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Introgression breeding through recombination in Oriental x Asiatic lily hybrids using *2n* gametes -a GISH analysis

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Introgression breeding through recombination in Oriental x Asiatic lily hybrids using *2n* gametes -a GISH analysis

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PREFACE

This is the report of my Major Thesis in plant breeding done at Plant Research International (PRI). This thesis is the last part of my study Plant Sciences and will finalize 5 years of student life. For this thesis I have chosen to make a combination between plant breeding and ornamental crops. I have especially chosen for this because these are the subjects I am most interested in and where I would really like to continue in after I graduate. In this thesis the important ornamental crop and also beautiful cut flower lily is used and multiple hybrids have been analysed for their genetic background and origination. This thesis project was done in 6.5 months and I have been guided and supervised by Dr. Ir. Jaap M. van Tuyl and Prof. Dr. Richard G.F. Visser.

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SUMMARY

Lilies are not only an important ornamental crop, but are also important for molecular cytogenetic research. In the past, hybrids have been made between the three most important lily sections, viz. the *Longiflorum* (L) section, the Asiatic (A) section and the Oriental (O) section, combining important horticultural traits and resistances. This was very promising and a great step forward was done by using unreduced or *2n* gametes and *in situ* hybridization techniques (such as FISH and GISH). These *2n* gametes have several advantages, because 1) F_1 sterility can be overcome, 2) intergenomic recombination can be accomplished and 3) genetic variation can be obtained instantly in the BC_1 generation. Using FISH and GISH these features can be made visible. All these developments together made research of interspecific lilies come as far as it is right now.

In this thesis a BC_1 and BC_2 population of Oriental x Asiatic lily hybrids is analysed by GISH. In this analysis chromosomes are counted, recombination determined and the restitution mechanism is recognised. What was found was that most BC_1 plants were triploid and all have been obtained via *first division restitution* (FDR). The BC_2 plants were found to be aneuploid, which was expected. All the results will be used for the understanding of the genetic background of lily hybrids and a part of the BC_1 will be used for the molecular marker project which started in January 2006.

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INTRODUCTION

1.1 The genus *Lilium*

Fig. 1 *Lilium*, lily

Lilies belong to the genus *Lilium* of the family Liliaceae. It is a monocotyledonous bulbous crop and is widely known as an important ornamental crop because of its wide variety in colours and shapes (Rhee, 2002). Lilies originate from the Northern Hemisphere, in Asia, Europe and North America. Wild species are found as far north as the Arctic Circle and as far south as the Philippine Islands and southern India (MacRae, 1998). The genome size of Liliaceae is one of the largest in the plant kingdom (Rhee, 2002). The lily plant naturally has 24 chromosomes (MacRae, 1998) (x=12) with 2 metacentric and 10 acro- or sub-acrocetric chromosomes (Rhee, 2002). The genus is divided into 6 sections of which three consist of our present cut flower cultivars (Barba-Gonzalez *et al.*, 2004). These three are:

a) *Leucolirion*, with Longiflorum hybrids (L – hybrids). These are the trumpet-shaped hybrids with white flowers and as the name already indicates they are "long flowered" (MacRae, 1998). Longiflorum hybrids have already been used in many crosses with Asiatic type lilies.

b) *Sinomartagon*, with Asiatic hybrids (A – hybrids). Asiatic hybrids are the most widely grown type of hybrid lilies around the world. Colours may vary from bright to soft and from white to all the warm shades (MacRae, 1998). Some of these hybrids are known to have a resistance against very important pathogens of lilies, *Fusarium oxysporum* (Straathof & Van Tuyl, 1994) and LMoV. This is not present in other sections.

c) *Archelirion*, with Oriental hybrids (O – hybrids). Since the beginning of the 1950s Oriental hybrids have been used in lily breeding (MacRae, 1990). They are known to be late-flowering plants with usually large and showy, bowl-shaped, flat or reflexed flowers. They usually have a sweet and strong fragrance. Flower colours can vary from white to pink or yellow. Most of these hybrids are resistant to

Botrytis elliptica (Barba-Gonzalez *et al.*, 2004) a pathogenic fungus that affect all lilies.

Lily species from each section have different valuable agronomic traits and crossing is therefore an important way of combining these traits. Crosses within a section are relatively easy to make, but problems occur when trying to make intersectional crosses. These impossibilities in making the intersectional crosses are mainly due to fertilization barriers.

