arms of the missing chromosomes and showed similar length and 45s rDNA locus (Xie, 2012).

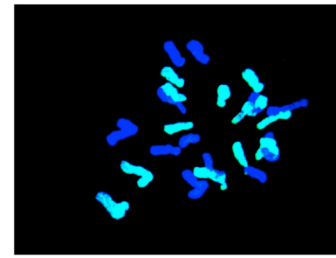

Figure 3. Chromosome pairing at metaphase I stage during meiosis of an interspecific LA hybrids. Blue represents chromosomes from A genome, green represents chromosomes from L genome. Some chromosomes could find partners and pair as bivalents (two chromosomes), and some chromosomes stay as univalents.

Some hybrids which show low frequency of bivalents could also be used in crossing, because they could also produce progenies. However, ploidy level test showed that the ploidy level of these progenies were increased and those hybrids are producing diploid gametes (unreduced gametes) (Khan et al., 2010). Most LA and OT cultivars are originated from unreduced gametes.

These crossovers, which happen during the meiosis of interspecific hybrids, could be successfully transmitted to the progenies by viable gametes, and plants with these recombination, unlike the crossing progenies from chromosome doubling, possesses intergenomic exchanges and hence, bring new variation in the lily world and materials combined traits from more than one section could be created.

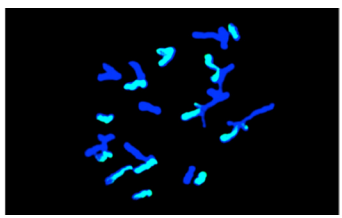

Figure 4. Abnormal chromosome pairing at metaphase I stage during meiosis of an interspecific LA hybrids. In this cell, four chromosomes paired as quadrivalents.

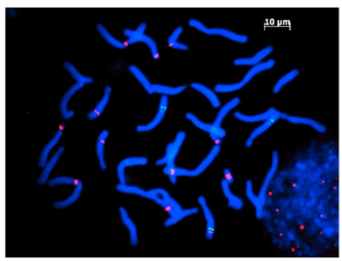

Figure 5. FISH revealed the small chromosomes in one progeny from a cross between an LA hybrids and its Asiatic parent (A) was indeed an isochromosome.

Conclusions

The fertility of distant lily hybrids relates on the success of chromosome association during meiosis. Abnormal phenomena in meiosis like multivalents, univalents, chromosome breakage and bridges could be found in interspecific lily hybrids, together with the crossovers, they all contribute the diversity of progenies when these hybrids are used for further crosses.

References:

Asano, Y., 1982. Chromosome association and pollen fertility in some interspecific hybrids of Lilium. Euphytica, 31(1): 121-128.

Khan, N. et al., 2010. Relevance of unilateral and bilateral sexual polyploidization in relation to intergenomic recombination and introgression in Lilium species hybrids. Euphytica, 171(2): 157-173.

Khan, N., Barba-Gonzalez, R., Ramanna, M.S., Visser, R.G.F. and Van Tuyl, J.M., 2009a. Construction of chromosomal recombination maps of three genomes of lilies (Lilium) based on GISH analysis. Genome, 52(3): 238-251.

Khan, N. et al., 2009b. Potential for analytic breeding in allopolyploids: an illustration from Longiflorum× Asiatic hybrid lilies (Lilium). Euphytica, 166(3): 399-409.

McRae, E.A., 1998. Lilies: a guide for growers and collectors. Timber Press. pp 235-247.

Van Tuyl, J.M. and Arens, P., 2010. Lilium: Breeding History of the Modern Cultivar Assortment. Acta Hort 900: 223-230.

Van Tuyl, J.M. et al., 1991. Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific Lilium crosses. Plant Sci, 74(1): 115-126.

Xie, S., 2012. A molecular cytogenetic analysis of chromosome behavior in Lilium hybrids. Wageningen UR, 115 pp.

Xie, S. et al., 2010. An assessment of chromosomal rearrangements in neopolyploids of Lilium hybrids. Genome, 53(6): 439-446.

Different Ways to Create Triploid Lilies

Shujun Zhou[1] and Jaap van Tuyl[2]

[1]Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, 866 of Yuhangtang Road, Hangzhou, Zhejiang Province, 310058, China

[2]Plant Breeding, Wageningen University and Research, Wageningen

1) Most modern intersectional lilies are triploid

Modern intrasectional lily cultivars, originating from normal hybridization within each taxonomical section in *Lilium*, are classified into Longiflorum (L), Asiatic(A), Oriental(O), Trumpet(T). They are usually diploid as their wild species ($2n = 2x = 24$). In addition, combing chromosome doubling and/or hybridization, breeders created tetraploid ($2n = 4x = 48$) and triploid ($2n = 3x = 36$) Asiatic cultivars. Since intrasectional hybridizations are usually compatible and their F1 hybrids are fertile due to normal meiosis, then it is reasonable to suggest that such hybrids possess similar or identical genomes. It is difficult to obtain wide F1 hybrids between different sections or groups. However, with cut style pollination and embryo rescue, many wide lily hybridizations have been produced and many new intersectional cultivars have been released (Asano & Myodo 1980; Van Tuyl *et al*. 1988, 1991; 2000). Because these wide F1 hybrids are highly sterile due to abnormal meiosis (Figure 1), it is regarded that they contain different genomes. Though the F1 intersectional hybrids are highly sterile, most of them can produce a small amount of 2n eggs or a few of them pro-

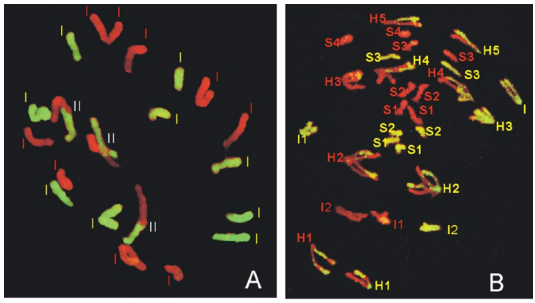

Figure 1. Abnormal meiosis of F1 LA wide hybrid. A: at metaphase I, only three bivalents (II) are formed by pairing between Longiflorum (yellow-green) and Asiatic (red) chromosomes. B: at anaphase I, bivalents are disjoined and become half-bivalents (H), simultaneously, the sister chromotids(S) of univalents are also divided. (See Zhou *et al*., 2008a for detail)

duce some amount of 2n pollen grain (Zhou 2007). This results in sexual ploypoloidization of their BC1 progenies and is the reason why, so far, most intersectional lily cultivars are triploid (Zhang *et al*., 2012).

2) Types of triploid lilies

Triploid lilies usually have strong stems, thick leaves and big flowers. Based on our investigation on lily cultivars using genomic *in situ* hybridization, so far, the popular types of triploid lily cultivars mainly are AAA, LLL, OOO, LAA (ALA), LLO, LOO (OLO), OTO (OOT), AOA and OAO (Figure 2). For example, LAA means that the cultivar contains one Longiflorum genome and two Asiatic genomes. It is expected that more new types of triploid lily cultivars, like LLR (Lim and Van Tuyl, 2004), L-Hen-Can, O-Hen-Can, and OLR, will be released in the near future.

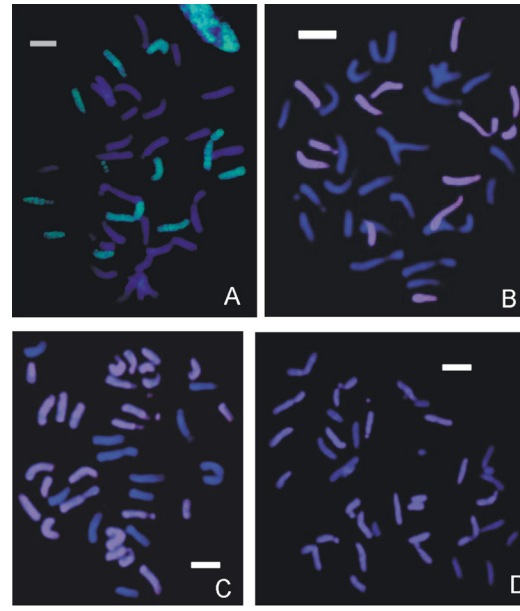

Figure 2. Four representatives of different types of triploid lily cultivars. A: 'Mombasa'(LAA) has 12 longiflorum (green) and 24 Asiatic (purple) chromosomes; B: 'Chiara (LLO)' has 24 longiflorum (purple) and 12 Oriental (pink) chromosomes; C: Terni (LOO) has 12 longiflorum (purple) and 24 Oriental (pink) chromosomes; and D: Candy Club (OOT) has 12 Trumpet (purple) and 24 Oriental (pink) chromosomes. Bar = 10 um. (See Zhang *et al*., 2012 for detail)

3) The ways to create triploid lilies

The first way or the common way to create triploids is to double the chromosomes artificially by using Colchicine or Oryzalin agent, and then to hybridize diploid with tetraploid or reciprocal. The second way is unilateral sexual polyploidization, i.e., lily distant F1 hybrids may produce small amount 2n gametes and result in triploid BC1 progenies.

Triploid Asiatic lilies generally result from diploid x tetraploid crosses because diploid F1 hybrids within Asiatic lilies are highly fertile and pro-

duce abundant haploid gametes. If you want to create triploid Oriental, Longiflorum or Trumpet lilies, diploid x tretraploid hybridizations are the only choice in most cases.

To create allotriploid lilies, like LAA, OTO, etc, you may take either or both of them depending what kind materials you have. For example, if you have a population of F1 LA hybrids, you may take the following strategy: first, test their pollen germination, if you find some of them have 2-15% of pollen germination, you may use them as paternal to hybridize with Asiatic lilies and produce triploid ALA; you may also use the LA hybrids as maternal to hybridize with Asiatic lilies, regardless of the LA hybrids' male fertility, it is highly possible to produce triploid LAA. It is also possible to double F1 LA hybrids' chromosomes to produce LALA, and then, make crosses between AA and LALA to produce ALA.

4) Advantages and disadvantages of the two normal ways producing triploid lilies

With the first way, it is easy to get many triploid lily seeds (or embryos which need to be rescued); however, you need to have tetraploid lilies. On the contrary, it saves time to get triploid lilies with the second way; however, the number of triploid seeds usually is quite limited due to low fertility of distant F1 hybrids. Amphidiploid lilies produce unanimous 2*x* gametes. Once you find a promising 2*x* x 4*x* combination, it is much easer to make mass propagation with seeds than with tissue culture or scaling. Distant F1 hybrids produce a variable percentage of 2n gametes. This makes triploid BC1 progenies variable and increases the chance of selection (Barba-Gonzalez *et al.*, 2006; Zhou *et al.*, 2008b). Based on our experience, most triploid LAA cultivars usually result from unilateral polyploidization, while LLO cultivars are derived from diploid x tetraploid hybridization (Zhang *et al.*, 2012).

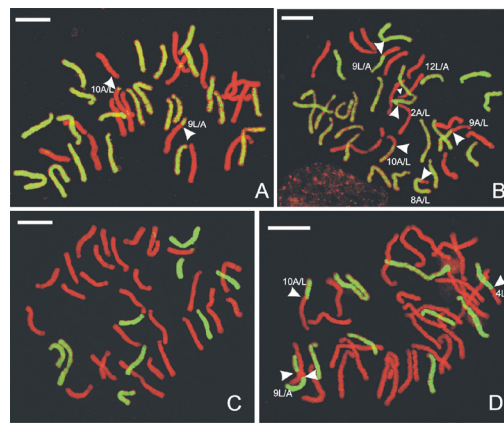

Figure 3. Pseudotriploid lilies produced through LL x ALALA in (A) and (B); and AA x ALALA in (C) and (D). Longiforum chromosomes are in green and Asiatic chromosomes are in red. All of them have 36 chromosomes, however, neither of L genome and A genome is euploid in any of them. (See Zhou 2007 for detail).

A different way to produce triploid lilies is through hybridization between diploid and allopentaploid, e.g., AA x ALALA and LL x ALALA (Zhou 2007). Allopentaploid can be created by hybridization between triploid (ALA) and tetraploid (LALA) (Lim *et al*., 2003). The similar phenomenon was also observed in another allopentaploid lily (AOAOA). The pentaploid lily can produce functional 2*x* pollen through abnormal meiosis and its progenies are triploid. However, such triploids usually contain aneuploid chromosome numbers of each genome, and thus, they are called pseudotriploid (Figure 3). Because this method of producing triploids is time-consuming and complex, is not the best choice for most lily breeders.

Main references

Asano Y, Myodo H (1980) Lily hybrids newly obtained by the technique combining cut-style pollination with embryo culture (II). Lily Yearbook North Am Lily Soc 33: 7-13

Barba-Gonzalez, Van Silfhout RAA, Ramanna MS, Visser RGF, Van Tuyl JM (2006) Progenies of allotriploids of Oriental x Asiatic lilies (*Lilium*) examined by GISH analysis. Euphytica 151: 243-250

Lim KB, Ramanna MS, De Jong JH, Jacobsen E, Van Tuyl JM (2003) Evaluation of BC2 progenies derived from 3*x*-2*x* and 3*x*-4*x* crosses of *Lilium* hybrids: a GISH analysis. Theor Appl Genet 106: 568-574

Lim KB and Van Tuyl JM (2004) A pink Longiflorum Lily cultivar, 'Elegant Lady' suitable for cut flower forcing. Korean J. Breeding 36(2): 123-124

Van Tuyl JM, Keijzer CJ, Wilms HJ, Kwakkenbos AAM (1988) Interspecific hybridization between *Lilium longiflorum* and the white Asiatic hybrid 'Mont Blanc'. Lily Yearbook North Am Lily Soc 41: 103-111

Van Tuyl JM, Van Diën MP, Van Creij MGM, Van Kleinwee TCM, Franken J, Bino RJ (1991) Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci 74: 115-126

Van Tuyl JM, Van Dijken A, Chi HS, Lim K-B, Villemoes S, Van Kronenburg BCE (2000) Breakthroughs in interspecific hybridization of lily. Acta Hort 508: 83-90

Zhang X, Ren G, Li K, Zhou G, and Zhou S. 2012. Genomic variation of new cultivars selected from distant hybridization in *Lilium*. Plant Breeding 131:227-230.

Zhou S (2007) Intergenomic recombination and introgression breeding in Longiflorum x Asiatic lilies. PhD thesis (ISBN 90-8504-637-8),

Wageningen University, The Netherlands
Zhou S, Ramanna MS, Visser RGF, van Tuyl JM (2008a) Analysis of the meiosis in the F1 hybrids of Longiflorum x Asiatic (LA) of lilies (*Lilium*) using genomic *in situ* hybridization. Journal of Genetics and Genomics 35: 687-695
Zhou S, Ramanna MS, Visser RGF, van Tuyl JM (2008b) Genome composition of triploid lily cultivars derived from sexual polyploidization of Longiflorum Asiatic hybrids (*Lilium*). Euphytica 160: 217-215

The Potential of Aneuploids for Selecting New Lily Cultivars

Shujun Zhou

[1]**Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, No. 866 of Yuhangtang Road, Hangzhou, Zhejiang Province, 310058, China**

Euploid means that a genotype has one or more full sets of chromosomes. In *Lilium*, diploid, triploid, tetraploid and pentaploid are all euploid. Different from euploid, apart from complete genomes, aneuploid lilies contain extra chromosomes, such as $2n = 27 = 2x + 3$, $2x = 30 = 2x + 6$. The extra chromosomes may cause imbalanced gene expression and result in morphological and physiological variations. This will greatly increase the chance of selection although most variations are usually unfavorable for organisms themselves. Hyacinth, a good example, has many aneuploid cultivars. However, except that some Patterson hybrid lilies are aneuploid (Stushnoff and Nelson, 1998), few other aneuploid lily cultivars are reported or released. In order to accomplish aneuploid cultivars, two important factors should be considered: 1) it is easy to produce to aneuploid; 2) it is easy to be vegetatively propagated. Based on a series of results reported so far, lily meets the two factors.

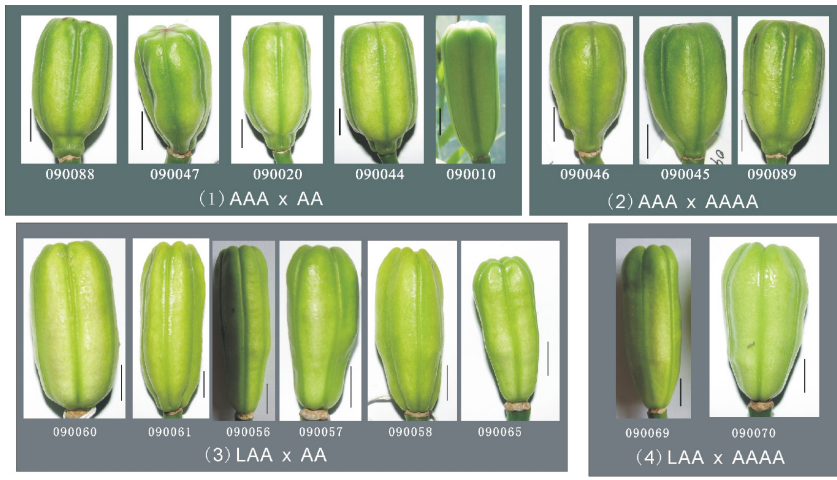

Figure 1. The developed fruits of $3x$ x $4x$ crosses in *Lilium*. (see Zhou et al., 2011 and 2012 for detail)

Table 1. The reported successful cases of *3x* x *2x*/*4x* in *Lilium*.

Hybridizations		Central cell	Sperm	Endosperm	Cross-ability	References
Maternal	Paternal					
LAA	AA	4A + 2L	A	A	+	Lim et al., 2003
LAA	AAAA	4A + 2L	2A	2A	++	Zhou et al., 2012
LAA	LALA	4A + 2L	L + A	L + A	+	Lim et al., 2003
AOA	AA	4A + 2O	A	A	+	Barba-Gonzalez et al., 2006
AOA	OAOA	4A + 2O	O + A	O + A	+	Barba-Gonzalez et al., 2006
LLO	LLTT	4L+2O	L+T	L+T	+	Xie et al., 2010
AAA	AA	6A	A	A	+	Zhou et al., 2011
AAA	AAAA	6A	2A	2A	++	Zhou et al., 2011
LAA	LL	4A + 2L	L	L		Zhou et al., 2012
LAA	OO	4A + 2L	O	4A + 2L + O	-	Zhou et al., 2012

Lily triploids, regardless of their male sterility, can be used as maternal to cross with appropriate diploid and tetraploid paternal to produce aneuploid progenies (Table 1, Figure 1). Most triploid plants are usually sterile and seedless, like triploid watermelon and banana, because triploids usually produce dysfunctional aneuploid gametes due to abnormal meiosis. Triploid

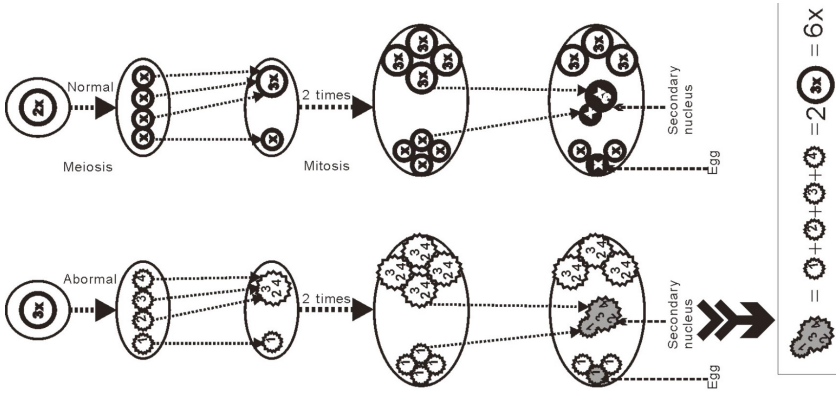

Figure 2. Triploid lilies produce aneuploid egg and *6x* central cell in their embryo sacs, because nuclear DNA amount of central cell is invariably twice that of its somatic cell based on diploid normal megasporogenesis of Fritillaria-type embryo sac. (See Zhou et al., 2011 or 2012 for detail)

lilies are also highly male sterile. However, they can be good seed parents because *Lilium* produce a tetrasporic embryo sac. From the normal megasporogenesis of tetrasporic embryo sac, Zhou (2007) deduced that triploid lilies produce embryo sacs with aneuploid eggs and hexaploid central cells (Figure 2), and thus, after double fertilization in 3*x* x 2*x*/4*x* hybridizations, the embryos are usually aneuploid but the endosperm is euploid (7*x*/8*x*). Euploid endosperm has balanced chromosomes and can develop well in 3*x*

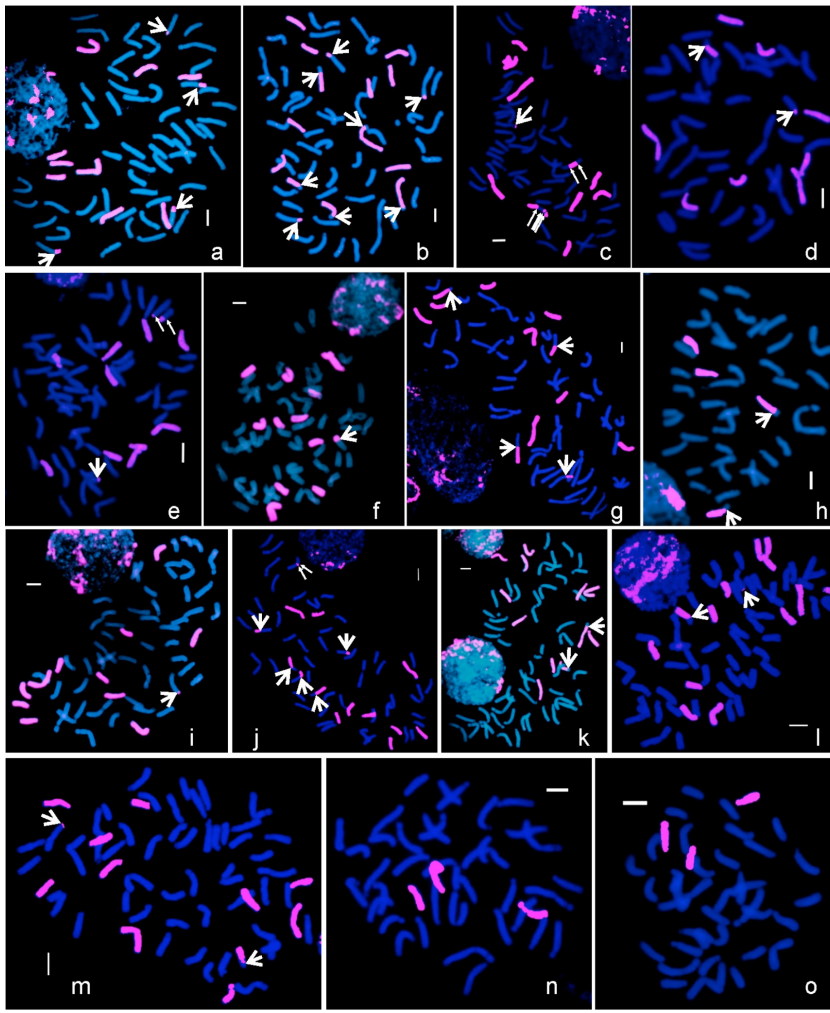

Figure 3. Aneuploid progenies obtained from LAA x AAAA, showing they contain different Longiflorum (pink) and Asiatic (blue) chromosomes. (See Zhou et al., 2012 for detail)

x 2*x*/4*x* and thus make aneuploid embryos be survived (Zhou *et al*. 2011). Many cases about 3*x* x 2*x*/4*x* hybridizations, including AAA x AA/AAAA, LAA x AA/AAAA/LALA, AOA x AA/OAOA, OTO x OO, etc., have been reported. Near all their progenies are aneuploid (Figures 3). These aneuploid progenies show a great variation in morphological traits. Figure 4 is a good example showing the variation caused by aneuploid lilies.

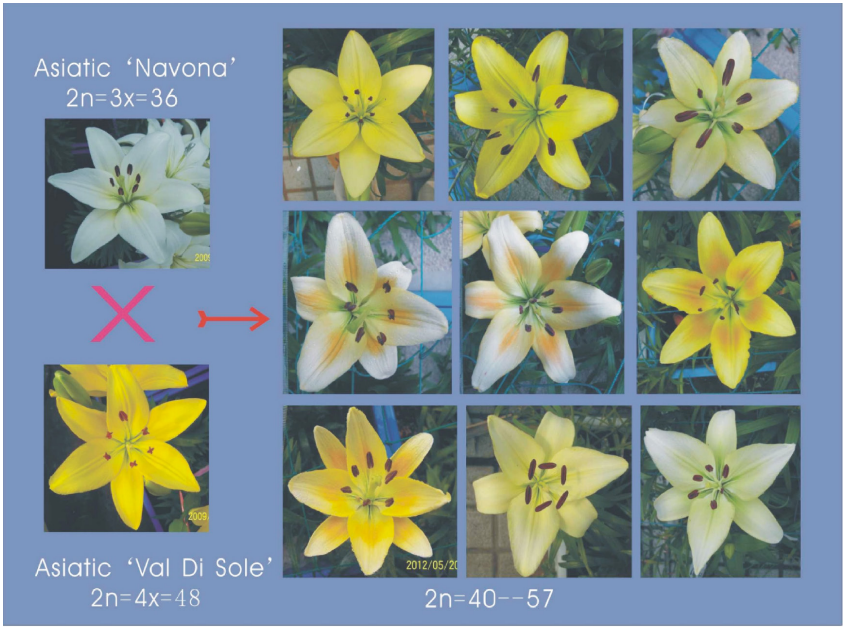

Figure 4. The variation of aneuploid (2n=40—50) progenies of 3*x* x 4*x*. The triploid is Asiatic lily 'Navona' and The tetraploid is Asiatic lily 'Val Di Sole'. Their progenies have different chromosome numbers, ranging 40 to 50. Their color, number of petals, etc are different each other.

Based on these reported cases, a hypothesis 'Five same genomes of endosperm are essential for its development in *Lilium*' was proposed to explain the success or failure of lily hybridization, and this hypothesis can be used to guide breeders to select parents for combining different lily genomes (Zhou *et al*. 2012). It is known that the breeders have combined two different genomes and created many new promising triploid lily cultivars (Zhou *et al*., 2008; Zhang *et al*., 2012). So, we hope that three or more different genomes are combined and more new lilies cultivars are released. We know these combinations are already produced but no cultivars are realeased yet. So, how to combine three different genomes? We know, generally, it is

not possible if breeders just make such hybridizations as LA x OO, LAA x OO, OT x AA, OTO x AA, etc, because their seed and pollen parents are incompatible. However, based on the new theory, it could be highly possible to combine L, A and O together by making hybridizations like LAA x OAOA or AOA x LALA, because their endosperm genome composition is 5A+2L+O and 5A+2O+L, respectively. "Five same genomes" would play a key role in the hybridizations. A reported example is the combination with L, O and T genome through LLO x LLTT hybridization, resulting in aneuploid seedlings (Xie et al., 2010).

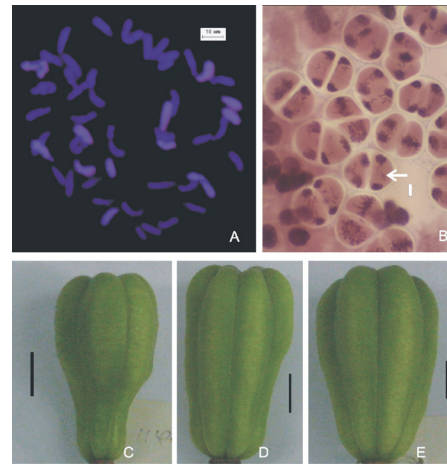

Figure 5. A: 'Honesty'(LAAA) contains 12 Longiflorum (pink) and 36 Asiatic (blue) chromosomes; B: its meiosis is abnormal; C, D and E: the fruits of LAAA x AAAA. (See Zhou et al., 2013 for detail)

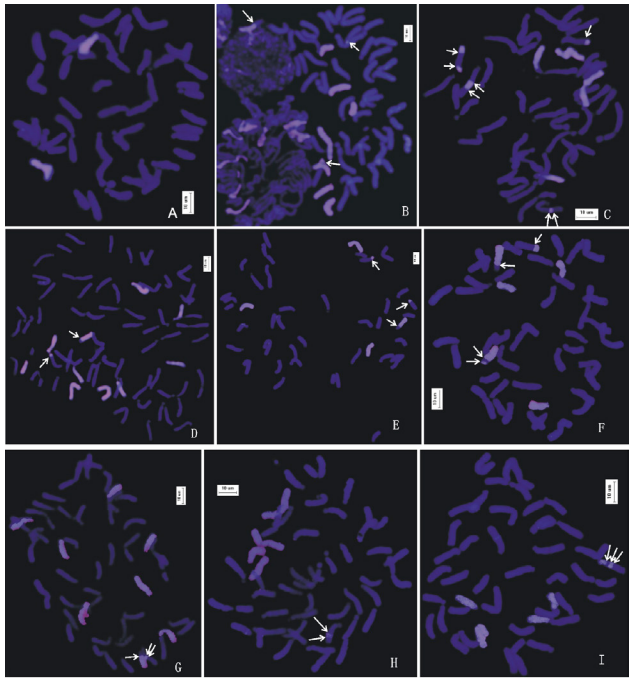

Figure 6. Aneuploid progenies of LAAA x AAAA, showing they have variable number of Longiflorum (pink) and Asiatic (blue) chromosomes. Arrows indicate the break points of the recombinant chromosomes. (See Zhou et al., 2013 for detail)

Odd-tetraploid can also be used as seed parents to hybridize with tetraploid pollen parents to produce aneuploid progenies (Zhou et al. 2013). 'Honesty', an LA cultivar, contains one L genome and three A genomes. It is coded as LAAA and called odd-tetraploid. It is male sterile, but it can be used as seed parent to cross with tetraploid Asiatic lily (AAAA) and produce aneuploid progenies (Figures 5 and 6)

From these examples, we can see it is not difficult to produce aneuploid lilies. Besides, we know, lily can be easily propagated by scaling or tissue culture. This makes it possible to multiply a promising aneuploid lily seedling until it forms a new cultivar.

Main references

Barba-Gonzalez, Van Silfhout RAA, Ramanna MS, Visser RGF, Van Tuyl JM (2006) Progenies of allotriploids of Oriental x Asiatic lilies (*Lilium*) examined by GISH analysis. Euphytica 151: 243-250

Lim KB, Ramanna MS, De Jong JH, Jacobsen E, Van Tuyl JM (2003) Evaluation of BC2 progenies derived from 3*x*-2*x* and 3*x*-4*x* crosses of *Lilium* hybrids: a GISH analysis. Theor Appl Genet 106: 568-574

Stushnoff C, and Nelson SH (1988) Sterility and incompatibility in the Patterson hybrid lilies. In Llies: *a Guide for Growers and Collectors*, McRae EA (author). Timber press, Portland, Oregon.

Xie S, Ramanna MS, Van Tuyl JM (2010) Simultaneous identification of three different genomes in *Lilium* hybrids through multicolour GISH. Acta Horticulturae 855: 299-303

Zhang X, Ren G, Li K, Zhou G, and Zhou S. 2012. Genomic variation of new cultivars selected from distant hybridization in *Lilium*. Plant Breeding 131:227-230.

Zhou S (2007) Intergenomic recombination and introgression breeding in Longiflorum x Asiatic lilies. PhD thesis (ISBN 90-8504-637-8), Wageningen University, The Netherlands

Zhou S, Zhou G and Li K. 2011. Euploid endosperm of triploid x diploid/tetraploid crosses results in aneuploid embryo survival in *Lilium*. HortScience 46:558-562.

Zhou S, Li K and Zhou G. 2012. Analysis of endosperm development of allotriploid x diploid/tetraploid crosses in *Lilium*. Euphytica 184:401-412.

Zhou S, Tan X, Fang L, Jian J, Xu P, and Yuan G. 2013. Study of The Female Fertility of an Odd-tetraploid of *Lilium* and Its Potential Breeding Significance. Journal of American Society of Horticultural Science 2013, 138(2):114-119.

Molecular Markers as a Tool for Parental Selection for Breeding in Lilium

Arwa Shahin, Paul Arens, W. Eric van de Weg, and Jaap M. van Tuyl

Wageningen UR, Plant Breeding, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

Introduction

Conventional breeding in lily is a slow process since lily has a long juvenile phase (2-3 years) and the selection often takes many cycles of breeding in order to combine desirable agronomic traits from different parents into a single cultivar. There is a need to improve the efficiency of conventional breeding. Molecular assisted breeding (MAB) is a very vital tool to speed up breeding and to understand the genetic of traits. The MAB concept is based on defining molecular markers linked (co-segregating) to the trait/gene of interest. This can be achieved by developing molecular markers for the targeted population/collection and then compare the segregation of these markers with the segregation of the phenotypic trait of interest (QTL mapping). This process helps to visualize if the trait is a mono- or polygenetic trait based on its segregation ratio in the segregating population and to find markers linked with this trait (trait-markers).

Developing trait-markers is of great advantage for breeders not only to speed up selection in progeny but also to select parents for breeding programs. Selecting parents that have the right genes/alleles by markers is mainly beneficial when the trait is controlled by recessive allele(s) and it improves breeding efficiency by increasing the number of progeny that has the desired trait. If a trait is controlled by recessive gene(s) then it only will be expressed when the recessive allele is present in homozygous state (aa). Thus, having trait-markers for the recessive trait to distingue between AA and Aa (have the same morphology) becomes very important to improve the efficiency of breeding programs.

In lily, several types of molecular markers were generated: AFLP (Amplified Fragment Length Polymorphism), NBS (Nucleotide Binding Site) profiling, DArT (Diversity Arrays Technology) markers (Shahin et al., 2011) and recently SNP (Single Nucleotide Polymorphism) markers and SSR Simple Sequence Repeats) (Shahin et al., 2012a; Shahin et al., 2012b; Smulders et al., 2012) and used to develop well saturated linkage maps for

lily (Shahin, 2012). These maps were used to map several horticultural traits: *Fusarium* resistance, virus resistance, and several ornamental traits (Shahin et al., 2011). Here we analyse two of the mapped traits in lily (flower spots and flower direction) to show the importance of understanding the genetics of these traits and developing markers for recessive traits that can guide the breeders to choose the right parents.

Material and Methods

Plant Material

Two mapping populations were used in this study. The first is LA population, which is a F1 population of 98 genotypes made from a cross between Longiflorum 'White Fox' x Asiatic 'Connecticut King'. The second is an AA population of 100 individuals (Straathof et al., 1996; Van Heusden et al., 2002). It is a backcross of 'Connecticut King' with 'Orlito' (='Connecticut King' x 'Pirate'). Cultivar 'Connecticut King', which is the common parent in both populations, is a well-known Asiatic cultivar. It has yellow, spotless, and up-facing flowers. The Longiflorum parent 'White Fox' has white, spotless, and out-facing flowers. 'Pirate' has orange flowers with spots. 'Orlito' has orange flowers with few spots.

Phenotypic data

Flower spots segregated in LA and AA populations. The number of spots on lily petals of the two populations was counted and classified into five groups: no spots, 1-10 spots, 11-20 spots, 21-30 spots, and >31 spots. Flower direction segregated in LA population and it was scored as out-facing/up-facing. segregation ratio of Flower spots and flower direction were tested using the Chi-square test with a significance threshold of P=0.05

QTL mapping

Spot number and flower direction were mapped using MapQTL 5.0 (Van Ooijen, 2004). A permutation test with 1000 replications (Churchill and Doerge, 1994) was carried out to establish the LOD threshold. For spot number, ^{10}Log transformation was performed to obtain normally distributed data for mapping.

Results and Discussions

Analysis and mapping of flower spots

The number of spots in the AA population varied between 0 and 44 (47 'no spots', 28 '1-10 spots', 18 '11-20 spots', 3 '21-30 spots', and 1 '>31 spots', Fig. 1A) and between 0 and 50 in LA population (65 'no spots', 17 '1-10 spots',

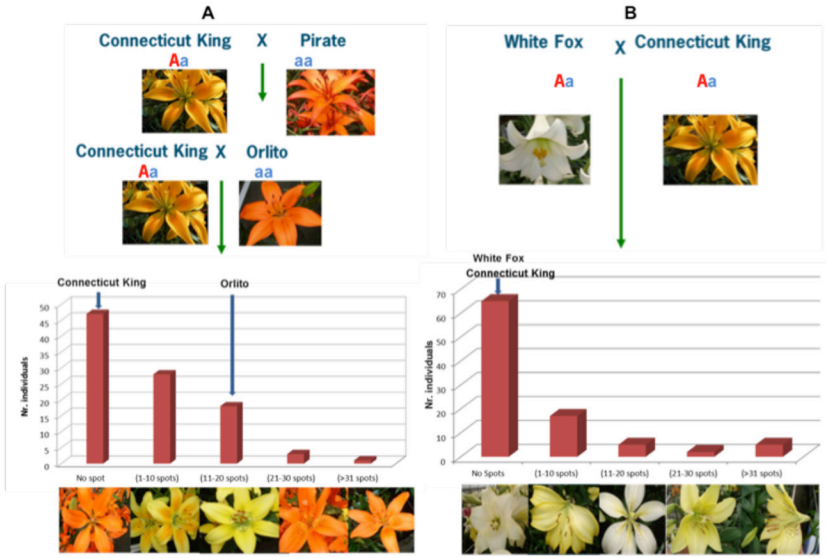

Figure 1: Flower spots segregation in AA (A) and LA populations (B).

5 '11-20 spots', 2 '21-30 spots', and 5 '>31 spots', Fig. 1B). Mapping this trait in both populations resulted in a very strong QTL on linkage group 11 (Fig. 2). In LA population, the QTL (LOD 19.4, threshold of 5) explained around 60.3% of the phenotypic variation; whereas, in AA population the QTL (LOD = 7.04; threshold of 4.6) explained 28.4% of the variation. In both populations no other QTLs that might have minor effects on the number of spots were detected.

The continuous distribution of spots indicates that several genes regulate this trait. However the presence of spots segregated in AA population as 1:1 (50 with spots: 47 no spots, X2= 0.047), and as 1:3 (29 with spots: 65 no spots, X2= 0.97) in LA population, thus there is a single major gene that controls the formation of spots whereas the number of spots may be controlled by other genes with a minor effect (Shahin et al., 2011). In this study, only one single locus was identified. This might be due to a single gene controlling both traits, or due to the involvement of two or more closely linked genes.

Spots segregated 1:1 in AA population and 3:1 in LA population, the latter indicating that both parents 'White Fox' and 'Connecticut King' are heterozygous for this locus (AaxAa, Fig. 1A,B), and the allele responsible for spots formation is recessive since these two parents have no spots. Consequently, if a breeder wishes to produce progeny that have no spots (spot free) then the best parents for this target are of AAxAA or AAxAa cross types (both parents have no spots) and all the progeny will be spot

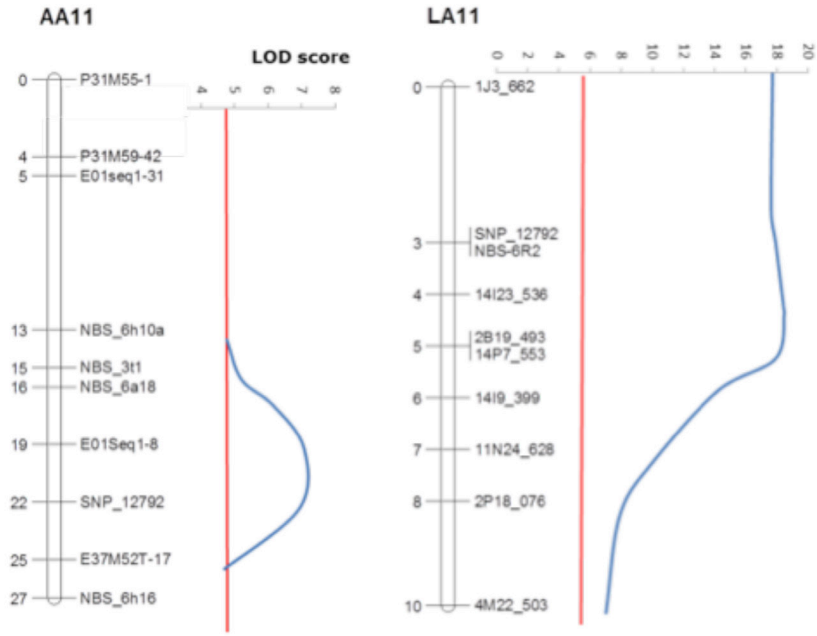

Figure 2. Mapping flower spots in AA and LA populations on linkage group 11. SNP marker (SNP_12792) co-segregated with spot formation and it is a common marker between the two populations

free. But, if a breeder wishes to generate progeny with spots, then there are several options: AaxAa cross type (both parents have no spots) will segregate as 3 no spots: 1 with spots, Aaxaa cross type (one parent is spot free, and the other has spots) will segregate as 1 no spots: 1 with spots, and the best cross will be aaxaa (both parents have spots) that all progeny will have spots. There is need to screen the candidate parents to know which alleles they have and thus predict the ratio of no spot/with spots progeny expected. Comparing the QTL for flower spots in both maps showed that the SNP marker (SNP-12792, Fig. 2) is linked to this trait and thus can be considered as a trait-marker that can be used for this target.

Analysis and mapping of flower direction

Flower direction is an economically important trait in the Longiflorum group, since the common out-facing phenotype leads to flower damage and quality losses in packaging and higher transport costs. Flower direction segregated in the LA population (67 out-facing: 28 up-facing, Fig. 3A). This

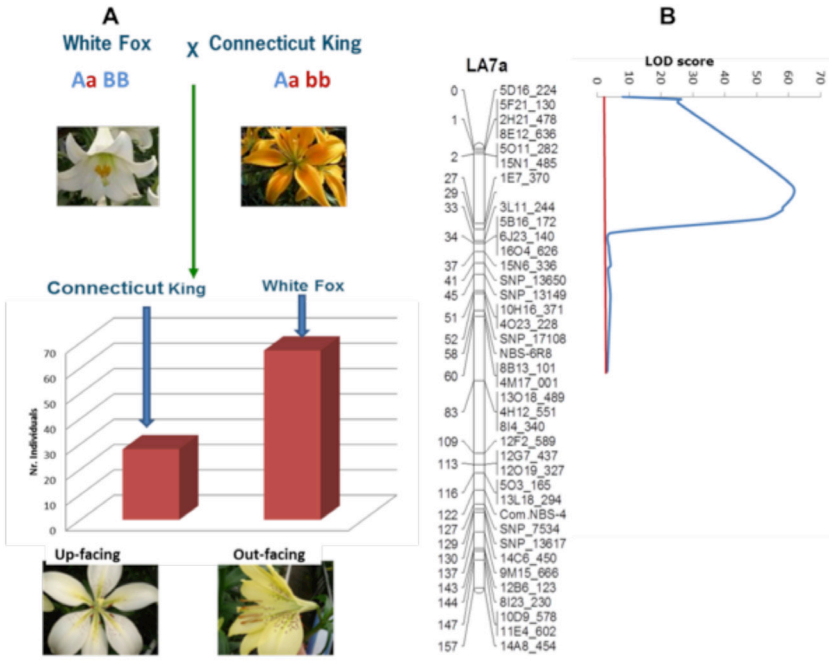

Figure 3. Mapping flower direction in LA population. **A)** Segregation of F1 population into two groups: up-facing and out-facing flowers, **B)** mapping of flower direction trait on the genetic maps of lily: mapped on the top of linkage group 7a with very high LOD value.

was mapped on LA7a (Fig. 3B). The QTL (LOD =61.11; threshold of 3.9) explained 94.8 of the phenotypic variation.