1.2 Genetic aspects and polyploidization

1.2.1 Fertilization barriers and hybrid sterility

The problems in making interspecific hybrids can be due to two types of barriers, viz. pre-fertilization or post-fertilization barriers. In the first case, this is caused by poor pollen tube growth due to stigmatic incompatibility (Asano & Myodo, 1977a; Asano, 1980). Post-fertilization barriers result in seeds without endosperm or very small embryo's that abort in early stages (Myodo, 1975; Asano & Myodo, 1977b). To overcome these barriers there have been some major changes in lily breeding by introducing special pollination techniques such as cut-style and intra-stylar pollination together with embryo-culture, both for pre- and post-fertilization barriers (Van Tuyl *et al.*, 2002). These were successful techniques and resulted therefore in many new intersectional hybrids. Later, even more techniques were introduced to get round the fertilization barriers, such as mentor pollen and ovary- and ovule culture and embryo rescue (van Tuyl *et al.,* 1991) and even more intersectional lily hybrids were produced. In fig. 2 a crossing polygon is given with successful crosses produced at PRI by van Tuyl *et al.* (2002).

Especially Oriental x Asiatic hybrids are nowadays interesting, because of their possibility to combine resistance to *Fusarium oxysporum* and viral diseases from Asiatic hybrids and the resistance to *Botrytis elliptica* from the Oriental hybrids (Barba-Gonzalez *et al.*, 2004).

As said, in the genus *Lilium* many successful interspecific crosses have been made, but unfortunately are sterile. In most cases this hybrid sterility is due to low chromosome pairing and irregular chromosome segregation during meiosis (Asano, 1982). In order to

overcome this sterility, the use of chromosome doubling agents such as colchicine and oryzalin were introduced. Fertility became restored, but due to autosyndetic chromosome pairing, recombination between the parental genomes is impossible (Ramanna & Jacobsen, 2003). Another way of overcoming this barrier is the use of *2n* gametes (Van Tuyl *et al.*, 1989; Ramanna & Jacobsen, 2003).