This trait segregates 3:1 in LA population which indicates the possibility of having one gene controlling this trait, and consequently assumes that the two parents are of AaxAa cross type. This is however not possible in this case because the two parents of the LA population are morphologically different for this trait: 'Connecticut King' has up-facing flowers while 'White Fox' has out-facing flowers. To explain this trait we proposed the following model: two genes are involved in controlling this trait (A and B). Having both genes in dominant phase (*i.e.* A- B-) is needed to have the out-facing flowers, while the presence of only one or none of these genes in dominant phase (*i.e.* A- bb, aa B-, or aa bb) is needed to have up-facing flowers.

Crossing 'White Fox' (Aa BB, out-facing) and 'Connecticut King' (Aa bb, up-facing) would result in four different allele combinations: three combinations have both genes in dominant phase (AA Bb, Aa Bb, Aa Bb, Fig 4A) and one has only one gene in dominant phase (aa Bb, Fig 4A). Consequently,

71 of the progeny is expected to have out-facing flowers (compared with 63 observed, X2=0.22) and 24 of the progeny is expected to have up-facing flowers (compared with 27 observed, X2=0.67).

This proposed model assumes that there are two loci (A and B) controlling this trait. However, in the QTL mapping of the trait only one locus was identified (Fig. 3). However, in our model for 'White Fox' and 'Connecticut King' (Aa BB x Aa bb) the second locus (B) does not segregate in the LA F1 population (*i.e.* all progeny have the same Bb combination, Fig. 4A), and consequently this locus cannot be mapped in this cross.

Having markers to screen for the two loci and knowing whether they are present in dominant or recessive phase is important to increase the ratio of up-facing flowers in the progeny. In the LA population, three quarters of the progeny have out-facing flowers (Fig. 4A). If the breeder's aim is to produce LA cultivars that have up-facing flowers, then the cross between 'White Fox' (Aa BB, out-facing) and 'Connecticut King' (Aa bb, up-facing) is not efficient. Crossing a parent that is heterozygote in both loci (Aa Bb) with a parent that is homozygous recessive for one locus and heterozygous for the second (aa Bb or Aa bb, Fig. 4B) will result in having half of the progeny out-facing and half up-facing flowers. Still, the ratio of having even higher number of up-facing flowers can achieved by crossing a parent heterozygous for both loci (Aa Ba) and a homozygous recessive for both loci (aa bb, Fig. 4C). In such cross, 75% of the progeny will have up-facing flowers. But, the highest number of up-facing flowers can achieved by crossing parents homozygous recessive for both loci (aa bb x aa bb).

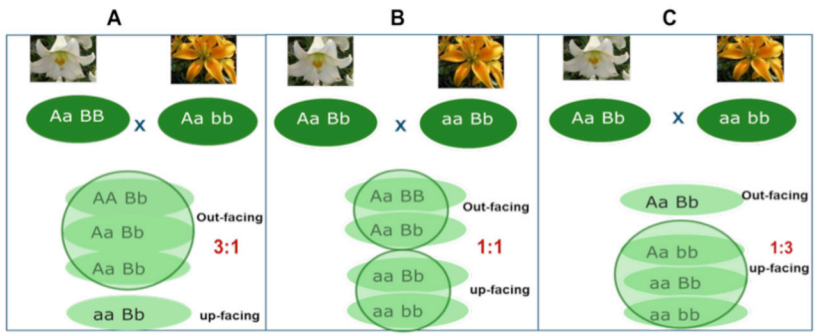

Figure 4. Selecting the right parents for breeding for flower direction trait. **A)** cross type that results in having three quarters of the progeny with out-facing flowers, **B)** cross type that results in having half of the progeny with out-facing flowers, **C)** cross type that results in having one quarter of the progeny with out-facing flowers.

To conclude, the availability of markers for recessive ornamental traits such as flower spots and flower direction is very useful to improve the efficiency of breeding programs. Such markers allow the identification of suitable breeding parents so that expression of the recessive trait can be either enhanced or repressed.

Acknowledgements

A special thanks for Alex van Silfhout and Teus Bleijenberg who took care of the lily populations.

Rererences

Churchill, G.A., and R.W. Doerge, 1994: Empirical Threshold Values for Quantitative Trait Mapping. Genetics **138**:963-971.

Shahin, A., 2012: Development of genomic resources for ornamental lilies (*Lilium* L.). PhD thesis, Wageningen UR, The Netherlands.

Shahin, A., T. Van Gurp, S.A. Peters, R.G.F. Visser, J.M. Van Tuyl, and P. Arens, 2012a: SNP markers retrieval for a non-model species: A practical approach. BMC Res Notes. **5**, 79.

Shahin, A., M. van Kaauwen, D. Esselink, J. Bargsten, J. van Tuyl, R. Visser, and P. Arens, 2012b: Generation and analysis of expressed sequence tags in the extreme large genomes *Lilium* and *Tulipa*. BMC Genomics **13**, 640.

Shahin, A., P. Arens, A.W. Van Heusden, G. Van der Linden, M. Van Kaauwen, N. Khan, H.J. Schouten, W.E. Van De Weg, R.G.F. Visser, and J.M. Van Tuyl, 2011: Genetic mapping in Lilium: mapping of major genes and quantitative trait loci for several ornamental traits and disease resistances. Plant Breed. **130**:372-382.

Smulders, M.J.M., M. Vukosavljev, A. Shahin, W.E.v.d. Weg, and P. Arens, 2012: High throughput marker development and application in horticultural crops. Acta Horticulturae **961:**547-551.

Straathof, T.h.P., J.M. Van Tuyl, B. Dekker, M.J.M. Van Winden, and J.M. Sandbrink, 1996: Genetic analysis of inheritance of partial resistance to *Fusarium oxysporum* in Asiatic hybrids of lily using RAPD markers. Acta Hort **414:**209-218.

Van Heusden, A.W., M.C. Jongerius, J.M. Van Tuyl, T.P. Straathof, and J.J. Mes, 2002: Molecular assisted breeding for disease resistance in lily. Acta Hort **572:**131-138.

Van Ooijen, J., 2004: MapQTL® 5, Software for the Mapping of Quantitative Trait Loci in Experimental Populations. Kyazma B.V., Wageningen, Netherlands.

The Effect of Sugar and ABA on the Longevity of Lily Flowers

Arwa Shahin[1], Alex van Silfhout[1], Francel Verstappen[2], Harro Bouwmeester[2], Jaap M van Tuyl[1], Paul Arens[1]

[1]Wageningen UR Plant Breeding,

Wageningen University & Research Centre

[2] Laboratory of Plant Physiology, Wageningen University

Introduction

Longevity of lily flowers is a very important trait since it has a direct implication on the commercial value of these flowers. The lifespan of a flower is terminated by senescence, *i.e.* wilting or abscission of whole flowers or flower parts. Flowers are either ethylene-sensitive and senescence is regulated by ethylene or ethylene-insensitive and senescence is not regulated by this hormone (Van Doorn and Woltering, 2008).

In lily flowers, the role of ethylene is unclear. Some studies showed that treatment with the ethylene inhibiter STS (Silver Thiosulphate) enhances the vase life of Asiatic hybrids lilies (Nowak and Mynett, 1985). However, other studies found that senescence of flowers is either ethylene-insensitive (Van der Meulen-Muisers, 2000), or that ethylene has little effect on the vase life of flowers (Elgar et al., 1999). Asiatic lilies processed through the Dutch and New Zealand auctions, nevertheless, have to be pre-treated with STS. The lack of clear results makes the benefit of treating cut lilies with STS debatable. Abscisic acid (ABA) is a candidate hormone that might regulate senescence in lily. Abscisic acid showed to have a secondary role during flower senescence in ethylene-sensitive senescence and might have a major role in ethylene-insensitive senescence.

Several non-hormonal substances are known to be involved in regulating flower senescence such as: calcium and sugars (Tripathi and Tuteja, 2007). Exogenous sugars usually delay visible senescence in flowers. In this study we investigated: the effect of exogenous sugar on vase life and dry weight of lily flowers, and which hormones present in lily flowers and how their concentrations between anthesis and senescence with and without sugar addition change with special emphasis on ABA. Consequently, the relation between ABA and senescence and the influence of exogenous sugar on ABA concentrations in the flower were investigated.

Materials and Methods

Plant material

Six lily (*Lilium* L.) genotypes belonging to the *Sinomartagon* section were used: species *L. bulbiferum* (2n=2x=24), cultivar 'Red Twin' (2n=4x=48) and four Asiatic hybrids; 891338-27, 891338-25, and 891338-1 resulting from crossing 'Connecticut King' with 'Orlito' and 921442-2 resulting from 'Fashion' x 'Montreux' (all 2n=2x=24) (Figure 1). Twelve bulbs (size 12-16 cm) of each genotype were used. Bulbs were grown in a standard pre-fertilized commercial potting soil under tunnel conditions. No additional fertilization was used and plants were irrigated daily. For harvest conditions and statistical analysis see (Shahin, 2012). Two treatments were used: 'Standard treatment' in which 6 inflorescences of each of the six genotypes were placed in tap water (1 liter) with 8-Hydroxy Quinolinol Sulfate (HQS), and 'Sugar treatment' in which 6 inflorescences of each genotype were placed in tap water (1 liter) with sugar (sucrose, 30 g) and HQS.

Figure 1: The genotypes used in lily vase life experiment: A) L. bulbiferum, B) cv. 'Red Twin', C) 891338-27, D) 891338-25, E) 891338-1 and F) 921442-2.

Flower longevity

Figure 2: Senescence of A) cv. 'Red Twin' and B) 921442-2 genotype

Flower longevity was defined as the time between anthesis and wilting (Figure 2A, B) of the flower. Genotype longevity was defined as the average longevity of all flowers per treatment. Flowers were collected at senescence and weighed before and after drying in oven (120 °C for 24 hrs) to determine dry weight/fresh weight ratios. Two flowers of each genotype were collected at anthesis and senescence for hormones' measurements. Collected flowers were pooled together and used for hormones extraction (Shahin, 2012) on a LC/MS/MS (Liquid Chromatography- Mass Spectrometry- Mass Spectrometry).

Results and Discussion

The effect of sugar treatment on flower vase life and dry weight

Vase life of each genotype was calculated as the average over all flowers in each treatment (Table 1). Vase life for all genotypes increased with the exogenous application of sucrose. Statistical analysis (ANOVA) showed that this increase in vase life due to sugar treatment was significant (Table 1) and explained 3 to 79 % of the increase in vase life (the variance explained by sugar / explained variance, Table 1).

Table 1: The average vase life of each genotype was calculated for the two treatments: standard and sugar (standard error 'SE' is included). The significance between the two treatments (significant when P value <0.05), and the explained variance were calculated using the ANOVA.

	Avg. Vase life Standard treatment ± SE	Avg. Vase life Sugar treatment ± SE	P value	Explained variance
L. bulbiferum	8.46 ± 0.7	10.52 ± 0.9	0.002	56 %
Red Twin	9.4 ± 0.6	10.4 ± 1.1	0.338	3 %
891338-1	10.36 ± 1	12.3 ± 1.3	0.001	35 %
891338-25	9.07 ± 0.54	12.08 ± 1.2	0.001	69 %
891338-27	9.19 ± 1	10.7 ± 0.99	0.001	79 %
921442-2	12.19 ± 0.7	14.5 ± 0.9	0.001	44 %

Similarly, dry/fresh weight ratios of flowers increased due to sugar treatment (Table 2) explaining 15 to 67 % of the increase in dry/fresh weight ratios (Table 2). Similar findings were recorded by Van Doorn (2001). Sugar delays senescence, and thus gives flowers longer time to grow and increase their sizes and weights. The variation in the explained variance percentages might be related to genotypes and their specific abilities to transport, mobilize, and manipulate sugars.

Vase life and dry/fresh weight ratio of cv. 'Red Twin' increased when applying sugar (30 gL^{-1}) but the increase was not significant (Table 1, 2). 'Red Twin' is a tetraploid cultivar and has almost a double size compared with the other five diploid genotypes used for this study. In a previous study, the addition of 10 gL^{-1} sucrose (with recurrent solution change) was not enough to increase vase life of the triploid lily cultivar 'Courier' (Arrom and Munné-Bosch, 2012). This might indicate that the amount of added exogenous sugar needed to extend the vase life of any genotypes might be linked to its ploidy level.

Table 2: The average ratio dry/fresh weight for the standard and sugar treatments (SE= standard error). The significance between the two treatments (significant when P value <0.05), and the explained variance were calculated using the ANOVA.

	Avg. dry/fresh weight (standard) ± SE	Avg. dry/ fresh weight (Sugar) ± SE	P value	Explained variance
L. bulbiferum	0.08 ± 0.02	0.12 ± 0.03	0.007	31 %
Red Twin	0.06 ± 0.01	0.07 ± 0.02	0.127	15 %
891338-1	0.07 ± 0.02	0.09 ± 0.01	<.001	17 %
891338-25	0.07 ± 0.01	0.1 ± 0.01	0.002	30 %
891338-27	0.07 ± 0	0.1 ± 0	0.002	24 %
921442-2	0.07 ± 0.01	0.11 ± 0.01	<.001	67 %

Hormone measurements

Hormones of lily flowers present at anthesis were measured using a sensitive chromatography method LC/MS/MS. Cytokinins were present in rather low concentrations at anthesis. Gibberellins (GA1, GA4, GA7, and GA9) were present in measurable quantities at both anthesis and senescence, however invariable in time. Auxins could be measured at anthesis although at low concentrations but were not measurable at senescence. The ABA (both

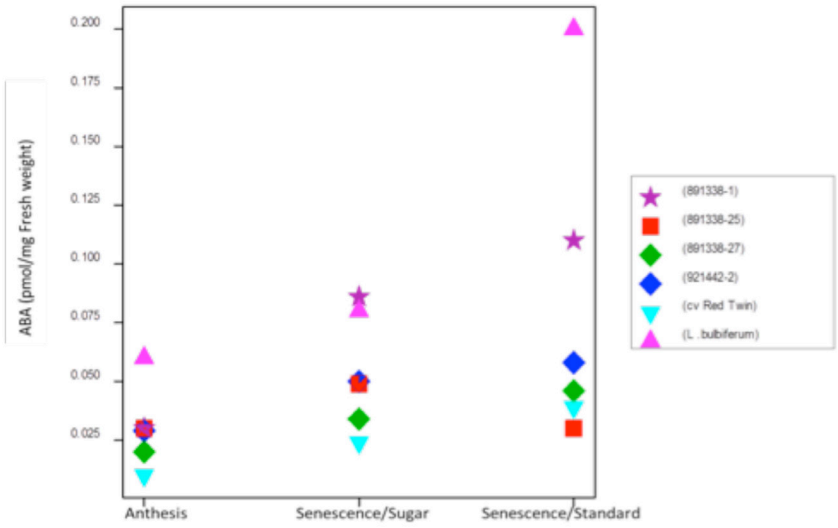

Figure 3: ABA concentration (pmol/mg FW) for six lily genotypes at two stages: anthesis and senescence (standard and sugar treatments).

ABA and ABA-GE: abscisic acid glucose ester) were present in measurable quantities. The ABA levels increased at senescence and responded to sugar treatment (Figure 3). A strong correlation (0.9) between vase life and ABA concentration was found.

ABA plays a major role in late seed development and adaptation to environmental stresses such as drought and other stress responses (León and Sheen, 2003). However, the exact role of ABA in flower senescence has not established in detail yet. Our measurements of ABA concentrations showed 2 to 3 fold increase in ABA concentrations from anthesis to senescence. Similar results were recorded in other ethylene-insensitive species (Aneja et al., 1999; Panavas et al., 1998). Accordingly, exogenous application of ABA hastened flower senescence (Borochov et al., 1976; Panavas et al., 1998) and induced many senescence-related changes in ethylene-insensitive daylilies, cocoa, and *Iris*. Thus, ABA is thought to be the primary hormonal regulator of flower senescence in these flowers (Aneja et al., 1999; Panavas et al., 1998; Zhong and Ciafré, 2011). The application of an inhibitor of ABA biosynthesis decreased ABA levels and extended vase life of cocoa flowers (Aneja et al., 1999). The results taken all together support the notion that flower senescence of ethylene-insensitive plants is possibly regulated by ABA.

Sugar and ABA interaction

Studies on the effects of ABA and sugar on a range of developmental processes have suggested interactions among signaling pathways (Finkelstein and Gibson, 2002). In our study, application of sugar delayed the increase in ABA levels compared with standard treatment (Figure 3). Similarly, sugar decreased ABA levels at anthesis in lily cultivar 'Courier' (Arrom and Munné-Bosch, 2012). Sugar and ABA showed to have opposite effects in rose flowers (ethylene-sensitive). While sugar prolongs vase life of rose, ABA in presence of ethylene shortens it (Borochov et al., 1976). Overall, the availability of sugar decreased ABA levels at flower senescence. This might indicate that sugar and ABA might have the same signaling pathway or interacting pathways.

Conclusions

In our study, we confirmed the important role of sugar in prolonging the vase life of lily flowers. The concentration of the plant hormone ABA increased at senescence compared with anthesis which might reflect a regulatory role for ABA in controlling vase life in lily. Interestingly, sugar treatment decreased ABA concentration, suggesting opposite effects of ABA and sugar on lily senescence and a possible interaction between their pathways.

References

Aneja, M., T. Gianfagna, and E. Ng, 1999: The roles of abscisic acid and ethylene in the abscission and senescence of cocoa flowers. Plant Growth Regul. **27:**149-155.

Arrom, L., and S. Munné-Bosch, 2012: Sucrose accelerates flower opening and delays senescence through a hormonal effect in cut lily flowers. Plant Sci. **188–189:** 41-47.

Borochov, A., S. Mayak, and A.H. Halevy, 1976: Combined Effects of Abscisic Acid and Sucrose on Growth and Senescence of Rose Flowers. Physiol. Plant. **36:**221-224.

Elgar, H.J., A.B. Woolf, and R.L. Bieleski, 1999: Ethylene production by three lily species and their response to ethylene exposure. Postharvest Biol. Technol. **16:**257-267.

Finkelstein, R.R., and S.I. Gibson, 2002: ABA and sugar interactions regulating development: cross-talk or voices in a crowd? Curr. Opin. Plant Biol. **5:**26-32.

León, P., and J. Sheen, 2003: Sugar and hormone connections. Trends Plant Sci. **8**:110-116.

Nowak, J., and K. Mynett, 1985: The effect of sucrose, silver thiosul-

phate and 8-hydroxyquinoline citrate on the quality of Lilium inflorescences cut at the bud stage and stored at low temperature. Sci. Hortic. **25:**299-302.

Panavas, T., E.L. Walker, and B. Rubinstein, 1998: Possible involvement of abscisic acid in senescence of daylily petals. J. Exp. Bot. **49**:1987-1997.

Shahin, A., 2012: Development of genomic resources for ornamental lilies (*Lilium* L.). PhD thesis, Wageningen UR, The Netherlands, p 106-120.

Tripathi, S.K., and N. Tuteja, 2007: Integrated signaling in flower senescence: An overview. Plant Signal Behav. **2**:437-445.

Van der Meulen-Muisers, J.J.M., 2000: Genetic and physiological aspects of postharvest flower longevity in Asiatic hybrid lilies (*Lilium* L.), Wageningen UR, The Netherlands.

Van Doorn, W.G., 2001: Role of soluble carbohydrates in flower senescence:a survey Acta Hort 543:179-183.

Van Doorn, W.G., and E.J. Woltering, 2008: Physiology and molecular biology of petal senescence. J. Exp. Bot. **59**:453-480.

Zhong, Y., and C. Ciafré, 2011: Role of ABA in ethylene-independent Iris flower senescence. International Conference on Food Engineering and Biotechnology, IPCBEE, ACSIT Press, Singapoore **volume9**

The Correlation of Lily Saponins Content and Resistance to *Fusarium oxysporum f.sp. Lilii*

Hongzhi Wu[a] Sixiang Zheng[a], Yufen Bi[b] Li Yi[a]

aThe Gardening Department of Horticulture College, Yunnan Agriculture University Kunming 650201, China;

bThe National Center for Agro-biodiversity, Yunnan Agricultural University, Kunming 650201, China;

Author introduction:

Professor Wu Hongzhi is a teacher/researcher of bulbous and ornamental plants who undertakes teaching of master students/undergraduates majored in ornamental plants and researches of the bulbous/wild flowers at the Gardening Department of Horticulture College of Yunnan Agriculture University.

Introduction

Lily (*Lilium* spp.) is one of the most important ornamental bulb crops worldwide. It is widely cultivated as cut flower, pot and garden plant. However the soil-born fungus *Fusarium oxysporum* f. sp. *lilii*, which is causing bulb rot disease, is one of the most serious threats in bulb and cut flower production of lily in China. Breeding for resistance to this disease is therefore an important goal. However, breeding for resistance in lily is limited by the long juvenile phase (2-3 years) and the fact that selection often takes many cycles of breeding in order to introgress desirable agronomic traits from different parents into a single cultivar. Therefore the development of some quick and efficient method can speed up the breeding process considerably.

The correlation of antifungal effects and the accumulation of total saponins from in tissue cultures plants has been shown in several studies (Soetan et al., 2006; Rosca-Casian et al., 2007; Rodriguez et al., 2007). Saponin content could be an indicator for the degree of *Fusarium* resistance in *Lilium* (Curir et al., 2003).

In this study, the total saponin content of lily genotypes was measured and compared with the known *Fusarium*-resistance, in order to find a correlation between these two characters.

Materials and Methods

Plant material

Lily genotypes were grown in tissue culture. Cai-74 is a variant of an Oriental selection which showed a significant resistance to *Fusarium* (Wu, 2008) compared to other genotypes. The highly resistant wild species '*L. dauricum*' and the susceptible Oriental *Lilium* cultivars 'Sorbonne', 'Siberia', 'Tiber' and the resistant OT cultivars 'Conca d'Or', 'Robina' were as references.

In field test for *Fusarium oxysporum* resistance, some breeding lines and cultivars with different resistance were used .

Field inoculation test

The *Fusarium oxysporum* f.sp. *lilii* used for inoculation was isolated from the rot bulb in oriental lily, and identified as the same pathogen by back inoculation to the health plant, which is the same cultivar that *Fusarium oxysporum* f.sp. *lilii* was isolated from at the beginning.

The tissue culture plants of all test materials were transplanted to the field, and inoculated the *Fusarium oxysporum* f.sp. *lilii* to the young plants when they grow out 2-3 new leaves. Before spraying the pathogen, small wounds were made with needle on the scales to help pathogen to invade into the plants, The pathogen was sprayed to the wound directly to make sure a successful inoculation. The inoculation was done in May 2007 in Kunming.

Extraction of total saponins content

For eliminating the influence of growing factors saponin contents was measured of bulbs cultured *in vitro* of the two outer layers of the scales were used for the test of saponins content. The diosgenin was used as a standard compound to determine the saponins content (from Sigma, pure content ≥99%).

The extraction of total saponins was carried out according to Ren et al. (2005) with minor modification. The bulb scales were dried to approximately 6% water in the drying oven at a temperature of 50°C, and grounded with an electric grinder. The powder was passed through a 0.8 mm mesh sieve. The small powder was defatted in petroleum ether (60-90°C) by ultrasonic wave processing for 1h, then the residue was extracted with 20 times methanol for 1h, and volumed to 50 ml with methanol. 20 ml of the methanol extracting solution was dried by 60 hot water, and the residue was extracted 4 times by water saturated n-Butanol, and the combined extraction with n-Butanol was evaporated under reduced pressure. The extracts were dissolved in methanol for measuring the saponin content. The content of total saponins was determined with UV-756 spectrophotometer at 408 nm.

Results and Discussion

Pathogen and its morphological characteristics

After purification, single spore of the pathogen was cultured in media PDA with the standard conditions: 25 , 12h light. Its culture traits and morphology was as follow:

Aerial mycelium was fluffy white. Small conidia looked like ellipse, 0~1 partition, and the size were 3.7~10.4μm×1.4~4.3μm; Large conidia looked like fusiform, slightly curved, evenly tapering towards the ends, 3~5 partitions, mostly 3 partitions, and the size were 11.0~30.8μm×2.7~5.0μm; sporulation cells and chlamydospores were not found. According to the classification system of Booth (Booth, 1971), and the description for *Fusarium oxysporum* (Lu, 2001; Zhang, 1988), the isolated pathogen was identified as *Fusarium oxysporum* f.sp. *lilii*. Spores and culture characters were shown in Fig. 1.

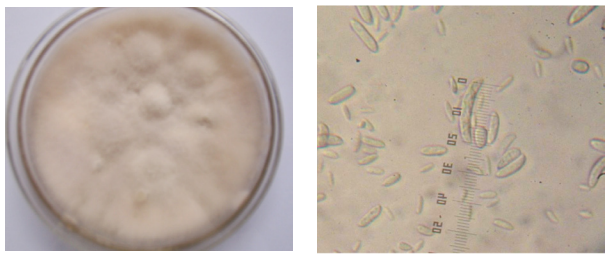

Fig.1: Spore and culture character of fungus isolated from *Lilium Oriental* a.Culture character of single spore; b. Spore type and size.

Except for the imported bulbs, all samples with rot were bulbs from main production regions in Yunnan province were infected by *Fusarium oxysporum* f.sp. *lilii*. The same symptoms were observed by back inoculation to the health plants, the same cultivars which the pathogen was collected and isolated from.

Field performance to *Fusarium oxysporum* f.sp. *liliii* inoculation

After 13 days of inoculation, some susceptible genotypes started to show disease symptoms. Firstly, it was leaf yellow, then the basal part of bulb dropped off. The plants died after inoculation 45~60 days (the death was judged by the death of the part above ground and the scales rot/drop off). The development of disease through some relatively disease-resistant genotypes was slower, and the typical symptoms took longer time to develop.

A high resistance was found in breeding line Cai-74. Over 90% plants of Cai-74 survived after inoculation 45~60 days, and their bulbs kept growing. Finally 71% plants of Cai-74 were immune to *Fusarium oxysporum* f.sp. *lilii*, and harvested 5 months later. The above results indicated that Cai-74 had a higher level of resistance to *Fusarium oxysporum* f.sp. *lilii* than that of suscep-

tible oriental Lilium in the field test. The field performance of resistant and susceptible *Lilium Oriental* to *Fusarium oxysporum* f.sp. *lilii* was showed in the fig. 2.

Fig.2 The field performance of *Lilium Oriental* after inoculation with *Fusarium oxysporum* f.sp. *lilii* a. scale off at early stage; b. comparison of plants of a resistant variant Cai-74 (left) and susceptible Oriental *Lilium* cultivar (right)

Saponin determination

Calibration curve for diosgenin

Diosgenin (7.2×10^{-2} mg/ml) prepared in methanol was used as standard for the saponins content. A calibration curve was prepared for quantitative determination of lily saponins. The amount of Diosgenin and its absorption measured with a spectrophotometer was used to determine the calibration curve. The absorption data for diosgenin and the calibration curve was shown in Fig. 3.

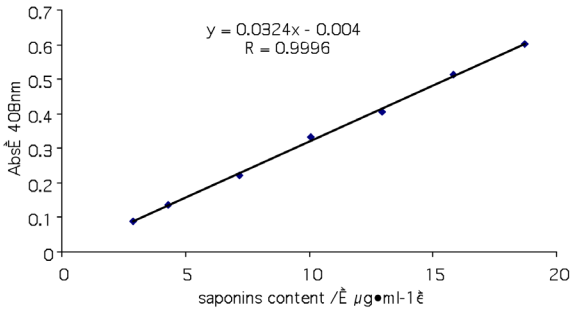

Fig. 3. The calibration curve of saponin content and absorbance.

Saponins content in lily bulbs

The bulb scale of tissue culture plants was used for the analysis of total saponins content. The wild species '*L dauricum*' is a highly resistant source to *Fusarium oxysporum* f.sp. *lilii* (Lim et al, 2003; Straathof et al, 1994). As presented in Table 1, the saponin content determined by spectrophotometry showed that the highly resistant wild *L. dauricum* had the highest level - 4.59 mg/g, followed by the resistant Cai-74 with 4.01mg/g. The resistant OT cultivars 'Conca d'Or' and 'Robina' had a higher saponins content - 3.70 mg/g and 2.83 mg/g, than the susceptible Oriental lily cultivars 'Sorbonne',

'Siberia' and 'Tiber' (Table 2). These results suggest that total saponins content in lily bulbs could possibly used as indicator for *Fusarium oxysporum* resistance in lilies.

In this study we showed that total saponins in lily bulb are positively correlated with resistance to *Fusarium oxysporum* f.sp. *lilii*. When these findings are proved on a larger scale this could be a method to speed up the breeding for resistance to this disease.

Table 2 Quantitative determination of lily saponin contents

Genotype	Field reaction to Fusarium	Abs (408 nm)			Saponins content of solution/ μg·ml-1	Saponins content of bulb scale / mg·g-1
		A1	A2	A3		
Cai-74	resistant	0.2201	0.2357	0.2327	7.2130	4.01
Siberia	moderate	0.1189	0.1005	0.1044	3.4599	1.92
Conca d'or	resistant	0.2218	0.2185	0.1946	6.6605	3.70
Sorbonne	moderate	0.1190	0.1307	0.1249	4.0677	2.26
Tiber	moderate	0.0941	0.1035	0.1141	3.3364	1.85
Robina	resistant	0.165	0.160	0.159	5.0988	2.83
L. dauricum	Highly resistant	0.2567	0.2571	0.2770	8.2654	4.59

Conclusions

A positive correlation was found between saponin content of lily cultivars and their resistance to Fusarium bulb rot.

Saponin content in the bulb could be an indicator for Fusarium resistance

Acknowledgements

The author would like to thank professor Long Chunlin and Wang Yuehu of Kunming Institute of Botany, China Academy Science for valuable advice and equipment support to determine saponins content, and professor Lei Jiajun of Shenyang Agricultural University for kindly giving *L. dauricum*.

Literature Cited

Booth C. 1971. The genus Fusarium. London, CIM

Curir, P., Dolci M., Pasini C. ,Capponi A., Galeotti F., Grassotti A. 2003. Saponin content in lily (Lilium Hybr.) as an indicator of the resistance degree against *Fusarium oxysporum* f.sp. *lilii*. Italus Hortus. Societa Orticola Italiana, Florence, Italy: 10(4),

Supplemento, 291-293.
Lim, J.H., Rhee, H.K., Kim, Y.J., Lim, K.B., Van Tuyl, J.M. 2003. Resistance to *Fusarium oxysporum* f.sp. lilii in *Lilium*. Acta Hort. 620:311-318.
Lu, J. Plant pathogenic fungi. China Agriculture Press, Beijing, China.
Rodriguez, D.J., Hernandez-Castillo, D., Angulo-Sanchez, J.L., Rodriguez-Garcia, R.J., Quintanilla, A.V., Lira-Saldivar, R.H. 2007. Antifungal activity in vitro of Flourensia spp. extracts on Alternaria sp., Rhizoctonia solani, and Fusarium oxysporum. Industrial Crops and Products, 25:111–116.
Rosca-Casian, O., Parvu, M., Vlase, L., Tamas, M. 2007. Antifungal activity of *Aloe vera* leaves. Fitoterapia. 78(3):219–222.
Soetan, K.O., Oyekunle, M.A., Aiyelaagbe, O.O., Fafunso, M.A. 2006. Evaluation of the antimicrobial activity of saponins extract of *Sorghum bicolor* L. Moench. African Journal of Biotechnology. 5 (23): 2405-2407.
Straathof, Th.P., Van Tuyl, J.M. 1994. Genetic variation in resistance to *Fusarium oxysporum* f.sp. *lilii* in the genus Lilium. Ann. appl. Biot., 125: 61-72.
Ren, F.L., Qiu, C.G., Lian, Y. 2005. Extraction process of total saponins from *Lilium brownii*. J. Cent. South Uni. (Science and technology). 36 (1): 69-72.
Wu, H. 2008. *Lilium* oriental breeding via 2n gametes and an analysis on resistance of their progenies to *Fusarium* bulb rot disease. PhD-thesis Yunnan Agricultural University, China, 99 pp.
Zhang, Z. 1988. Plant pathogenic fungi. Sichuan Science and Technology Press, Chengdu, China.Tables

The Outcome of Lily Breeding Research at RDA, Rep. of Korea

Ju Hee Rhee1* and Kang Yun-Im2

1National Agrobiodiversity Center, RDA

2National Institute of Horticultural & Herbal Science, RDA

Suwon, 441-440, Rep. of Korea

Introduction

Lily was one of the strategic flowers for export to Japan since the beginning of 1990. Commercial bulbs have been imported from the Netherlands and cut flowers have been exported to Japan. Cut flowers have been produced on acreage of more than 200 ha per year in Korea since it was 84ha in 1990. In 2012, cut lily export to Japan was US$ 30 M; it was the percentage of 35.8 of total flower export (US$ 84 M) in Korea. Japan, more than 90% of cut flowers imported lilies are imported from Korea and it takes advantage of close distance between Korea and Japan (MIFAF, 2013). The major production areas are highland Gangwon province for summer season and southern Jeju province for winter season (Rhee 2007).

Most of the cut flowers produced in Korea have been bulbs from the Netherlands except for *L. x formolongi* 'Augusta' imported by seed from Japan. Oriental lily cultivars developed in the Netherlands show many physiological disorders during the hot summer season. Recently, lily bulbs imported in Chile and New Zealand often appeared to be virus infected and cut flower quality deteriorated. Therefore it was necessary to breed new lily cultivars suitable for Korea's climate and environmental conditions and Asian flower market's demands.

Interspecific hybridization and polyploidization are applied as one of the most important sources of evolution and domestication of ornamental plants (Van Tuyl and Lim, 2003). Interspecific hybrids have the potential to capture hybrid vigor as well as combine traits that do not occur within a single species (Volker and Orme, 1988). Because a breeder always wants to add new characteristics to current cultivars, interspecific hybridization is indispensable to combine diverse gene pools. Thus interspecific hybrids have excellent potential to extend qualitative and quantitative traits such as flower type, plant phenotypes, and other single dominant traits from parent species or improve vase life. In lily, interspecific hybridization has greatly contributed to commercial Longiflorum-Asiatic (LA) and Oriental-Trumpet

(OT), which are increasingly important in the flower market.

Korea's climate consists of a long, cold winter and hot summer makes it difficult to produce the high-quality cut flowers and bulb of lily. It is necessary to have a short forcing period and a short bulb production cycle. Cultivars derived from *L. x formolongi* have upright-facing flowers, a short generation period from seed to flowering, good plant height and are seed propagated. The cultivation area of 'Raizan' and 'F1 August', *L.x formolongi* cultivars bred in Japan, has been expanding in Korea, Japan and China (Rhee et al., 2005). Korea's climate and environmental conditions in order to generate the appropriate new varieties, interspecific hybridization and polyploidy breeding techniques have been applied intensively.

Lily breeding goals of RDA were to shorten the breeding generation and bulb production period of Oriental hybrids, diversify the flower color of *L. longiflorum* and *L. x formolongi* cultivars, have a mild scent of Asiatic hybrids, and introduce the resistance against virus, *Fusarium* and *Botrytis* etc. Lily breeding in Korea has been focused on interspecific hybridization and polyploidization to create new varieties adapted to the Korean climate and environmental conditions. To overcome the sterility of interspecific hybrids obtained, *in vitro* chromosome doubling was done to obtain tetraploids. *In vitro* pollen germination trials were done with the converted tetraploids to verifty fertility restoration.

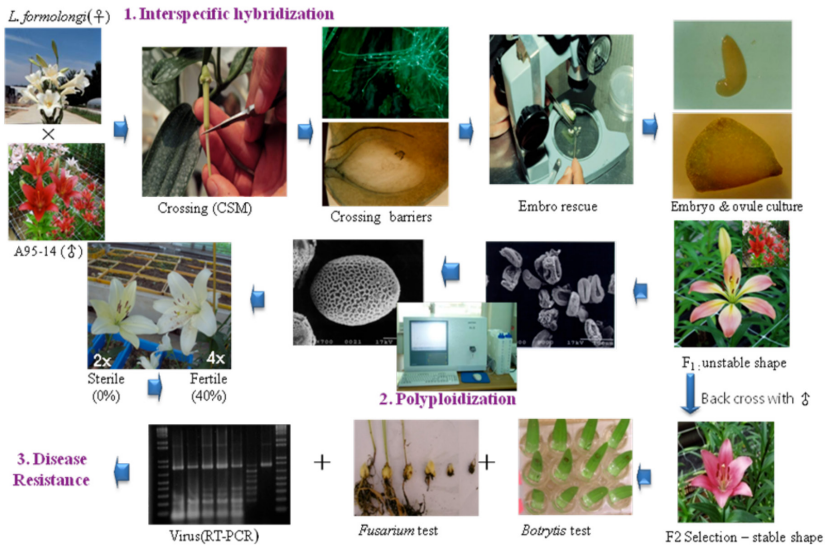

Fig. 7. Diagram the 3 kinds of breeding technology system for lily breeding established at RDA.

Interspecific Hybridization

Emasculation was conducted one day before anthesis. Stigmatic (SP) and cut style pollinations (CSM) were employed. Immature seed pods were harvested after one month; embryo and ovule culture was conducted to rescue the immature embryos (Rhee, 2002). New interspecific hybrids such as FA, FAA, FO, OA, OH and OTO, have been obtained by CSM and ovule culture (Figure 7 and 8).

The FA hybrid 'Supia' was developed from the cross between *L. formolongi* 'Raizan' with white petaled, trumpet shaped and upward-facing, and *L.* Asiatic 'A95-14' with red petaled. 'Supia' shows trumpet shaped, small, upward-facing flowers, is early flowering with no fragrance and is susceptible to virus. The FAA hybrid 'Pink Pearl' was obtained by crossing 'Supia' with Asiatic 'A95-14. 'Pink Pearl' had bigger flowers than the father, upward-facing flowers and little spots (Figure 7, 8 and Table 1).

The FO hybrid 'FO03-16' was obtained by *L. formolongi* 'Raizan' with Oriental 'O54' as male. 'FO 03-16' was characterized by deep pink, big flowers, broad leaf shape, sterility of pollen and a little fragrance. The advantages of FO hybrids compared to Oriental hybrids are short forcing period and short bulb production duration. It is difficult to obtain progeny as male or as female parents (Table 1 and Figure 8). And it should be converted to a tetraploid plant in order to obtain the next generations.

The OA hybrid 'OA 05-1' was obtained by crossing the Oriental hybrid 'Casa Blanca' with the Asiatic hybrid 'Pepper'. It had a weaker fragrance than Oriental hybrids, upward-facing flowers and a shorter forcing period than Oriental hybrids (Figure 7 and 8).

The OH hybrid 'OH02-1' was obtained by crossing the Oriental hybrid 'Casa Blanca' with *L. henryi*. Flower size was 17.5cm. It was characterized by downward-facing flowers and very strong waxy flowers and leaves (Figure 7). It is difficult to become a commercial cultivar but it is highly considered utilization as disease resistance materials.

The OTO hybrid was developed from the cross of the OT hybrid 'Avocado' and the Oriental hybrid 'Acapulco'. 'Flesh Party (OTO-08-3)' shows Oriental flower-shape, large flower size, semi outward-facing flowers, sterile pollen and has a weak fragrance. It is strong environmental adaptability and disease resistance (Figure 7, 8 and Table 1).

In Vitro Chromosome Doubling

In order to overcome the sterility of the interspecific hybrid 'Supia', *in vitro* colchicine, oryzalin and caffeine treatments were applied on scales. Four tetraploids were detected in the different oryzalin treatments (0.003 and

0.005%) and three in the colchicine treatments (0.5 and 0.1%). No tetraploids were obtained in any of the caffeine treatment. Low concentrations (0.003% to 0.005%) of oryzalin were more effective in inducing tetraploidy than the high concentrations of colchicine (0.1% to 0.005%) and caffeine (0.005% to 3%). Pollen fertility of interspecific 'Sinavro (FA)', was recovered up to 40% on tetraploid level (Figure 7).

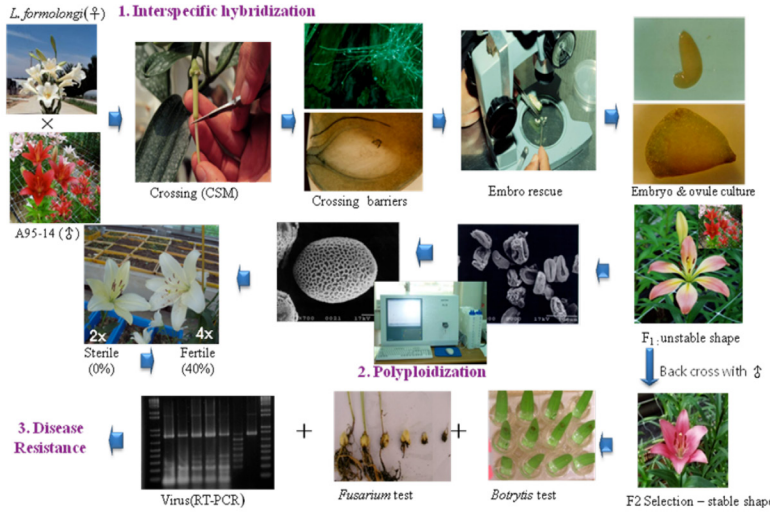

Fig. 8. Successful interspecific hybrids (FA, FAA, FO, OA, TO, OT, OTO) obtained by crossing between distantly related lily groups at RDA. .

Disease Screening

Screening for *Botrytis elliptica* resistance using the leaf-tip test as described by Beers and Van Tuyl (2005) showed resistance groups resulted from Oriental hybrids and cultivars derived from Oriental hybrids such as OH hybrids, FO hybrids and OT hybrids (Figure 7). *Fusarium* test as described by Straathof and Van Tuyl (1996) was conducted by using in vitro bulb of LA progenies between 'White Fox', highly susceptible female parents and 'Connecticut King', highly resistant male parents. It was ranged from partially resistant to completely susceptible (Figure 7).