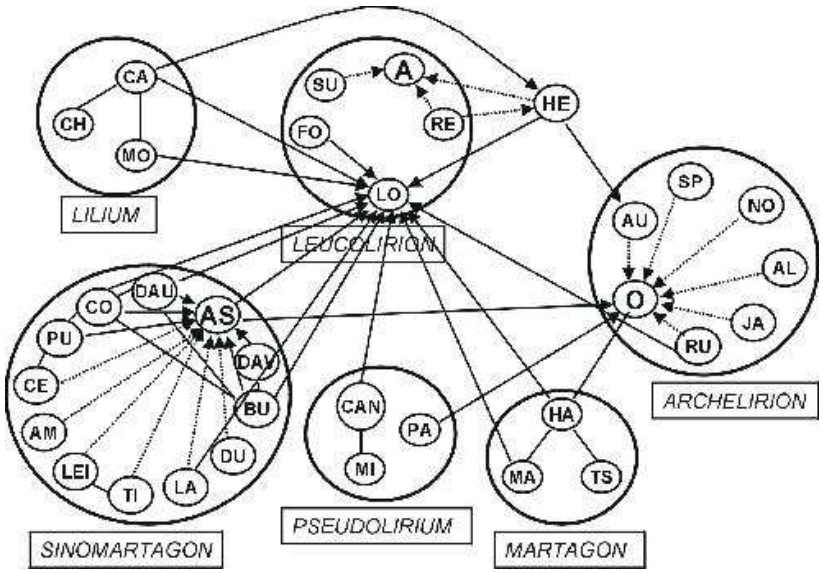

Fig. 2 Crossing polygon of the genus *Lilium* showing all successful intersectional crosses of the genes *Lilium* developed at Plant Research International, Wageningen University and Research Centre, the Netherlands. In this figure the connection between the Asiatic, Aurelian and Oriental hybrid groups (large ellipses) are shown by dotted lines. In successful crosses between species (small circles) of different sections (large circles) the arrows point towards the female parent. Abbreviations: A: Aurelian hybrids; AL: *L. alexandrae*; AM: *L. amabile;* AS: Asiatic hybrids; AU: *L. auratum*; BU: *L. bulbiferum*; CA: *L. candidum*; CAN: *L. canadense;* CE: *L. cernuum*; CH: *L. chalcedonicum*; CO: *L. concolor*; DAU: *L. dauricum*; DAV: *L. davidii*; DU: *L. duchartrei*; FO: *L. formosanum*; HA: *L. hansonii*; HE: *L. henryi*; JA: *L. japonicum;* LA: *L. lankongense*; LEI: *L. leichtlinii*; LO *L. longiflorum*; MA: *L. martagon*; MI: *L. michiganense*; MO: *L. monadelphum;* NO: *L. nobilissimum*; O: Oriental hybrids; PA: *L. pardalinum*; PU: *L. pumilum*; RE: *L. regale*; RU: *L. rubellum*; SP: *L. speciosum*; SU: *L. sulphurenum*; TI: *L. tigrinum*; TS: *L. tsing-tauense.* (Van Tuyl *et al.*, 2002; www.liliumbreeding.nl).

1.2.2 Use of 2n gametes

"Unreduced" or "2n" gametes are gametes which have a somatic chromosome number. They have already been discovered in the early '40s and have been attributed to be the origin of polyploid plant species (Ramanna & Jacobsen, 2003). These polyploids, originated by means of *2n* gametes are denominated sexual polyploids and because their occurrence was thought to be sporadic, their use for the production of polyploids was disregarded. Artificially induced polyploids, by means of chemicals such as colchicine and orazylin, were therefore preferred. Problem though, is that the artificially induced

polyploids have fixed heterozygosity and are not able to create large genetic variation (fig. 3). As discussed before, this is due to autosyndetic chromosome pairing in the allopolyploids resulting from the mitotic polyploidization.

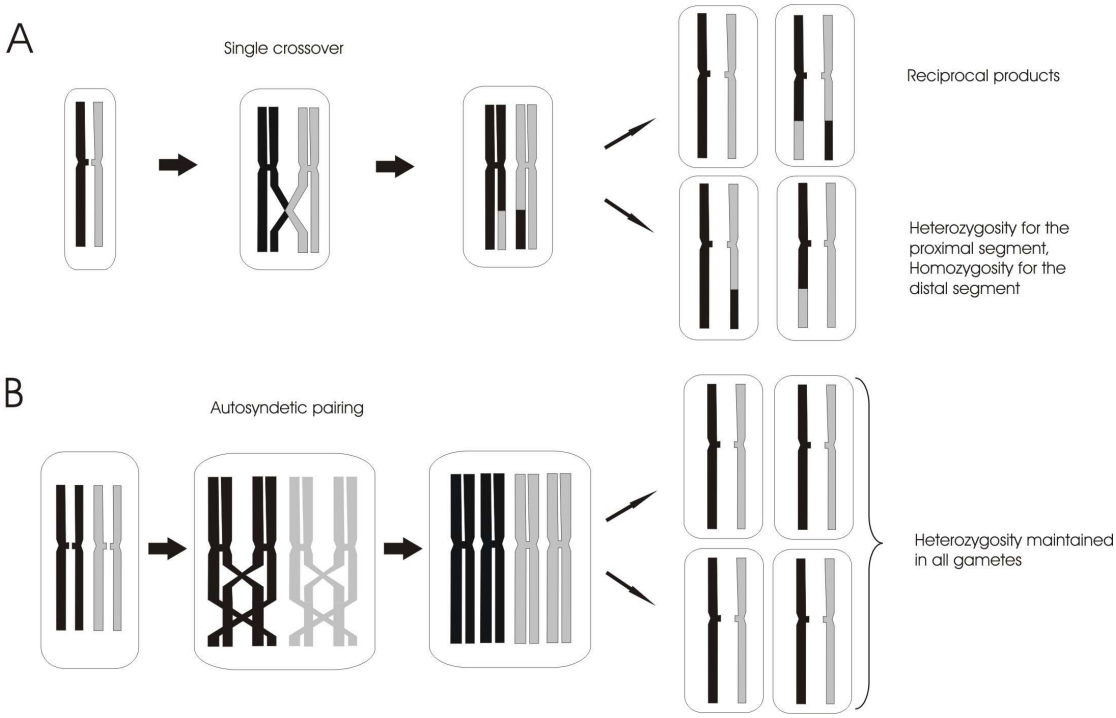

Fig. 3 (**a**) Consequences of single crossover in OA lily hybrid after sexual polyploidization with FDR (**b**) Somatically doubled allotetraploid showing autosyndetic chromosome pairing resulting in no intergenomic recombination.