If a new lily variety was developed at RDA, *in vitro* meristem culture was conducted to make virus-free explants. The multiplex RT-PCR method was used to detect viruses such as LSV, LMoV, CMV and etc (Figure 7). *In vitro* micropropagation of virus-free new variety derived from multi-shoot formation and rapid propagation by using in vitro shoot cluster and 2nd addition of liquid medium.

Lily varieties developed at RDA

Since lily breeding started in the first of 1990 at RDA, Korea, for the first time, 7 Asiatic varieties such as 'Yeji', 'Sabi', 'Hyewhoa' and etc. were released in 1998. Since then, every year, diverse Asiatic lily varieties for cut flower and pot flower were released. Forty-one Asiatic lily varieties were developed from 1998 to 2012. Breeding of Oriental lily was more difficult than that of Asiatic lily because of long breeding generation from seed to flowering time and bulb production period. Five Oriental lily varieties were bred such as 'Saerona', 'Casa Dream and etc. Breeding of Asiatic hybrids and Oriental hybrids were conducted by stigmatic pollination method. On the other hand, FA, FO, and OTO varieties were conducted by interspecific hybridization techniques with cut style pollination and embryo rescue. Since FA (*L. x formolongi* x Asiatic hybrids) varieties, namely 'Supia', 'Doran' and etc. was released from 2000, twenty-nine FA varieties were bred till 2012. FO (*L. x formolongi* x Oriental hybrid) variety, 'Hanuri' was released in 2001. And OTO (OT hybrid x Oriental hybrid) variety, 'Flashy Party', was developed in 2011. Eight-seven Lily varieties were released at NIHHS, RDA from 1998 to 2012 (Table 1).

Fig. 1. Heugjinju(A)

Fig.2. Cream star(FA)

Fig. 3. Pink Pearl(FA)

Fig. 4. Casa dream(O)

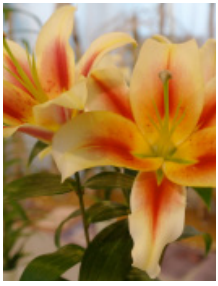

Fig. 5. Flash party (OTO)

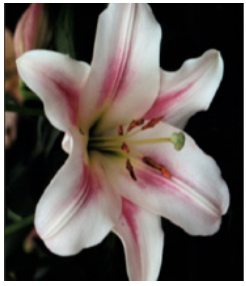

Fig. 6. Hanuri(FO)

Group	No. of cultivars	Name of cultivars (released year)
Asiatic hybrids	41	Yeji, Sabi, Hyewhoa, Gaya, Dasom, Soho, Garam (1998), Whanggum, Midang, Seonyu, Soya, Sara (1999), Mirr, Yaeseom, Haerang, Ena, Gisl (2000), Hongwhoa, Ahyun (2001), Maro, Bomi (2002), Yena (2004), Yeeun, Yeri, Yesol, Heugjinju, Joara, (2005), Pinky smile, Sunny gold, Yeu, Black eye (2006), Apricot king, Asian flame (2007), Lovely girl, Orange girl (2008), Daphne (2009) Yellow carpet, Orange pond (2010), Orange belt, Red flame (2011), Orange sunup (2012)
FA hybrids	29	Supia, Doran, Sinavro, Haewool (2000), Migreen (2001), Goyo, Eunbi (2002), Salmon Bowl (2004), Pink Pearl, Green Star (2005), Cherry Pink, Honey bowl (2006), Orange crown, Glory pink, Fanfare (2007), Apricot star, Golden center, Red star, Cream star (2008), Bonanza, Peach honey, Diana, Glossy orange (2009) Pink pond, Yellow pond, Rain purple (2010), Scarlet giant (2011), Purple crystal, Golden harmony (2012)
Oriental hybrids	5	Saerona (2004), Swan beauty (2006), Pacific wave, Pink lady (2007), Casa dream (2008)
FO hybrid	1	Hanuri (2001)
OTO hybrid	1	Flesh party (2011)
Total	87	

Table 1. Lily varieties have been released at RDA, Korea from 1998 to 2012.

Conclusions

Since lily breeding started in the first of 1990, 87 lily varieties were developed and registered at RDA, Korea from 1990 to 2012. Forty-one Asiatic hybrids named 'Yeji', 'Lovely Girl', 'Daphne', 29 FA hybrids, 1 FO hybrid named 'Hanuri', 5 Oriental hybrids (like 'Saerona', 'Swan beauty', 'Casa Dream'), and 1 OTO hybrid have been released by the Korean lily growers (Table 1).

'Supia', FA (*L. formolongi* 'Raizan' x Asiatic 'A95-14') and 'Pink Pearl', FAA (FA 'Supia' x Asiatic 'A95-14') were released and registered in Korea. 'Hanuri', FO (*L. formolongi* 'Raizan' x Oriental 'O54'), and 'Flash Party', OTO (OT 'Avocado' and Oriental 'Acapulco') were released new varieties. But 'OA 05-1', OA (Oriental 'Casa Blanca' x Asiatic 'Pepper'), and 'OH 02-1' OH (Oriental 'Casa Blanca' x *L. henryi*) are not released because of the lack of commercial value as cut flowers.

Tetraploid forms of FA-hybrid 'Supia' were obtained by using *in vitro* oryzalin and colchicine treatment. No tetraploids were produced after caffeine treatment. The pollen fertility of 'Sinavro (FA)' was restored up to 40% on the tetraploid level while pollen of diploid was sterile.

Screening for *Botrytis elliptica* resistance was examined by using the leaf-tip test. Resistant lily group were Oriental hybrids and cultivars derived from Oriental hybrids. New lily varieties that were bred, were conducted in meristem culture and multiplex RT-PCR. Only virus-free explants were used for *in vitro* microporopagation.

Some lily varieties developed at RDA, Korea, were sold out to small bulb companies and big lily farmers. It will be propagated, cultured as a cut flower and sold domestically and exported abroad for the flower market.

Acknowledgements

This study was carried out with the support of "Cooperative Research Program for Agricultural Science & Technology Development (Project No. 200803A01081274)", Rural Development Administration, Republic of Korea and "Research Program for Agricultural Science & Technology Development (Project No. PJ008245)", National Institute of Horticultural and Herbal Science, Rural Development Administration, Republic of Korea.

Literature cited

Beers, C.M., Barba-Gonzalez, R., Van Silfhout, A.A., Ramanna, M.S., and Van Tuyl, J.M. 2005. Mitotic and meiotic polyploidization in lily hybrids for transferring *Botrytis* resistance. Acta Hort. 673:449-452.

Ministry for Food, Agriculture, Forestry and Fisheries (MIFAFF). 2013. 2012 the present condition of floriculture cultivation.

Rhee H.K., Kim Y.J., Kim K.J., Lim J.H., Cho H.R., Goo D.H., Ko J.Y., Kim J.M. 2000. Breeding of the light pink lily 'Seonyu' with early-flowering. Kor. J. Hort. Sci. Technol. 18:302

Rhee H.K., Kim Y.J., Kim K.J., Lim J.H., Cho H.R.. 2001. Breeding of the orange Asiatic lily 'Mirr' with cup type. Kor. J. Hort. Sci. Technol. Suppl. (I). p. 124.

Rhee, H.K. 2002. Improvement of breeding efficiency in interspecific hybridization of lilies. Ph.D. Thesis, Seoul National University. 118p.

Rhee H.K., Lim, J.H., Kim, Y.J., and Van Tuyl, J.M. 2005a. Improvement of breeding efficiency for interspecific hybridization of lilies in Korea. Acta Hort. 673:107-112.

Rhee, H.K., H.R. Cho, J.H. Lim, H.K. Shin, B. H. Han, M. S. Kim, Y.J. Kim, and Y.J. Na. 2005b. A new oriental lily 'Saerona' with strong fragrance and light pink and white color. Kor. J. Breed. 37:255-256.

Rhee H.K., Cho H.R, Lim J.H., Shin H.K., Kim M.S., Kim Y.J., Kim H.J., Kim J.M., Na Y.J. 2005c. A new Asiatic lily 'Yena' with bright pink for bedding flower. Kor. J. Hort. Sci. Technol. 23:471-474.

Rhee H.K., Cho H.R., Lim J.H., Kim Y.J. 2006a. A new Asiatic lily, 'Maro', with spotted deep orange petals. Korean J. Breed. 38:145-146.

Rhee H.K., Cho H.R, Lim J.H., Goo D.H., Kim Y.J. 2006b. A new Asiatic lily, 'Bomi', with unspotted bright yellow petals. Korean J. Breed. 38:51-52.

Rhee H.K., Lim J.H., Cho H.R., Kim Y.J., 2006c. A new FA interspecific hybrid lily, 'Eunbi' with unspotted light orange petals. Kor. J. Breed. Sci. 38: 215-216

Rhee H.K., Cho H.R., Lim J.H., Shin H.K., Kim Y.J., Na Y.J. 2006d. A new FA interspecific hybrid lily, 'Salmon Bowl'with orange colored petals. Kor. J. Breed. Sci. 38:53-54.

Rhee H.K., 2007a. Horticulture in Korea, Lily. ISHS. pp.274-278.

Rhee H.K. 2007b. A new Asiatic lily 'Sabi' with deep yellow flower for bedding. Kor. J. Hort. Sci. Technol. 25:291-294.

Rhee H.K., Cho H.R., and Lim J.H. 2007c. 'Ahyun', an Asiatic lily with light orange petals. Korean J. Breed. 39:84-85.

Rhee H.K., Cho H.R., Lim J.H., Kim M.S., Choi S.Y. 2008b. An Asiatic hybrid Lily, 'Joara', with unspotted orange petals. Korean J. Hort. Sci. & Tech. 26 (Sup. II):100-103.

Rhee H.K., Cho H.R., Lim J.H., Kim K.J. 2009a. An Asiatic Lily New Cultivar 'Hyehwa' with unspotted orange petals. Korean J. Breed. Sci. 41:583-586.

Rhee H.K., Cho H.R., Lim J.H., Kim M.S., Park S.K., Joung H.Y. 2009b. An

Asiatic Hybrid Lily 'Apricot King' with Unspotted Light Orange Petals. Flower Res. J. 17:36-39.

Rhee, H.K., H.R. Cho, J.H. Lim,M.S. Kim, S.K. Park, and S.Y. Choi. 2009c. A New Oriental Lily 'Swan Beauty' with Strong Fragrance and White Petal for Cut Flower. Kor. J. Hort. Sci. Technol. 27:160-162.

Rhee H.K., Cho H.R., Lim J.H., Kim M.S., Park S.K., Joung H.Y., Shin H.K. 2009d. 'Glory Pink' a FA intersectional lily with unspotted pink petals. Flower Res. J. 17: 40-43.

Rhee H.K., Cho H.R., Lim J.H., Kim M.S., Park S.K., Shin H.K., Joung H.Y. 2010a. 'Asian Flame' an Asiatic hybrid Lily with unspotted Red Petals. Kor. J. Hort. Sci. Technol. 28:519-521.

Rhee H.K., Cho H.R., Shin H.K., Lim J.H., Kim M.S. 2010b. An Asiatic hybrid Lily 'Yesol' with unspotted light yellow petals. Kor. J. Breed. Sci. 42:659-663.

Rhee H.K., Cho H.R., Kim M.S., Park S.K, Lim J.H. 2011. A FA intersectional hybrid lily 'Golden Center' with light yellow petals. Kor. J. Breed. Sci. 43: 509-512.

Rhee H.K., Cho H.R., Lee J.S., Han B.H. 2012. An Asiatic hybrid Lily 'Daphne' with small flower and light pink and ivory bicolor petals. Kor. J. Breed. Sci. 44:643-647

Straathof, Th.P., J.M. van Tuyl., B. Dekker, M.J.M. van Winden, and J.M. Sandbrink. 1996. Genetic analysis of inheritance of partial resistance to *Fusarium oxysporum* in Asiatic hybrids of lily using RAPD markers. Acta Hortic. 414:209-218.

Van Tuyl, J.M. and Lim, K.B. 2003. Interspecific hybridization and polyploidization as tools in ornamental plant breeding. Acta Hort. 612:13-22.

Volker, P.W. and Orme, R.K. 1988. Provenance trials of *Eucalyptus globulus* and related species in Tasmania. Australian Forestry 51(4):257-265

Lilium pumilum DC. on the roof of the world

Nan Tang, Daocheng Tang & Jaap van Tuyl

Plateau Flower Research Institute, Qinghai University, China

Introduction

I was born in Xining, the capital city of Qinghai province (China), located at an altitude of 2295 meters on the Qinghai-Tibet plateau. From an early age, my favorite place was my father's garden. I never thought that flowers would become of such importance in my life. I followed the steps of my father Daocheng Tang, who works as the leader of Plateau Flower Research Institute (PFRI) in Qinghai university, started my research on lilies and tulips. About ten years ago, my father and his colleagues started collecting *L. pumilum* in Qinghai.

Qinghai-Tibet Plateau

The Qinghai-Tibet plateau, known as 'the roof of the world', is the highest and largest plateau. It has an area of 2,500,000 square kilometers and an average altitude of 4,500 meters. Qinghai province, located on the northeastern part of Qinghai-Tibet Plateau, where the average altitude is over 3,000 meters. The province has an area of 722,300 square kilometers and its topography is diverse and complicated. It has an unique plateau continental climate, such as strong solar radiation, low in oxygen, dry, windy and cold. The annual hours of sunshine are above 2500 h. Solar radiation is high as it is located in the mid-latitude area, the total amount of radiation is 140-180 Kcal/m^2, rank only second to Tibet in China. The average annual temperature is -5.7°C - 8.6°C and there are large regional variations. The average temperature ranges from 5.3°C to 20°C in the hottest month, and from -17°C to -5°C in the coldest month. The whole province has low precipitation throughout the year. The annual precipitation varies from 15 to 750 mm in different regions, but in most parts it is below 400 mm.

Fig. 1. Representative of *L. pumilum* habitats (Location: Menyuan)

Lilium pumilum

It is known that China is one of the dis-

tribution centers of *Lilium* in the world. About half of the lily species are widely distributed throughout the country, especially the Sichuan, Yunnan and Tibet areas. Due to the unique geographical and climate condition, *Lilium pumilum* DC. (Coral lily) is the only wild *Lilium* species that distributes in this region (Figure 1 and 2). Although it was recorded in 'Flora of China' that *L. lancifolium* Thunb. (Tiger lily) also distributed in Qinghai, we haven't find one plant in the wild during these years. We only found a few plants in some farmers' courtyards. *L. pumilum* has a wide geographical distribution in Qinghai-Tibet plateau, especially in the southeastern part. There is an old song describing the beautiful scene when large amounts of the orange-red flowers all blooming on the mountain slope. Besides the beautiful flowers, the bulb is edible and has great medicinal values. It is rich in starch, protein, flavone and other alkaloid, which is good for our health. Also it helps in soothing the lung and contributes to the relief of a cough or asthma. Therefore, this is a valuable resource in Qinghai-Tibet plateau. Followed the 'Flora of Qinghai' and with the help of local people, till now, a total of 28 *L. pumilum* natural populations were collected (Figure 3).

Fig. 2. *L. pumilum* on a grass slope and a glimpse of its natural habitat (location: Huzhu)

According to the records, *L. pumilum* is distributed in places where the altitude is between 1900-3000 m. However, we haven't found a single plant at the altitude below 2000 m. The altitude of the distribution region is rising over the years which might be due to climate changes and human interruption. *L. pumilum* can be found in various habi-

Fig 3. A closer look at *L. pumilum* and its surrounding plants in natural habitat

tat environments such as shrubs, grass slopes, forest margins, mountain, or even cliff. It has very high adaptability to all kinds of habitats. The annual average precipitation of the distribution region ranged from 240 mm to 550 mm. The annual average temperature is between 2.3°C to 7.5°C and the annual hours of sunshine is 1546 -2876 h. We did a series of studies on the soil properties of different natural populations. It was found that generally *L. pumilum* prefers alkalescent and fertile soil, but it doesn't have a strict requirement for water content, nitrogen content or soil porosity. The soil properties of different populations are significantly different. Meanwhile, the plant community of the habitat was investigated. In a total of 34 families 49 genus plants were found in the plant communities in the habitats of *L. pumilum.* In most communities, *Stellera chamaejasme*, *Allium chrysanthum*, *Potentilla fruticosa* and different species of genus *Pedicularis* are the dominant species. At this moment, we don't know exactly the relationships between these dominant species and *L. pumilum, b*ut apparently these plants are easily recognizable and very helpful on finding *L. pumilum* in the wild.

Population genetics

Habitat heterogeneity and natural selection often result in genetically distinct ecotypes within a species. This is also noticed in *L. pumilum* populations in Qinghai-Tibet plateau. A total of 41 morphological traits were evaluated, among which hairy flower bud is one of the most distinctive and easy visible characteristic. Flower bud is glabrous in some populations, however, others are piliferous (Figure 4). July 2012 Jaap van Tuyl visited Qinghai to observe the natural populations of *L. pumilum* in Quinhai province (Figure 5).

Genetic analysis was performed using both morphological traits and molecular markers. Although different populations showed different gene diversity, the total diversity at the species level is rather high. The overall population differentiation was considerably high,

Fig. 4 Variations in morphology of L. pumilum flower buds.

which indicated the populations are isolated. The 28 populations were clustered into four groups. It was observed that the clustering of populations has a certain correlation with annual mean precipitation. The investigated *L. pumilum* populations showed very strong genetic structure. There is little or no admixture between populations, which verified that the populations are highly isolated and there is no interbreeding between them. Although migration of plant populations can occur through dispersal of pollen and seed, a number of factors such as fragmented habitat can be the barrier to gene flow between populations. Qinghai-Tibet plateau is a large mountainous area with various ecosystems such as alpine, forest, grass. Alpine is the major geographic barrier which is difficult to overcome. Thus the populations are reproductively isolated since their pollen and seed dispersal is limited by the complicated topography which was formed during the uplift of the plateau.

Fig. 5. Jaap van Tuyl visited Qinghai in 2012 to observe the natural populations of *L. pumilum* in Quinhai province. From left to right: Xiaoling Xing, Daocheng Tang, Jaap van Tuyl, Nan Tang, Xiuting Ju

As previously mentioned, *L. pumilum* is a promising plant. It can be used as breeding material for its ornamental values as well as high disease and abiotic resistance. It also can be grown as food or used for extraction of chemical substances. However, just because of this, large amounts of *L. pumilum* have been illegitimately harvested by local people over the years which makes the propagation under threat. Thus the distribution of *L. pumilum* is decreasing year after year. Nowadays it is hard to see a mountain slope full of the red-orange *L. pumilum* flowers. It is urgent to take some measures to protect the wild resources. PFRI has already started making efforts on the preservation of *L. pumilum* in Qinghai. At the meantime, crossings were made between *L. pumilum* and some Asiatic lily cultivars as well as some LA hybrid cultivars. We hope that in the coming years we will obtain some hybrid plants that have beautiful flowers and can grow well in a plateau environment.

Lily Breeding in Nanjing Forestry University

Mengli Xi and Jisen Shi

Key Laboratory of Forest Genetics and Biotechnology of the Ministry of Education, Nanjing Forestry University, Nanjing 210037, People's Republic of China

China is the origin of by far the most *Lilium* species widely distributed across the land (Long and Zhang, 1998). According to a survey, there are 47 species and 18 varieties in China, accounting for more than half of the total species of *Lilium* of the world, 36 species and 15 varieties of which are native to China (Wang and Tang, 1980). In the late eighteenth century, *Lilium* germplasm resources of China was introduced into Europe, which has made great contributions to *Lilium* breeding in the world (Wu et al., 2006; Zhao and Wang, 2000). Lily occupies a very important position in today's flower industry in China. According to the statistics of the Ministry of Agriculture, fresh cut flower production of lily increased from 2006 to 2010 year by year.

China is rich in resources of wild lily, but its history of commercial cultivation of ornamental lily is relatively short, with its breeding research lagging seriously behind. It is a "bottleneck" that China lacks new lily cultivars with its own intellectual property rights, and bulbs of commercial ornamental lily depend mainly on import. One possible solution to this problem is to take advantage of China's resources of wild lily: combine the conventional breeding methods with the modern biotechnology breeding methods and develop new, surprising lily cultivars with intellectual property rights.

In order to fully exploit, conserve and utilize the Chinese resources of wild lily germplasm, from 2002 on, our group has been collecting wild lilies in area such as Sichuan, Yunnan, Chongqing, Shaanxi, and so on, to investigate the wild lily resources.. For the cultivars, we bought them from the market. Now, 26 species of wild lily and 40 cultivars have been collected and preserved in Nanjing Forestry University, China. We have established an efficient tissue culture technology system for wild lily species and commercial cultivars. The genetic relationship of the lily resources which we have collected was studied with reference to pollen morphological traits, ISSR, AFLP, SRAP molecular markers, and ITS sequences. From 2004 to 2009, we carried out crossed and applied embryo rescue, and established a technology system of obtaining hybrids from distant hybridization or closely related hybridization. We have obtained a large number of distant hybrids. Through years of selection and field observation , we have selected some

genotypes which possess excellent traits, such as colour, height, and resistance.

Lilium regale is native to China. It is naturally distributed in the dry valley of Minjiang River at elevation from 760 m to 2200 m in Sichuan province, China. *Lilium regale* with white flowers grows mainly in grasses, low bushes, and crevices, its distribution is, therefore, dispersed. It has a very strong adaptability to environment, and is also quite resistant to virus. It is the most important parent for disease resistance and saline-alkaline tolerance. . Therefore, over the world, research institutions and companies which engaged in lily breeding possessed *Lilium regale*. In 2005, we went to the Sichuan Minjiang River Basin to investigate the habitat, and to analyze the genetic diversity of *Lilium regale*. We found that there are significant differences among populations.