Sexual polyploids, on the contrary, are much more interesting for breeding because intergenomic recombination between the parents can be present (fig. 3). A second advantage of *2n* gametes is the ability to achieve introgression due to this recombination (Van Tuyl *et al.*, 2002). It was actually only much later when Harlan & de Wet (1975) showed that the occurrence of *2n* gametes may be sporadic, but does occur in all plant species (Bretagnolle & Thompson, 1995). Since then, for a few crops, *2n* gamete introgression breeding has been successfully applied, for example in *Medicago* (Veronesi *et al.*, 1986), *Primula* (Skiebe, 1958) and *Solanum tuberosum* (Mendiburu & Peloquin, 1971).

Even though the occurrence of *2n* gametes appeared to be regular, it still can be very hard to detect them. Various ways of detecting the *2n* gametes have been used, such as

pollen size examination (Ramanna, 1983), flowcytometry (Van Tuyl *et al.*, 1989) and progeny analysis (Bingham & McCoy, 1979). The production of *2n gametes* is thought to be controlled genetically, but because it is also influenced by the environment (Ramanna & Jacobsen, 2003) plants which produce *2n* gametes may not always give consistent results. It is also possible to increase or induce *2n* gametes, for example by genetic selection, high solar level, low temperature, heat, caffeine treatments of immature flower buds or N_2O treatments (Barba-Gonzalez *et al.*, 2006; Lim *et al.*, 2005).

The origin of these *2n* gametes can be found in a deviating meiosis. This process is called meiotic nuclear restitution and can occur during either micro- or megasporogenesis. There are different types of restitution mechanisms recognized and the two most important mechanisms are called first division restitution (FDR) and second division restitution (SDR). The differences in these mechanisms are found in the meiotic stage of which nuclear restitution occurs. Besides these, a third mechanism called indeterminate meiotic restitution (IMR) was found in lily hybrids by Lim *et al.* (2001).

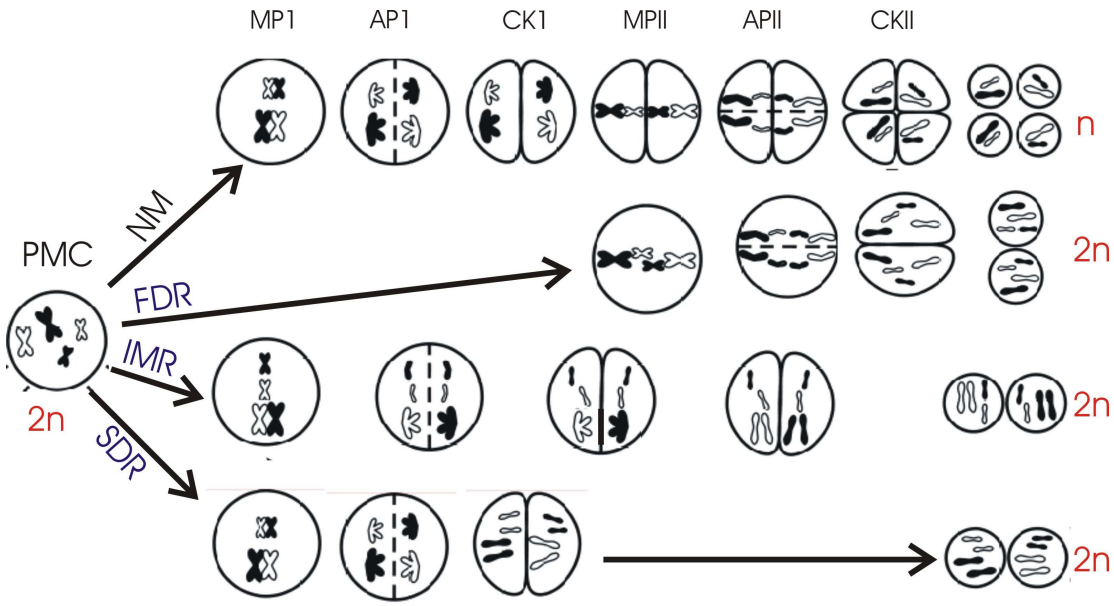

Fig. 4 Schematic overview of meiotic restitution. PMC: Pollen Mother Cell, NM: Normal Meiosis, FDR: First Division Restitution, IMR: Intermediate Meiotic Restitution, SDR: Second Division Restitution, MPI&II: Metaphase I&II, API&II: Anaphase I&II, CKI&II: Cytokinesis I&II.