From 2006 on, we have selected *Lilium regale* as male parent, and Oriental lily as female parent. We carried out the interspecific crosses using normal pollination of *Lilium regale* pollen on the stigma of Oriental lilies. After 40 days of pollination, we harvested the capsules, and then obtained hybrids through ovary slice culture. We cultured the seedlings of hybrids to small bulblets. When their circumference reached 6-8 cm, they were transferred to cold storage (4°C) for 2 months to break dormancy, and planted in a greenhouse for selecting excellent genotypes. The greenhouse observation indicated that the hybrids of Oriental lily and *Lilium regale* have clear heterosis. The hybrids have abundant variations of flower colour with a reduced amount of pollen. Plant height of hybrids is significantly greater than that of the parent, with enhanced resistance and weakened scent. Further the hybrids bloom 10-20 days earlier than most of the Oriental lilies. These hybrids are especially suitable for garden cultivation, and can be used as cut flowers as well. We describe four excellent hybrids which we selected in Nanjing, China.

Nanlin-1 (Figure 1): This genotype is derived from crossing the white Oriental lily 'Constanta' and *Lilium regale*. . The blossom season of this genotype is in early June. It has white flowers with nice fragrance. The fila-

Figure 1. Nanlin-1

ments and styles of the flowers are green. The length of the anther is 3.41 cm, and the color of the pollen is orange. With the bulbs the circumference of which are 6-8 cm, the average height is 52 cm, leaf length 15.5 cm, leaf width 2.4 cm, growing one or two 14.5 cm diameter flowers. As for the 12-14 cm circumference bulbs, the average height of the plants is 106 cm, leaf length 17.1 cm, leaf width 2.6 cm, growing three to four 15.6 cm diameter flowers.

Nanlin-2 (Figure 2): This genotype is derived from crossing Oriental lily 'Marco Polo' and *Lilium regale*. . The blossom season for this genotype is in early June. Nanlin-2 has pink flowers with nice fragrance. The filaments and styles of the flowers are green. The length of the anther is 3.02 cm, and the color of the pollen is orange. With the 6-8 cm circumference bulbs, the average height of the plants is 58 cm, leaf length 19.5 cm, leaf width 1.4 cm, and each grows one flower, whose diameter is 16.5 cm. With the 12-14 cm circumference the bulbs, the average height of the plants is 131 cm, leaf length 21.5 cm, leaf width 1.6 cm, and each grows three to five flowers. The diameter of the flower is 18.9 cm.

Figure 2. Nanlin-2

Nanlin-3 (Figure 3): This genotype is derived from crossing Oriental 'Acapulco' and *Lilium regale*. The blossom season of Nanlin-3 is in early June. Its flowers are red and with nice fragrance. The filaments and styles of the flowers are green. The length of the anther is 3.94 cm, and the color of the pollen is orange.

Figure 3. Nanlin-3

With the 6-8 cm circumference bulbs, the average height of the plants is 85 cm, leaf length 20.5 cm, leaf width 1.48cm, and each grows one or two flowers. The diameter of the flower is 14.8 cm. With the 12-14 cm circumference bulbs, the average height of the plants is 155 cm, leaf length 21.3 cm, leaf width 1.9 cm, and each grows 3-5 flowers. The diameter of the flower is 15.6 cm.

Nanlin-4 (Figure 4): This genotype is derived from crossing Oriental 'Acapulco' and *Lilium regale.* . Its blossom season is in early June. It grows pink flowers with nice fragrance. The filaments and styles of the flowers are green. The length of the anther is 3.96 cm, and the color of the pollen is orange. With the 6-8 cm circumference bulbs, the average height of the plants is 88 cm, leaf length 18.5cm, leaf width 1.6cm, and each grows 1-2 flowers. The diameter of flower is 16.4cm. With the 12-14 cm circumference bulbs, the average height of the plants is 165 cm, leaf length 20.5 cm, leaf width 1.8cm. Each grows 3-4 flowers, the diameter of which is 17.6 cm.

Figure 4. Nanlin-4

Acknowledgements

More than ten funds have support our lily breeding research, including the"948" project of the State Administration of Forestry of China (2013-4-34) and Jiangsu Agriculture Science and Technology Innovation Fund CX(12)2018.

Long Y. Y., Zhang J. Z. 1998, The Protection and Utilization of Lilium Resources. Journal of Plant Resources and Environment, 7(1):40-44.

Wu Z. H., Shi J.S., Chi J., Xi M.L., Hu F.R., Jiang F. X. 2006, Research Advances on Resources and Breeding of Ornamental Lily, Journal of Nanjing Forestry University (Natural Science Edition) 30(2) : 113-118.

Wang F.Z., Tang J. 1980, Flora of China (Vol 14): Liliaceae (1). Beijing: Science Publishing House.
Zhao X.Y., Wang S.D., Chen X.X. 2000, Lily, Beijing: Agricultural Publishing House.

Development of Lily Production in Northeast China

Lianwei Qu,

Shenyang, China

I am a grower and breeder of lily and was born in a village in Northeast China. I work as an associate researcher in the Department of Horticulture of Liaoning Academy of Agricultural Sciences in Northeast China. Growing flowers to make the world colourful and breeding flowers to create new varieties has always been my hobby, as well as my career now. When I was young I was very interested in ornamental plants, that is why I studied at Shenyang Agriculture University.

Fig 1. Lily cultivar 'pollenless white'

I worked for a lily company in Dalian city as soon as I graduated from Shenyang Agriculture University in 2000. Although I have been growing lilies for thirteen years, it doesn't feel long for people who live in flower developed countries, like the Netherlands and America, for China it's not a short time. China's lily industry was gradually formed in the mid to late 80s, has only twenty years of history (Mu 2009). It is in this lily company that I learned more about lily cultivation and I experienced the prosperity of lily cut flower trade.

In the 1998's, the lily cut flowers price was $4.8 each, approximately what wholesale lily cut flowers (ten lilies) are selling for today! When I attended to the lily company in 2000, the price of each lily flower was about 3 and the company produced approxi-


Fig. 2. Fantasy Land

mately 200,000 lily cut flowers per year. In this case lily growers could have huge profits. I remember that my company leader was quite protective of their valuable lilies and hired two guards to protect his lilies during the night.

Fig. 3. Lily cultivar Pretty in Pink

Since the huge market demand and the high profit, the production of lily bulbs and cut flowers have made a significant increase (since 2000 an average annual growth rate more than 20%) both in area and output in Northeast China, especially in Liaoning province. Liaoning is the primary regions of lily cut flowers production and lily bulbs production in China, as well as the first place to engage in lily cut flowers production region. Liaoning's lily industry is starting in the 80's. After the rapid development of 90's, it is becoming a certain scale when the beginning of the 21st century. My company expanded from five greenhouses in 1998 to ten greenhouses in 2000. At present the company has a base of 100 hectares for flower cultivation, of which 60 ha of greenhouse.

Fig. 4. Planting of lily bulbs in the field

In 2005 I left the company and entered the Flower Institute of Liaoning Academy of Agricultural Sciences located in Shenyang, the capital city of Liaoning province, where I started my career as a flower breeder. The director of our flower institute is Researcher Dongsheng Yin who did lily breeding work in the plant breeding group of Wageningen University under the guidance of Dr. Jaap van Tuyl in 2004. After Researcher Dongsheng Yin returned to China, he set up the lily breeding group where I am working now. Few years later Zhigang Wang and I also worked in the plant breeding group of Wageningen University for one year under supervision of Dr. Jaap van Tuyl. Due to our lily breeding group was learning from the periods in

Wageningen, we made great achievements in lily breeding and lily cultivation techniques. Our institute has 34 persons working on flower research, specially focus on lily breeding, lily culture in lab, lily propagation and lily cultivation techniques. The 'pollenless white' was the first cultivar released in 2009 (fig. 1). In 2013 there were two cultivars that were registered successfully in The Royal Horticulture Society (RHS) in the UK (fig. 2 and fig. 3). In terms of lily propagation and cultivation, we have a lot of cooperation with flower companies supported by Liaoning provincial government (fig. 4 and fig. 5).

Fig. 5. Harvesting of lily cut flowers

Nowadays, there are more than 800 hectares used to produce lily bulbs in Liaoning province, but in 2005, the number was only 100 hectares nationwide (Buschman, 2005). Liaoning is famous for the biggest bulbous-flower breeding and propagation production base in China, has the largest areas of energy-conserving sunlight greenhouses used to cultivate flowers (5789.7 hectares), has some national key scientific research institutions like The National Engineering Research Center for Horticulture, The Oriental Lily Chrysanthemum R&D and Promotion Center, The Sub-center of National Flower Engineering Research Center, etc.

Oriental (O, *Archelirion*) hybrids ('Siberia' and 'Sorbonne') are the most predominantly cultivated bulbs, ranking approximately 80% of 60 million bulbs imported per year from The Netherlands. More than 240 million bulbs are produced per year in Liaoning province (Jia, 2008) and the demand of lily bulbs is in average 180 million per year, along with more than 50 million lily bulbs imported from the Netherlands, according 27.8% of the total demand.

The rapid development of the lily industry is due to the proper climate and developed transportation. The coastal region is especially well suited for lily production (Zlesak and Anderson 2009). Liaoning has an extensive coastline of 2,178 kilometers in length (12 percent of China's total), has now been completed ten ports, formed in Dalian, Yingkou as the center, Dandong, Jinzhou for the two wings, a more reasonable distribution of ports which is needed to the export and transport of lily products.

More factors that favoured the development of the lily industry, are

the richness in germplasm resources, cheaper labour force resources and abundant governmental investment in agriculture. Liaoning province in Northeast China, as the biggest bulbous-flower breeding and propagation production base in China, plays a significant role in national flower industry.

References:

Mu, D. 2007. The technical problems of lily production in China. Flowers Trees Potted Landscape, (09): 31-33.

Buschman, J.C.M. 2005. Globalisation - Flower - Flower Bulbs - Bulb Flowers. Acta Hort, 673: 27-33.

Zlesak, D. and Anderson, N. 2009. Inside West Easter Lily Production. North America Lily Society Yearbook, pp. 92-95.

Jia, H.Q, Song, G.Z and Hao, D.T. 2008. The Production Situation of Lily Bulbs and Development Suggestions in Liaoning Province. Liaoning Agricultural Sciences, (2): 41-42.

Studies on *Lilium lancifolium* in China

Zhigang Wang & Huihua Zhang, Shen-Yang, China

Introduction

L. lancifolium THUNB. (tiger lily*; L. tigrinum*) is one of the most widely distributed *Lilium* species, which can be used for landscaping, gardening or as pot lily. The bulb of *L. lancifolium* is also known in China as a traditional material for making food or medicine. This species has habitats in most areas of East Asia. It is very tolerant to the different environmental conditions in China, Korea and Japan, which provide possibilities for extensive use and research by local residents and scientists. Some basic facts have been clarified by Japanese and Korean scientists, such as *L. lancifolium* is a polyploid complex with diploid and triploid types involved (Noda, 1978). In this article, some research results about *L. lancifolium* performed within China, mostly published in Chinese will be summarized.

1. Genetic Studies on *L. lanclfolium*

All *L. lancifolium* collected in China were reported to be triploid till now, and it is commonly believed that triploid *L. lancifolium* is sterile. While two out of over one hundred *L. lancifolium* plants were observed to give seedpods after being preserved in Harbin Normal University and showed to be sterile for many years. The chromosome number of the seeds was 27, 29 or 34, separately, 79% of which was 29. After sowing, all the seeds germinated quickly and normally.

Due to the morphological similarity between *L. leichtlinii* var. *maximowiczii* Baker and *L. lancifolium* except for the bulbils of the latter, they were thought to be close relatives. But a study on the karyotype of these two species revealed that the karyotype of *L. leichtlinii var. maximowixaii* was 3A while *L. lancifolium* was 3B, and the karyotype equation was 2n=4m(2SAT)+lost+10t and 2n =6m(3SAT)+12st+15t+3T, respectively (Yue, et al., 2006), they were distantly related in genetic relationship.

Cytogenetical research together with morphological investigation also revealed genetic diversity between different geographical populations of triploid *L. lancifolium*. The populations closely distributed also had a close genetic relationship, but the nearest populations were not most closely related to each other, which showed there were still some other factors affected the genetic diversity (Yue and Lei, 2006).

2. Breeding Studies on *L. lanclfolium*

In order to develop new hybrids between *L. lancifolium* and other crossing parents, crosses were made and embryo rescue techniques were applied at the Flower Institute of the Liaoning Agricultural Academy of Sciences (LAAS) and Shenyang Agricultural University (SYAU). Four pollination methods were used to study the cross-compatibility of *L. lancifolium* with six wild species and three Asiatic cultivars. Fruits were obtained from seven of the nine combinations when *L. lancifolium* were used as the female parent and embryos were obtained from six of the nine combinations. Cut-style pollination could overcome species-incompatibility between *L. lancifolium* and *L. concolor* var. buschianum Baker, *L. concolor* var. megalanthum Wang et Tang or *L. cernuum* Korn. After pollination, the pollen tubes of compatible combinations reached the basal part of the style in 48-72 h., and finally arrived at the ovule in ovary in 72-96 h (Pang et al., 2009). The embryo rescue method for the progenies derived from the combination were set up subsequently (Lei et al., 2009).

Fig 1. Hybrids derived from *L. lancifolium* and Asiatic cultivars

Also a start was made with the development of molecular markers. An AFLP reaction system was established by optimizing some factors including enzyme digestion, ligation, pre-amplification and selective amplification. 12 pairs of primer combinations which gave stable and polymorphic bands were selected (unpublished). This system will

be used to analyze a population derived from the combinations between *L. lancifolium* and some Asiatic cultivars

3. Studies on Propgation of *L. lanclfolium*

L. lancifolium was collected from four regions in Qinghai Province in Northwest China. The effects of vernalization time on germination rates of bulbils harvested from the collections were studied. The results showed that vernalized under 4 for 45 d was the best for germination and growth of the bulbils. The effects of same treating time varied between collections from different areas (He & Ma, 2010).

In vitro propagation methods for scale and bulbil of *L. lancifolium* were developed as well. The regeneration ability of external scales was better than the middle ones. The medium containing MS+6-BA1.5mg/L+NAA0.2mg/L could induce more and stronger shoots. The best proliferation medium was MS+6-BA1.0mg/L+NAA0.2mg/L (Guo & Lei, 2006).

4. Expectation

L. lancifolium is known by researchers and breeders for several interesting characteristics such as cold tolerance, strong vigor, virus and *Fusarium* resistance. In cold remote areas of Northeast China, local residents collect wild *L. lancifolium* to green their garden and it can survive the long winter with temperatures colder than -20 for a few weeks. Besides, the bulbils formed in the axils of *L. lancifolium* can germinate easily and give large quantity of plantlets covering the surrounding land. This phenomenon inspires the author that *L. lancifolium* could be used to make hybrids for poor villages which cannot afford expensive landscaping materials and labors. With the help of *L. lancifolium*, this type of lily hybrids will have the characteristics as follows: cold tolerance; *Fusarium* and virus resistance; self-propagation and good ability of covering the land.

References

Guo, Haibin and Jianjun Lei (2006). The Bulb Scale and Bulblet Culture in vitro of *Lilium lancifolium* Thunb. Chinese Agricultural Science Bulletin 22(2): 72-743.

He, Changfang and Fugui Ma (2010). Effect of Vernalization Time on Germination Rate of Bulbil for *Lilium lancifolium* Thunb. Northern Horticulture 16: 89-91.

Lei, J. (Jiajun), Lan Pang, Yifei Lin and Jingzhu Guan (2009). Embryo culture of interspecific hybrids from *Lilium lancifolium* Thunb. Journal of Shenyang Agricultural University, 40(1):84-87.

Noda, S. (1978). Chromosomes of diploid and triploid forms found in the natural populations of tiger lily in Tsushima. Bot. Mag. Tokyo 91: 279-283.

Pang, L. (Lan), Jiajun Lei and Yifei Lin (2009). Study on Interspecific Hybridization Compatibility of *Lilium lancifolium*. Northern Horticulture 6: 188-191

Yang, L. (Liping), Bing Ding, Xianghuan Liu and Xiaofang Zhang (1996). Cytogenetic diversity in *Lilium* in Northeast China. Journal of Northeast Forestry University 24(5): 19-23.

Yue, L. (Ling), Jiajun Lei, Xinying Hu (2006). Study on karyotypes of Study on Karyotypes of *L. leichtlinii* var. maximowiczii Baker and *L. lancifolium* Thunb. Heilongiiang Agricultural Sciences 6: 41-43.

Yue, L. (Ling) and Jiajun Lei (2006). Study on the Genetic Diversity of *Lilium lancifolium* Thunb. Journal of Anhui A. Sci. 34(22): 5802-5803, 5852.

Development of Lily Production in Yunnan, China

Xue Wei Wu[1,2], Li Hua Wang[1,2], Jing Hong Yang[1,2], Ji Wei Ruan[1,2], Su Ping Qu[1,2], Ji Hua Wang[1,2]

[1]Flower Research Institute (FRI), Yunnan Academy Agricultural of Science (YAAS), Kunming, China.

[2] National Engineering Research Center for Ornamental Horticulture, Kunming, China.

Yunnan province is located in South-West of China (latitude 21° 8' 32"-29° 15' 8" N., longitude 97° 31' 39"-106° 11' 47" E, altitude 76.4 m (Yuanjiang) to 6,740 m (Deqin)), and has special stereo-scopic topography, climate and has developed a special biosphere. Although Yunnan province has a long history of growing flowers, commercial production of cut flowers started only in late 1980s. The first farmer who grew cut flowers in the Dounan village build a nice house and bought a three-bedroom apartment in Kunming with the income he earned from flower sales. Inspired by their fellowman in the same village getting affluent, many Dounan villagers also decided to grow cut flowers. The flower cultivation area expanded quickly to 100 Ha in the village. In less than five years time, Dounan Village became one of the production and wholesale centers for cut flowers in China. Now, Dounan flower and Kunming International Flower Auction are well known in the world.

1. Genus Lilium in Yunnan.

Excluding the section Martagon, about 25 species and 9 varieties of *Lilium* are found in the wild habitat in Yunnan Province. They are *Ll. brownii, brownii var. viridulum, wenshanense, sulphureum, sargentiae, lophophorum, lophophorum var. linearifolium, nanum, souliei, henrici, henrici var. maculatum, bakerianum var bakerianum, amoenum, pinifolium, nepalense,nepalense var. burmanicum, napelense var. ochraceum, wardii, taliense, duhartrei, lijiangense, papilliferum, davidii, fargesii, stewartianum, habaense, lankongense, primulinum, and lancifolium.* Eight species of *Lilium (Ll. Wenshanense, lophophorum var. linearifolium, bakerianum var. rubrum, amoenum, pinifolium, lijangense, habaense, lankongense)* are endemic species outside Yunnan (Wu and Xiong 2006). Most of them are found North of Yunnan. For example, North West of Yunnan, Lijiang, Xianggelila, Gongshan, which is on the East of India and

Myanmar, South of Tibet, and West of Sichuan Province. Many of them in the wild habitats are to become extinct for several years of drought in a row.

2. Lily research situation in Yunnan

Lily research in Yunnan is the earliest in China for a rich germplasm of wild lilies. Kunming Botanical Institute (KBI) collected over 30 wild lily species in 1950's. In the 1980's, a researcher of KBI began to make new varieties by crossing, and got 10 Asiatic hybrids (not registered). Unfortunately, they lost all lily germplasm material after the researchers retired. In the end of the1990's, Flower Research Institute (FRI), Yunnan Academy Agricultural of Science (YAAS) began to collect lily material for lily breeding. They had more than 45 wild lily species. Yunnan Agricultural University and Yunnan University began the lily research in 2003. There are 16 new varieties using crossing, which have been registered in the Ministry of Agriculture of China by FRI.

Lily classification, speciation and evolution have been studied by morphology, palynology, karyotype and molecular analysis. Owing to diverse weather conditions, the wild lily is ample in variation *Lilium bakerianum* is widely distributed in pinus forests and grasslands along the hillside and near the streams in Yunnan. They grow at 1,200 to 3,500 metres above the sea level. The bulb of *L. bakerianum* is broadly ovoid to subglobose, scale is white, ovate or ovate-lanceolate. Their heights of the type range are from 5-130cm. Leaves scattered, linear or linear-lanceolate, papillose at margin and on midvein abaxially, sometimes white pubescent on both surface. Flower 1-5, nodding or suberect, campanulate. Six varieties have been recognized; *L. bakerianum, L. bakerianum var. bakerianum, var. yunnanense, var rubrum, var aureum, and var. delavayi.* In a given variety, flower shape and color, and distribution of spots varied significantly, and investigation of these variations at the molecular level is required. *Lilium bakerianum* is rich in color, and is white, light-pink, pale rose-pink, purple-red, pale yellow, yellow, brownish yellow or purplish yellow, yellowish green, pale yellow-green, greenish, or pale green. The spots of tepals vary from number, size and density. Morphological characteristics such as shoot, leaves, stem, tepals, and the presence of spots in tepals of 6 varieties of *L. bakerianum* are showed in Fig. 1

3. Development of lily production in Yunnan.

In Yunnan, growers began to plant Asiatic lily for potted flowers during the 1980's in Dounan village. Yunnan cut lily flower production started from 1990's, in Dounan and Yuxi. The production areas were slowly increased until a sharply improvement in 2000. In 2005, Aziying town was rewarded

Fig. 1. Different flower shape, color, and the distribution of spots in 6 varieties of *L. bakerianum*.

a wonderful name - lily town of China - because of the rapid rising of the cut lily production area.

From 2007 to 2011, Yunnan cut lily production increased, with area 1133, 1353, 1433, 1593, and 1867 ha, respectively; and the value of lily product are 0.56, 0.54, 0.93, 1.28, and 1.33 billion yuan RMB, respectively (Yunnan flower industry office, China, 2012). The cut lily produced in Yunnan are mainly exported to South East Asia, and sale to Beijing, Shanghai and Guangzhou etc.

In earlier lily flower production, the main product was Asiatic hybrids, but soon was replaced by Oriental hybrids. In current status, the production pattern of cut lily now is mainly based on Oriental hybrids, supplemented with OT hybrids, Longiflorum hybrids, and Asiatic hybrids. In terms of the cut flowers production model, farmers, collaborative organization and professional companies yearly produce cut lily seasonally. In detail, farmers use plastic greenhouses to produce cut lily in summer, while professional companies produce cut lily in winter and spring in a plastic greenhouse or glasshouse, which combines with a heating system. A few years ago, some lily growers planted cut lily in higher altitude (more than 2000 metre) in summer and in lower altitude (less 700 metre) in winter, where lily bulbs had been planted in open fields without protecting except a shading net.

Lily bulb production began in the 2000's. In the beginning, some lily

growers imported the small size (circumference about 6-9cm or 9-12cm) lily bulb from Netherlands. After the lily bulb grew to big size (circumference about 14-16cm or 16-18cm), growers used them for producing cut lilies. Then lily growers were looking for some area where lily bulbs grow fast with higher quality. Some growers found the suitable area of lily bulb are Zhaotong, Lijiang and Xianggelila where the overwhelming majority of wild lily grew. Then some Netherlands companies came to Yunnan, and lily bulbs were produced in cooperation with a Chinese firm.

References

Haw S.G. 1986. The Lilies of China, Timber Press, USA, pp 172.

Liang S.Y., Zhang W.X. 1984. Pollen tetrads in Lilium with a discussion on the delimitation between L. sempervivoideum and its ally. Acta Phytotaxonomica Sinica, 22 (4), 297-300.

Liang S.Y., Minoru N.T. 2000. Lilium Linnaeus. Flora of China, 24, 135-149.

Wang F.Z., Tang .J, Liang S.Y. 1986. Notes on Chinese Liliaceae XII. Acta Botanica Yunnanica, 8 (1), 51-52.

Wang L., Gu Z.J., Gong X., Xiao D.J. 1993. Cytological study on Liliaceae. Journal of Systematics and Evolution, 31 (6), 549-559.

Wu X.W., Wang L.H., Wu L.F., Qu S.P., Suh J.K., Wang J.H. 2013. Native Species of the Genus Lilium and the Closely Related Nomocharis in Yunnan, China. Floriculture and Ornamental Biotechnology, 28-38.

Wu X.W., Li S.F., Xiong L. 2006. Distribution situation and suggestion on protecting wild lilies in Yunnan Province. Journal of Plant Genetic Resources, 7 (3), 327-330 (In Chinese).

Wu X.W., Zhang Y.P., Qu Y.H. 2008. A new cultivar of Lilium Oriental hybrid "Yun Xia". Acta Horticulturae Sinica, 35 (8), 1245 (In Chinese).

Wu X.W., Wang L.H., Zhang Y.P. 2011. Eight genus lilum pollen morphology and cluster analysis. Jiangsu Agricultural Science, 39(3): 197-199 (In Chinese).

Wu Z.Y., Chen S.K. 1997. Yunnan Zhiwu Zhi, Science Press, Beijing (In Chinese).

Zhao X.Y., Chen X.L., Li D.M., and Liu K.F. 1996. Resources and research situation of the genus Lilium in China. Acta Hort., 414:59-68.

Zheng S.X., Wu X.W., Liu F.H. 2009. Distant hybridization of two species of Lilium sulphureum and Oriental lily 'Ruyi'. Journal of West China Forestry Science, 38 (4), 5-8 (In Chinese).

Lily Genetic Modification at Wageningen UR

Frans Krens & Bernadette van Kronenburg-van de Ven

Wageningen University & Research Centre Plant Breeding

Introduction

Every living organism has the genetic information that defines its characteristics and appearance residing on nucleic acids such as desoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Specific traits are organized in gene(s) and genes are located on chromosomes. All the chromosomes together are called the genome of an organism. Genetic modification is the deliberate introduction of new genes into the genome, so in one of the chromosomes, of a recipient organism. If such an organism is a plant this can be achieved by two methods, mainly. The first is a man-made one called particle gun bombardment in which very small gold particles, smaller than a plant cell, are coated with DNA carrying the genes of interest. These particles are subsequently propelled into plant cells present in a particular plant organ or tissue by use of a particle gun. The particle gun makes use of a suddenly released Helium gas flow that carries the particles towards the plant tissue with enough power to ensure penetration of cells. In the cell sap the DNA soaks free from the particles and finds its way to the cell nucleus in which the chromosomes are located. At a certain frequency the DNA gets incorporated into one or more of the chromosomes.

The second procedure is a natural one. *Agrobacterium tumefaciens* is a soil-born bacterium responsible for the disease crown gall. The crown gall disease is characterized by the formation of large tumors on plants, mostly in the root-stem transition area. Research has revealed that this disease is caused by the transfer and stable integration of DNA of the bacterium into the genome of the recipient plant. The genes that *Agrobacterium* transfers are engaged in the production of plant hormones, leading to the tumors, and in the synthesis of specific nutritional compounds by the plant-tumor cells that the *Agrobacterium* can utilize but other bacteria cannot. It was found that the genes the bacterium transfers can be removed and replaced by genes that man would like to integrate into a plant. This transferred piece of DNA is called T-DNA and *Agrobacterium* is nowadays considered as the working-horse in plant genetic modification. Because crown gall tumors were never found on monocot species, it was originally thought that monocots could not be transformed (= genetically modified) by *Agrobacterium.*

Early research
Agrobacterium-mediated gene transfer

Genetic modification research on lily at Wageningen University and Research Centre (Wageningen UR) started in the eighties of the previous century. As with any new crop in which gene transfer studies were explored in those days the first experiments were done with constructs containing a selectable marker gene and a reporter gene in order to monitor DNA transfer efficiencies. Because Hooykaas-Van Slogteren et al. found in 1984 that *Agrobacterium tumefaciens* was also capable to transfer its T-DNA into monocots such as *Chlorophytum capense* (Liliaceae) and *Narcissus* cv. 'Paperwhite' (Amaryllidaceae), Langeveld et al. (1995) tried several different *Agrobacterium* strains for identification of the one most suitable for transformation of lily. They inoculated *in vitro* grown plantlets and sterilized stem segments of soil-grown bulbs of lily cv. 'Harmony' with wild-type *Agrobacterium* strains carrying an additional T-DNA piece with a *gus* intron gene. A *gus* intron gene is a so-called reporter gene; it reports to us whether or not gene transfer has been successful or not. Its presence can be easily visualized by biochemical staining; if it is present, the transformed tissue stains blue (Figure 1). To allow for the transformed cells to divide and grow into transformed tissue and plants, one needs to provide them with conditions to favor their growth above the growth of non-transformed cells. This is done by transferring a selection gene; this is a gene giving either resistance to antibiotics or to a herbicide. Unfortunately, the efficiencies found proved to be disappointingly low and the emphasis of WUR transformation research in lily shifted towards particle gun bombardment. All thinkable parameters were tested both from the plant's perspective and from the bombardment protocol perspective. Several cultivars representing different sections of *Lilium* were taken as well as different plant parts and organs ranging from roots, scales, leaves, callus and pollen. Particles of different sizes and material (tungsten versus gold), Helium pressure, distance, number of shots fired and different plasmids (DNA molecules) were just some of the parameters concerned with the protocol that were tried. In the end some successes were obtained and can be presented.

Figure 1. Transgenic, GUS positive lily material, from left to right root, leaf and scale; top GM lily, bottom non-GM control

Particle Gun Bombardment

Van der Leede-Plegt et al. (1992) bombarded lily (*Lilium longiflorum*) pollen with constructs containing the *gus* reporter gene under control of several different promoters and found gene expression in individual lily pollen grains when the TR2' mannopine synthase gene promoter was used. The idea was to use bombarded pollen for pollinations yielding genetically modified offspring and this was achieved by using bombarded *L. longiflorum* cv. 'Gelria' pollen in crosses with *L. longiflorum* cv. 'Indian Summer'. Three transgenic seedlings were obtained and used in further crossing with cv. 'White American' (Van der Leede-Plegt et al. 1997). However, despite the success in demonstrating gene transfer, also here efficiencies were not sufficient for further applications. Using *Lilium longiflorum* cv. 'Snow Queen' (SQ) scales bombardment with a plasmid carrying the gene giving resistance to the herbicide phosphinothricin as selection gene and the *gus* gene as reporter seven individual transgenic lines were regenerated and their transgenic nature was confirmed by GUS staining (Lin & Krens 1995, pers. comm.) The plants could be maintained for several years keeping their newly introduced trait and flowers were formed on these lines. Crossings could not be made because the permit of the Dutch government did not allow this. Unfortunately, reproducing the conditions in subsequent gene transfer experiments did not result in more successes. Other experiments, however, did give positive results, but always with varying efficiencies and poor reproducibility. Transgenic lines from SQ but also from the Oriental hybrid 'Star Gazer' with *bar* as selectable marker gene and *gus* as reporter gene both under control of the 35S promoter were produced.

After the initial studies on determining the best conditions for gene transfer and stable transformation, new experiments were performed targeted towards the introduction of particular traits of interest to lily as an ornamental crop. Generally for any ornamental crop, traits such as flower color, flower morphology and disease resistance are considered to be important. Langeveld et al. (1997) introduced by means of particle bombardment using a DNA molecule containing a hygromycine (antibiotic) resistance gene, *hpt*, as selectable marker and *gus* as reporter gene, the gene responsible for the production of the coat protein of the *Lily Symptomless Virus* (LSV). Introduction of such coat protein genes yielding a lot of coat protein in plant cells but without the virus itself (or its genetic material) are known to be able to give virus resistance. The plant material consisted of bulb scales from *in vitro* grown plants and bulblets of SQ. The transgenic nature was confirmed by long term expression of the *gus* reporter gene in the individual lines and by crosses taking the GM pollen to pollinate *Lilium longiflorum*

cv 'Gelria'. The transgenic lines were tested in the greenhouse for resistance by inoculating the plants with aphids and screening later with an ELISA for the presence of viruses and disease development. Ultimately, the most promising lines were tested in a field trial. Here, the resistance proved to be unstable and insufficiently high. In another line of research aimed at altering flower morphology, Benedito et al. (2005) introduced the zinc-finger transcription factor gene *SUPERMAN* (*SUP*) from the model plant *Arabidopsis thaliana* under control of the promoter of the petunia floral specific gene *FBP1* (*FLORAL BINDING PROTEIN 1)*. This means that they introduced a gene involved in determining flower shape and took care that this gene could only be expressed (= active) in petals and stamens. In Petunia, introduction of this gene led to malformed flowers without affecting the style (Kater et al. 2000). Although in lily the presence of the gene was confirmed by multiple molecular techniques, no effect on the floral phenotype could be observed.

Recent research

The marker-free system using Agrobacterium

Stimulated by Dutch lily breeders who wanted to fully explore possibilities and impossibilities of genetic modification for further improvement of lily and then primarily of the Oriental hybrids and by publications of other groups especially those of Hoshi et al. (2004), we concentrated once again on the use of *Agrobacterium*. This also because Dutch breeders wanted to incorporate a new development of the ongoing transformation research using marker-free systems, i.e. the production of genetically modified crops devoid of any antibiotic resistance marker genes or any other undesired gene sequences (Schaart et al. 2004). The general applicability of this system was tested using a model vector, pRCNG (Schaart et al. 2004), which carried a *hpt* gene for selection on hygromycin, which in our hands is the best selectable agent in lily. Transformation of lily SQ and Oriental hybrids proved possible and the marker-free system was effective. Next, specialized DNA constructs, the so-called pMF –vector –based system (Schaart et al. 2011), were made suitable for use in lily transformations by the introduction of a *hpt* selectable marker gene (hygromycin resistance) between the recombination sites. This would enable selection for transformants in lily and ensure removal of the antibiotic resistance marker when desired. As traits-of-interest flower color and insect resistance were chosen. First, at the request of the Dutch breeders a series of twenty lily cultivars encompassing longiflorums, Oriental hybrids, LO's, LA's and OT's, were tested for their callus forming ability and subsequently for their regeneration potential. As explants the flower organs, filaments and styles, were taken for callus induction, largely

according to Hoshi et al. (2004). Next, transformation capacity was assessed using *Agrobacterium tumefaciens* strain AGL0 equipped with the binary vector pCAMBIA1301 with *hpt* and *gus* intron for eight cultivars. From seven, transgenic plants could be obtained but at varying transformation efficiencies per genotype (Wang et al. 2012).

Flower color

For flower color manipulation, genes coding for enzymes producing a yellow-colored plant compound called aureusidine, capable of turning white flowers yellow (*AmAS1* and *Am4'GT;* Ono et al. 2006), were cloned into the pMF vector and introduced into a selection of white-flowered longiflorums and Orientals. In addition, a construct was made in which an extra gene sequence was added aimed at knocking out an endogenous lily gene, *CHI* (chalcone isomerase), thought to be competing for the substrate of *AmAS1*. However, despite the demonstration by molecular tools of the presence and of expression of the introduced genes, no alteration in flower color was observed in blooming plants of the many transgenic lines that were generated (Wang & Krens, pers. comm).

Insect resistance

For resistance against aphids as vectors for virus transmission, a dual approach was conceived and carried out. Linalool, a volatile compound, is known to be a deterrent of insects as was demonstrated in potato and chrysanthemum (Jongsma 2004) and proteinase inhibitors, interfering with digestibility of proteins in the mig-gut of insects, have been proven to reduce fecundity and population built-up of aphids in *Arabidopsis* upon feeding (Ceci et al. 2003; Carrillo et al. 2011). The Linalool Synthase gene from strawberry and the gene coding for the sea anemone proteinase inhibitor, Equistatin, were cloned into pMF and introduced in lily cultivars. Many putative transgenic lines were generated and using PCR and RT-PCR molecular analysis indications were obtained for the transgenic nature of 27 independent transformation events (lines) covering eight cultivars, seven Orientals and one OT. Not all putative transgenic lines were molecularly analyzed. Biochemically, it was not possible to confirm the presence of linalool or equistatin, hence aphid resistance assays were performed in the greenhouse on as many lines as possible encompassing both the 27 molecularly analyzed lines as well as more than 50 non-analyzed lines. Twenty-four lines, six analyzed and eighteen non-analyzed, from six cultivars showed statistically significant resistance levels, however, for some a rather large variation was observed between replicates and two controls showed some resistance as well. Further tests and confirma-

tions are required before we will be absolutely sure that the genetic modification of these lilies is responsible for the change in susceptibility to aphids.

Conclusion

In this paper we concentrated on the efforts of Wageningen UR Plant Breeding and Table 1 summarizes the lily cultivars with which we had success in the past with lily genetic modification. Of course, also elsewhere in the world researchers are working on transformation of lily and were able to report successes, e.g. Watad et al. (1997), Mercuri et al. (2003), Hoshi et al. (2004); Ogaki et al. (2008); Liu et al. 2011; Núñez de Caceres et al. (2011). Recent overviews of the research on transformation of bulbous crops and other new biotechnological tools are given by Cohen & Krens (2012), Kamo et al. (2013) and Krens & Kamo (2013). It is clear from our experience and from the scientific literature on this topic that genetic modification of lily is feasible but that frequencies are still rather low and that examples of commercial applications are not yet available. The latter is also due to the stringent legislation for market introduction of GM crops in Europe.

Table 1. List of lily cultivars transformed at Wageningen UR Plant Breeding

Cultivar	*L. longiflorum* , diploid
Snow Queen	*L. longiflorum* , diploid
White Fox	*L. longiflorum* , diploid
Gelria	Oriental hybrid, diploid
Star Gazer	Oriental hybrid, diploid
Sorbonne	Oriental hybrid, diploid
Barbados	Oriental hybrid, diploid
Marrero	Oriental hybrid, diploid
Santander	Oriental hybrid, diploid
Lake Carey	Oriental hybrid, diploid
Cherbourg	Oriental hybrid, diploid
White Express	Oriental hybrid, diploid
Sheila	Oriental hybrid, diploid
Paradero	Oriental hybrid, diploid
Montezuma	Oriental hybrid, diploid
Burlesca	Oriental hybrid, diploid
Robina	OT, hybrid, triploid

References

Benedito VA, BCE van Kronenburg-van de Ven, JM van Tuyl, GC Angenent & FA Krens. 2005. Transformation of *Lilium longiflorum* via particle bombardment and generation of herbicide-resistant plants. Crop Breed Appl Biotechnol 5:259-264.

Carrillo L, M Martinez, F Alvarez-Alfageme, P Castanera, G Smagghe, I Diaz & F Ortego. 2011. A barley cysteine-proteinase inhibitor reduces the performance of two aphid species in artificial diets and transgenic *Arabidopsis* plants. Transgenic Res 20:305-319.

Ceci LR, M Volpicella, Y Rahbe, R Gallerani, J Beekwilder & MA Jongsma. 2003. Selection by phage display of a variant mustard trypsin inhibitor toxic against aphids. Plant J 33:557-566.

Cohen A & FA Krens. 2012. Genetic Transformation in the Breeding of Flower Bulbs. Floriculture and Ornamental Biotechnology 6 (SI1): 24-34 (Print ISSN 1749-0294).Cohen 2012

Hooykaas-van Slogteren GMS, PJJ Hooykaas & RA Schilperoort. 1984. Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. Nature 311:763-764.

Hoshi Y, M Kondo, S Mori, Y Adachi, M Nakano & H Kobayashi. 2004. Production of transgenic lily plants by *Agrobacterium*-mediated transformation. Plant Cell Rep 22:359-364.

Jongsma M. 2004. Novel genes for control and deterrence of sucking insect pests. ISB News Report November:1-4.

Kater MM, J Franken, A van Aelst & GC Angenent. 2000. Suppression of cell expansion by ectopic expression of the *Arabidopsis SUPERMAN* gene in transgenic petunia and tobacco. The Plant J 23:407-413.

Krens FA & K Kamo. 2013. Genomic tools and prospects for new breeding techniques in flower bulb crops. Acta Hortic. 974:139-147.

Kamo KK, FA Krens & M Ziv. 2012. Biotechnology for the Modification of Horticultural Traits in Geophytes. In: Ornamental Geophytes; from basic science to sustainable production. R Kamenetsky & H Okubo (eds.), CRC Press, Taylor & Francis Group, Boca Raton, pp 159-195.

Langeveld SA, MM Gerrits, AFLM Derks , PM Boonekamp & JF Bol. 1995. Transformation of lily by *Agrobacterium*. Euphytica 85:97-100.

Langeveld SA, S Marinova, MM Gerrits, AFLM Derks & PM Boonekamp. 1997. Genetic transformation of lily. Acta Hortic 430:290.

Liu J, J Zhang, B Xu, C Jia, J Zhang, G Tan & Z Jin. 2011. Regeneration and production of transgenic *Lilium longiflorum* via *Agrobacterium*

tumefaciens. In Vitro Cell Dev-Plant 41:783-788.
Mercuri A, L de Benedetti, S Bruna, R Bregliano, C Bianchini, G Foglia & T Schiva. 2003. *Agrobacterium*-mediated transformation with rol genes of *Lilium longiflorum* Thunb. Acta Hortic 612:129-136.
Núñez de Caceres FF, MR Davey & ZA Wilson. 2011. A rapid and efficient *Agrobacterium*-mediated transformation protocol for *Lilium*. Acta Hortic 900:161-168.
Ogaki M, Y Furuichi, K Kuroda, DP Chin, Y Ogawa & M Mii. 2008. Importance of co-cultivation medium pH for successful *Agrobacterium*-mediated transformation of *Lilium x formolongi*. Plant Cell Rep 27:699-705.
Ono E, M Fukuchi-Mizutani, N Nakamura, Y Fukui, K Yonekura-Sakakibari, M Yamaguchi, T Nakayama, T Tanaka, T Kusumi & Y Tanaka. 2006. Yellow flowers generated by expression of the aurone biosynthetic pathway. Proc Natl Acad Sci USA 103:11075-11080.
Schaart JS, FA Krens, KTB Pelgrom, O Mendes & GJA Rouwendal. 2004. Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol. J. 2:233-240.
Schaart JG, FA Krens, AMA Wolters & RGF Visser. 2011. Transformation methods for obtaining marker-free genetically modified plants. In: Plant Transformation Technologies. CN Stewart, A Touraev, V Citovsky & T Tzfira (eds.), Wiley-Blackwell, Oxford, pp 229-242.
Van der Leede-Plegt LM, BCE van de Ven, RJ Bino, TPM van der Salm, AJ van Tunen. 1992. Introduction and differential use of various promoters in pollen grains of *Nicotiana glutinosa* and *Lilium longiflorum*. Plant Cell Rep 11:20-24.
Van der Leede-Plegt LM, BCE van Kronenburg-van de Ven, J Franken, JM van Tuyl, AJ van Tunen & HJM Dons. 1997. Transgenic lilies *via* pollen-mediated transformation. Acta Hortic 430:529-530.
Wang Y, B van Kronenburg, T Menzel, C Maliepaard, X Shen & F Krens. 2012. Regeneration and *Agrobacterium*-mediated transformation of multiple lily cultivars. Plant Cell Tiss Organ Cult 111:113-122.
Watad AA, DJ Yun, T Matsumoto, X Niu, Y Wu, AK Kononowicz, RA Bressan & PM Hasegawa. 1997. Microprojectile bombardment-mediated transformation of *Lilium longiflorum*. Plant Cell Rep 17:262-267.