In FDR, the entire chromosome complement is divided equationally before the cell is in a telophase I and cytokinesis stage and then a dyad is formed (fig. 4) (Ramanna & Jacobsen, 2003). In SDR, first meiotic division occurs normally up to telophase I and a

dyad is formed. A problem arises then in the second division. The chromatids do divide, but the nuclei restitute in each of the two cells of a dyad (fig. 4) (Ramanna & Jacobsen, 2003). The third mechanism, also known as indeterminate meiotic restitution (IMR), is actually a combination of both FDR and SDR (Lim *et al.*, 2001). In this mechanism some of the univalents divide equationally during the first meiotic division (as in FDR) and some bivalents disjoin reductionally before telophase I (as in SDR) (fig. 4) in a single division and restitute. The meiotic process and the three restitutional mechanisms are shown in fig. 4.

All three mechanisms are responsible for the production of *2n* gametes, but the genetic consequences are different (Ramanna & Jacobsen, 2003). As can be seen in fig. 4 FDR gametes will be identical to each other and to the mother cell, when no recombination occurs. But if recombination does occur, then for each recombinant chromosome heterozygosity can be maintained for the part of the chromosome which is proximal to the centromere and the first crossover. Looking at the SDR mechanism, gametes can also be highly heterozygous, but the chromosomes need to be assorted randomly and the gamete consists of chromosomes from both parents in different proportions. If recombination is present, heterozygosity is maintained for the part of the chromosome distal to the 1st crossover (in the recombinant chromosomes). In the case of IMR a part of the chromosome complement divides equationally (as in FDR) and the other part of the chromosomes disjoin normally as in SDR (fig. 4) (Ramanna & Jacobsen, 2003). This implies that the parental gene combination is largely preserved in chromosomes that divide equationally (i.e. FDR) and genetic variation occurs for those pairs of chromosomes that disjoin and divide (as in SDR) due to crossing-over as well as chromosome assortment. As a result of this, in the case of IMR homozygosity can be achieved for both proximal as well as distal regions of chromosomes of the complement. The net result of IMR will be different from both FDR as well as SDR. All three mechanisms are responsible for *2n* gamete production and can result in different degrees of recombination.

1.2.3 Detecting chromosomal recombination by GISH

Genomic *in situ* hybridization (GISH) is an effective technique for detecting chromosomal recombination. GISH allows the direct visualization of DNA, or parts of chromosomes, by hybridizing a labelled DNA probe to the DNA of the target chromosomes. When the DNA from one of the parents is used as a probe, it can be used to identify the chromosomes

from that specific parent in a hybrid (Schwarzacher & Heslop-Harrison, 2000). With the use of special staining techniques this can be made visible and it is possible to discriminate between the two parents and count the number of recombinant events. In lily this technique has already been used intensively and many studies on chromosomal behaviour and recombination have already been done. In this research we will use this same kind of technique for the analysis of recombination.

1.3 Current developments: Innovative molecular breeding techniques for resistance breeding in lily

One of the current developments in lily breeding is nowadays the introduction of molecular breeding techniques for a successful incorporation of disease resistances in commercially successful lily cultivars. This has been fitted into a new project which started in January 2006 by Plant Research International in cooperation with the Dutch breeding companies De Jong Lelies, Vletter and Den Haan. Also researchers from the National Institute for Horticultural Research in Korea are involved in this project.

Because many important lily cultivars lack resistance to *Fusarium oxysporum*, Lily Mottle Virus (LMoV) and *Botrytis elliptica*, several ways of introducing these resistances have already been developed. But because it takes a long time before resistance symptoms become visible, fast selection methods are needed, but are not yet available. In order to improve this, molecular markers are an excellent way of increasing the speed of the process. Here fore, highly valuable lily material will be tested for their resistance against pathogens by means of earlier developed techniques. The genetic inheritance of resistance will then be analysed by 2 new DNA marker technologies, NBS-profiling and DArT and will finally be visualized by chromosome painting (GISH). With these techniques it should be possible to detect which fragments are transferred to the progeny and which fragments might contain the genes carrying the resistance. The DNA markers will be converted into PCR markers, which can easily be applied in lily breeding and resistant plants can then be selected fast and efficiently.