Plant cytogenetics: terms and techniques

Nadeem Khan

Introduction

Genetics is a branch of biology that studies the science of genes and heredity. Genes are DNA sequences which are packed in small thread like structures called chromosomes located within the nucleus of a cell. Cytogenetics refers to the microscopic analysis of these chromosomes in individual cells. Genomics refers to the detailed analysis of the entire genome (the total chromosomes number) of an individual at molecular level. Cytogenetics study is considered an essential component of the important scientific work to identify the individuals within a species based on their chromosomal organization. Cytogenetics is particularly useful in the identification of a plant the at chromosomal level as various plant species have different chromosomal make up in their genome constitution. In the following paragraphs efforts are made to define basic terms used in cytogenetics as well as different techniques performed in the laboratory for cytogenetic analysis of a plant species.

Allopolyploidy

When a polyploid plant possesses more than two complete sets of chromosome derived from more than one plant species.

Amphidiploid

Plants obtained by doubling of chromosomes number of F1 interspecific hybrid.

Anaphase

It is the stage of mitosis or meiosis in which the chromosomes are split and the sister chromatids move to opposite poles of the cell.

Aneuploidy

A condition in which a plant containing one extra chromosome (trisomy) or lacking one chromosome than normal paired chromosome (monosomy). Or the condition when a plant does not contain an exact multiple of diploid chromosomes.

Autopolyploidy

A condition in which a polyploid plant possessing more than two sets of chromosomes derived from a single species.

Banding pattern

A series of dark and light stripes across a chromosome which are produced by treating (staining) the chromosomes with different chemicals in a laboratory. There are a number of different staining techniques which produce different patterns e.g., G-banding, R-banding, Giemsa-C banding etc.

Bilateral sexual polyploidization

The plants obtained after pollination of 2*n* producing pollen with 2*n* producing egg. Here both parents contribute 2*n* number of chromosomes and the offsprings are tetraploid (4x).

Centromore

The constricted region almost in the middle of a chromosome that separates the short arm of the chromosome from the long arm is called centromere. Based on the position of centromere the chromosomes are classified in three different groups

Metacentric: centromere is in the middle or almost middle of the chromosome

Acrocentric: centromere close to one end of the chromosome

Submetacentric: intermediate position of the centromere on the chromosome

Acentric: chromosome fragment without a centromere.

Dicentric: chromosome having two active centromeres.

Chiasma

The point of physical contact of two chromosomes at which crossing over takes place during recombination. (Figure 1)

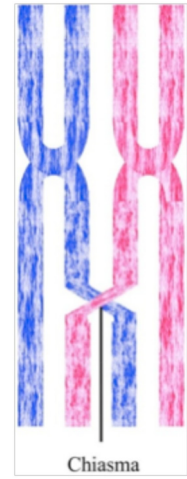

Fig. 1 Chiasma formation between chromosomes of two different species (Homoeologous chromosomes)

Chromatid

Chromatid is one-half of the two identical copies of a replicating chromosome which are joint together at centromere. The two chromatids of a chromosome are identical to each other and are called sister chromatids. (Figure 2)

Chromosome

The DNA of an organism is packed in thread like compact microscopic structures in the nucleus of a cell called chromosomes. (Figure 2)

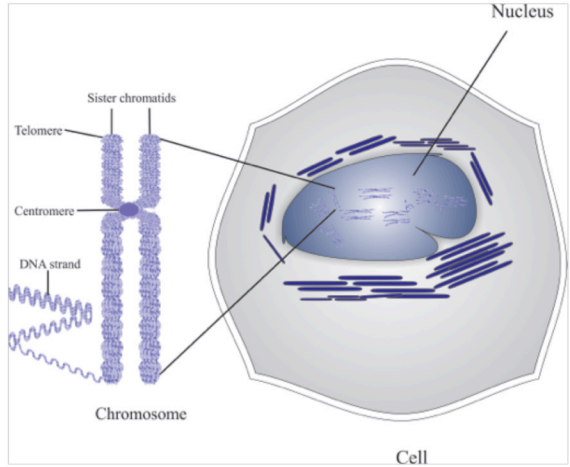

Fig. 2 Cell containing the chromosomes in the nucleus. The chromosome shows the centromere (the central portion where short and long arms of chromosomes are attached), the telomere (two ends of the chromosomes) and the DNA double strand which makes the whole chromosome in compact state.

Chromosomal aberration

The change of normal structure or number of chromosomes including e.g., duplications, inversions, translocations, aneuploidy, polyploidy and others deviation from the typical pattern.

B chromosome

Supernumerary chromosome present in some plant species. They are usually smaller than normal chromosomes and behave abnormally during mitosis and meiosis.

Crossing over

When chromosome pair join together, the two chromosomes may exchanges material i.e., part of one chromosomes cross over and exchanges its part with the corresponding part of other chromosomes. (Figure 1)

Cytogenetics

The microscopic study of chromosome structure, function and its behaviour.

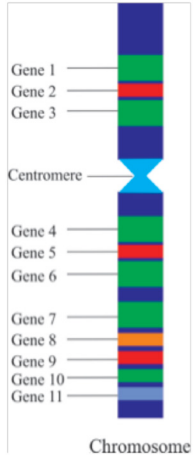

Fig. 3 Cytogenetic mapping: Different colours show the location of different genes on the chromosomes on both sides of centromere (short and long arm).

Cytogenetic mapping

The relative positions of genes on a chromosome and a measurement of the distance between them is called gene mapping. (Figure 3)

Diploid

A plant containing two copies of each chromosome is called diploid and the condition is called diploidy.

Diploid (2n) gametes

Diploid gametes contain somatic or diploid number of chromosomes (2*n* number of chromosome).

DNA (Deoxyribo Nucleic Acid)

A structure resembling a long winding staircase that contains all of the genetic instructions needed to develop and maintain a plant. DNA is contained in the nucleus of each cell in thread like structures called chromosomes.

Euploidy

Normal paired set of chromosomes is called euploidy. For example, diploid, triploid etc.

Flourescence in situ hybridization

This technique is used to identify individual chromosome based on specific DNA markers used as probes or localization of specific DNA markers on the chromosomes.

Gamete

A haploid reproductive cell of a plant such as sperm (or **pollen**) and egg (oocyte). (Figure 4)

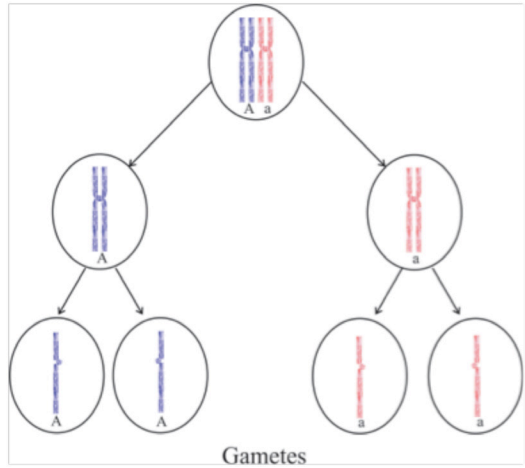

Fig. 4 Gamete formation (the pair of chromosomes separate resulting into the formation of haploid gametes i.e., they carry only one copy of each chromosome. An **Aa** individual therefore produces two kinds of gametes: **A** and **a**).

Gene

It is a piece of DNA located on chromosome that contains the information for a specific function or characteristic.

Genetic marker

It is a gene or DNA sequence located on a specific position on a chromosome.

Genetic variation

The differences among individuals of the same species due to differences in their genetic composition or due to different environmental conditions in which they were raised.

Genome

A genome is the set of haploid chromosome which contains all the genetic information of an organism. For example lily has 12 chromosomes and all these chromosomes constitute the lily genome.

Genomic *in situ* hybridization

When total genomic DNA of a plant species (the entire nuclear DNA) is used as a probe DNA in *in situ* hybridization experiment, it is called Genomic *in situ* Hybridization (GISH). (Figure 5)

Genotype

The total genetic information of an organism that is used for the physical appearance of that organism in later stages.

Haploid (n) gametes

They are reproductive cell called pollen or egg. Haploid gametes contain half the number of chromosome than their diploid parents i.e., a haploid cell contains only a single set of chromosomes (*n* number of chromosomes). (Figure 4)

Heterozygous

A diploid organism having two different alleles (genes) located on the same position of a chromosome. (Figure 6A)

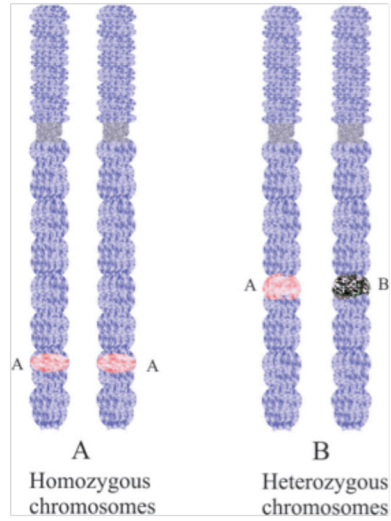

Fig. 6 The chromosome with two identical different genes at the same location i.e., Homozygous chromosomes (A). The chromosomes with two different genes at the same location i.e., Heterozygous chromosome (B)

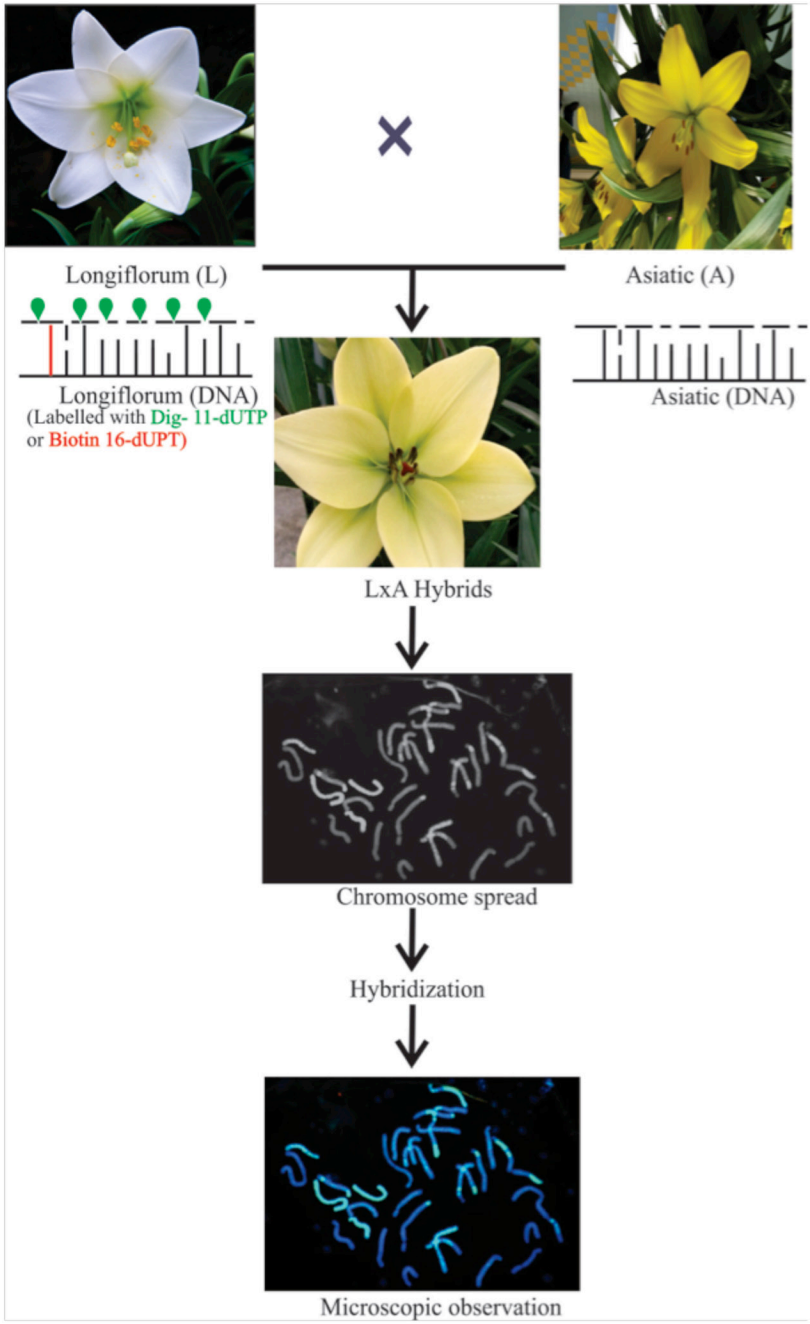

Fig. 5 The schematic representation of in situ hybridization (GISH/FISH) technique for chromosome identification and chromosomal recombination.

Homoeologous chromosomes

The chromosomes of an organism which are similar in size and position but derived from two different species are called homoeologous chromosomes. (Figure 7B)

Homologous chromosomes

The chromosomes which are similar in position, structure and function are called homologous chromosomes. (Figure 7A)

Homozygous

A diploid individual having two similar alleles (genes) located on the same position of a chromosome. (Figure 6B)

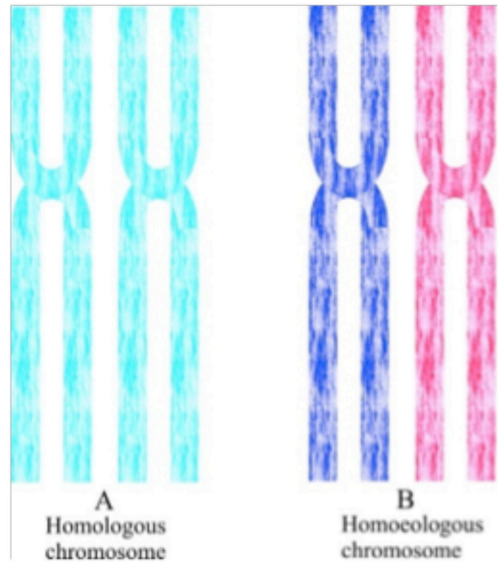

Fig. 7 The two chromosome of an offspring from the same origin are called Homologous chromosomes (**A**) while they are called Homoeologous when they come from different species (**B**).

Hybrid plants

The progeny of plants obtained from two genetically dissimilar parents.

Hybridization

The formation of a hybrid plants as a result of combination of parent plants from different groups. The term is applied to the progeny from matings of male and female plants within species (intraspecific) as well as to those between species (interspecific).

Introgression

The insertion of chromosomal segments from one species into another species through interspecific hybridization in a successive backcross breeding program.

Karyotype

Karyotype is the full set of chromosomes of a plant species. This term is usually used for images showing the arrangements of chromosome pairs

with respect to size and the position of centromere.

Meiosis

It is a process of cell division with two phases resulting four haploid cells (gametes) from a diploid cell. In meiosis I, the already doubled chromosome number reduces to half to create two diploid cells each containing one set of replicated chromosomes. Genetic recombination between chromosome pairs occurs during meiosis I. In meiosis II, each diploid cell creates two haploid cells resulting in four gametes from one diploid cell.

Metaphase

The stage of mitosis at which the chromosomes become more compact and align in the middle of the cell.

Mitosis

Cell division results into two identical daughter cells with the same chromosome number as the mother cell.

Mitotic recombination

During mitosis, sister chromatids freely exchange pieces without changing anything in genetic material because they are identical. Very rarely, and by chance, homologous chromosomes come very close to each other and exchange material as in meiosis which results in a recombinant chromosome.

Nucleolar organizer region (NOR)

Area of chromosome containing a number of genes encoding ribosomal RNA, located in the secondary constriction.

Phenotype

The outlook appearance of a plant species for a given trait or characteristic e.g., plant height, flower colour, etc.

Ploidy

The number of chromosome complement in a cell of a plant is called ploidy.

Pollen grain

The microspores of seed plants. It germinates to form the male gametophyte.

Pollination

The process in which pollens are transferred from an anther to a receptive stigma.

Polyploid

A plant containing more than two copies of complete chromosome set is called polyploid and the condition is called polyploidy.

Progeny

The offspring obtained after the combination of one female and one male plant.

Prophase

It is the first stage of mitosis, during which the chromosomes condense and become visible with a light microscope.

Recombination

A process in which the pieces of one chromosome cross overs and recombine to with another chromosome. The new chromosome is called recombinant chromosome and this process is called recombination**.** (Figure 8)

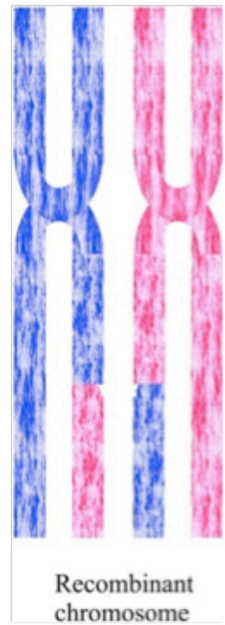

Fig. 8 The recombinant chromosomes resulted from over crossing of chromosomal segments from two different species.

Somatic cells

All the cells of the plant body except the reproductive cells i.e., pollens and eggs.

Telomere

The structure at the tip or end of the chromosome is called telomere.

Telophase

It is the final stage of mitosis or meiosis during which the chromosomes of daughter cells are grouped in new nuclei.

Unilateral sexual polyploidization

The plants obtained after pollination of one parent contributes diploid (2*n*) gametes with other parent contributing only haploid gametes (*n*). In the case the resultant progeny would be triploid (3x).

NALS Officers, Directors and Committees 2010

Officers

Terms expiring at the end of the Annual Meeting 2011
President: Larry Diehl
1st Past President: Delores Nelson
2nd Past President: Linda Smith
Canadian Vice President: Ieuan Evans
U.S. Vice President: Marianne Casey
Executive Secretary: Stephanie Sims
Treasurer: Vacant
Secretary: Sandy Venton
Executive Committee:
The Executive Committee includes the five elected Officers, together with the preceding elected presidents.

Board of Directors

Terms expiring 2011
Ron Chiabotta, Katharina Durand, Robert Jonckheere, Warren Summers

Terms expiring 2012
Richard Bayerl, Brian Bergman, Bruce Richardson, Jan Stein

Terms expiring 2013
Kathy Andersen, Tricia Kincheloe, Deborah Petrie, Jocelyn Thayer

Committees

The Presidents' Policy and Procedure Committee

Chair: Warren Summers
Members: All past and present Presidents and all past and presents Vice Presidents.

Publications Committee

Yearbook Editor: Calvin Helsley
Members: Maureen Barber, Larry Diehl, Stephanie Sims
Quarterly Bulletin Editor: Larry Diehl
Quarterly Bulletin Color: David Sims
Yearbook Indexing: L. Maureen Barber
Web-master: Richard Bayerl

Research Trust Committee
Chair: Arthur Evans
Members: Kathryn Andersen, Warren Summers, Robert Gilman, Jim Ault, Larry Diehl

Nominating Committee
Chair: Charlie Kroell
Members: Margaret Driver, Ed Czarnecki, Kevin Fry, Joe Nemmer

Educational, Meeting, and Conferences
NALS 2010 Mid-Atlantic and Ohio Regional Lily Societies Show: 2010 Lily Odyssey - Excursion to Mars!. Show Chair: Marianne Casey

Publicity and Public Relations
Chair: Marianne Casey

Advertising
Chair: Joe Nemmer

Competition and Judging
Awards & Standards: Kathryn Andersen and Ron Chiabotta
Judges Training and Accreditation: Linda Smith
Judges Training Coordination: Kathryn Andersen and Ron Chiabotta
NALS Show Schedule: Kathryn Andersen
RHS Liaison: Kathryn Andersen

Other Services

Seed Exchange: Michael Homick and Paul Machado
Membership Chair: Stephanie Sims
Historian and Archivist: Michael Homick and Paul Machado
Regional Representative Coordinator: Francine Nelson
Library, Slides, and Round Robin: Janet E. Vinyard
Lily Popularity Poll: Katharina Durand
NALS Medal Conservator: Carolyn Hawkins
Bookkeeper: Deborah Youngwirth, CPA

Regional Society Presidents

Alberta Regional Lily Society: President - Kevin Frey
Canadian Prairie Lily Society: President - Margaret Driver
Garden Club of Virginia: Lily Test Chairman - Mary Nelson Thompson
Golden State Lily Society: President - Beatrice O'Keefe
Iowa Regional Lily Society: President - Randy Hull
Manitoba Regional Lily Society: President - Nigel Strohman
Michigan Regional Lily Society: President - Theresa Fodale
Mid America Lily Society: President - Dennis Fitzgibbons
Mid-Atlantic Regional Society: President - Marianne Casey
New England Lily Society: President - G. Robert Meyer
North Star Lily Society: President - Jo Ann Hall
Ohio Lily Society: President - Kathleen Higgins
Ontario Regional Lily Society: President - David Maltby
Ozark Regional Lily Society: President - Calvin Helsley
Pacific Northwest Lily Society: President - Linda Stirling
Potomac Lily Society: President - John Lydon
South Saskatchewan Lily Society: President - Joann Liebe
Victoria Lily Society: Co-Presidents - Bryce Fradley and Beth Ruskowski
Wisconsin Regional Lily Society: President - Sandy Schley-Zelm
Wisconsin-Illinois Lily Society: President - Woody Imberman

Affiliated Lily Societies

Species Lily Preservation Group: President - Barbara Small