This DNA marker technology is an innovative way of analysing plant material and has not yet been used in ornamental bulbous crops before. This project has therefore not only

scientific importance, but also commercial breeding companies can benefit greatly from this research.

RESEARCH PROJECT

2.1 Accomplished research

In the eighties, extensive research has been done on hybrids between *Longiflorum* x Asiatic lilies (LA hybrids) by Asano and their backcross derivatives. This appeared to be very successful, because intersectional crosses succeeded and horticultural traits could be combined resulting in a faster growth and bigger flowers. Now many commercial LA-cultivars are on the market (more than 1000 ha in the Netherlands).
In 1998, then an even more important breakthrough was the ability to perform GISH analysis on LA hybrids (Lim *et al.*, 1998). This made it possible to discriminate the chromosomes of the different genomes in the hybrids and their progenies and it was shown that mitotically doubled hybrids were not able to create intergenomic recombination, whereas sexually doubled hybrids did have intergenomic recombination. It also became possible to predict which restitutional mechanism was responsible for the production of the *2n* gametes. Besides this and perhaps even more important was that another restitution mechanism was detected called indeterminate meiotic restitution (IMR), besides FDR and SDR (Lim *et al.*, 2001). Finally this GISH analysis appeared to be very useful and is therefore also used in other lily hybrid analyses.

In 1994 a cooperative project with the Dutch Lily Breeding Companies was started in which Oriental x Asiatic groups of lilies (OA hybrids) were obtained. These hybrids were not able to be produced until then, but are again very interesting. This is because of the possibility of combining the 2 biggest hybrid groups with their desirable horticultural traits and resistances to different pathogens. More or less 1000 seedlings were produced under which the OA hybrids 951502-1 and 952400-1, which were able to produce *2n* gametes in a high frequency. These and many others were then used to produce the first allotriploid AOA (3x) hybrids by backcrossing with the Asiatic group of lilies (AA). Besides these also mitotically doubled OA hybrids were used. This was very successful, but again the AOA hybrids obtained by mitotically doubled OA hybrids appeared to have no intergenomic recombination, because of autosyndetic chromosome pairing in the parental allotetraploids induced as a result of the mitotic polyploidization (fig. 3).

Then in 2003, Barba-Gonzalez used both mitotically doubled OA hybrids as the sexually doubled OA hybrids 951502-1 and 952400-1 to produce AOA hybrids after backcrossing with AA. Then reciprocal crosses (3x - 2x and vice versa) were made with these new AOA hybrids and both progeny as well as the parents were analysed by GISH. This research confirmed that there is a difference in ploidy level of the progenies of the 3x – 2x crossings and their reciprocals. Also recombination was found in the gametes produced by these F_1 OA hybrids, when the AOA hybrid was derived from sexual polyploidization (951502-1 and 952400-1), but (almost) not through mitotic polyploidization. In only two cases recombination was found in triploid hybrids which were achieved by mitotic polyploidization.

Latest research done at PRI by Barba-Gonzalez (2006) was the induction of *2n* gametes by a treatment of laughing gas (N_2O). In here, hybrids which were not able to produce *2n* gametes before suddenly did produce *2n* gametes (viz. 951301-5 and 969023-2). This was not demonstrated before and, obviously, opens new prospects in further lily breeding.

Furthermore an interesting discovery was made in genotype 022171-1 (Barba-Gonzalez *et al.*, 2005c (in press in Euphytica)). This genotype originated from a cross between AA x AOA and after GISH analysis it was shown that only one Oriental chromosome was introduced into its genome. This was not yet observed in other crosses before and new crosses with this genotype have been made. This type of monosomic addition of alien chromosomes can be useful in genetic mapping. All these results are very interesting and should be used in further development of lily breeding.

2.2 Thesis objectives

The first objective of this thesis is the analysis of the allotriploid, AOA population 022605 chosen for molecular marker analysis (described in chapter 1.3). As was pointed out earlier, in Barba-Gonzalez (2005b) crosses have been made with genotype 951502-1 (OA) as a male parent and a progeny (022605) is obtained. Some of this 022605 population is already analysed (Barba-Gonzalez *et al.*, 2005b) and ploidy level, number of chromosomes from O and A genomes and the number of recombinant chromosomes have been determined.

The second objective of this thesis is the analysis of AOA-hybrids derived from new 2n-producing OA-hybrids. Besides genotype 951502-1, another important OA hybrid has been discovered which is able to produce *2n* gametes at a relative high frequency (viz. 969023-2) after it was treated with laughing gas (N_2O) (Barba-Gonzalez *et al.*, 2005d). It seemed that treating the plants with N_2O results in an induction of *2n* gametes and with this genotype crosses have been made. So far nothing is yet known of this progeny. The progeny, populations 042978, 044118, 044194 and 044224, will therefore be analysed and the ploidy level, the number of O and A chromosomes and the percentage of recombination will be determined. Information and knowledge about these crosses is very important and the results will be used for further breeding purposes.

The last part of this thesis involves the analysis of some BC_2 populations (042616, 042620 and 042622). These populations have all been obtained by backcrossing three genotypes of the 022605 population to a diploid Asiatic cultivar. An overview of these BC_2 populations can be found in fig. 5. These BC_2 populations will be analysed to gain knowledge about the inheritance and transmission of recombinant chromosomes.

MATERIAL AND METHODS

3.1 Plant material

A BC_1 population and a BC_2 population have been analysed for their chromosome constitution. The BC_1 population (022605) was already produced at PRI (Barba-Gonzalez *et al.*, 2004) and has resulted from a cross between the diploid Asiatic cultivar "Amarone" as female parent and *2n* pollen producing OA hybrid 951502-1 as male parent. This population has especially been chosen for the marker project described in *1.3*. Besides these also two other BC_1 genotypes have been analysed, viz. 044194-2 and 044224-3. These genotypes have been obtained by a cross between the female parent 980072 and the male *2n* pollen producing OA hybrid 969023-2.

The BC_2 populations (042616, 042620 and 042622) which are analysed are obtained by crosses between triploid AOA hybrids from the BC_1 population described above as female parent and Pollyanna as a male parent. A more detailed overview is given in fig. 5.

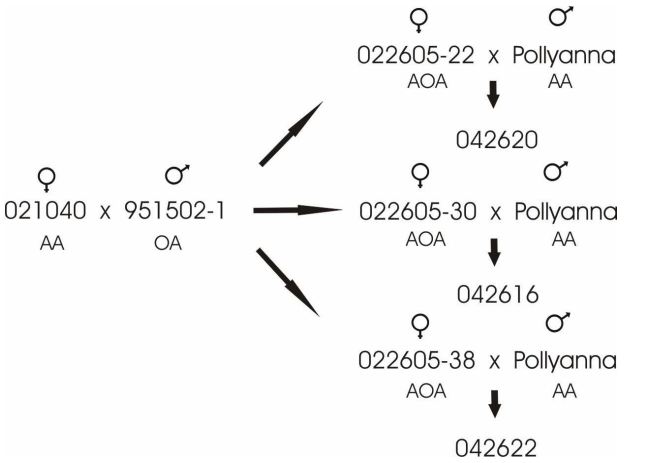

Fig. 5 BC_2 populations (042620, 042616 and 042622) and their parents.

All the bulbs have been grown in a greenhouse and transferred to tissue culture for GISH analysis under standard climate conditions.

3.2 Propagation of the plant material

Propagation of the bulbs was done the following way. The bulbs were washed thoroughly, peeled and again washed only this time with water and soap. After this the bulb were sterilized in 2% NaOCl for 30 minutes. The parts were then washed 3 times in sterilized water and cut in small pieces. Finally the parts were planted in propagation medium. The

medium consisted of 2.2 g/l MS salts (+MS vitamins), 50 g/l sucrose and 4 g/l gelrite. The pH was adjusted to 5.8.

The plants which were already in tissue culture were simply transferred to new propagation medium. Hereby every individual bulb scale was planted out in a tube. The medium used for this propagation is the same as described above. Both methods were used in order to grow root tips needed for the GISH analysis.

3.3 Mitotic chromosome preparation

Root tips were collected and incubated in 0.7 mM cyclohexamide for 5 to 6 h and then fixed in ethanol - acetic acid solution (3:1) and stored at 4°C until further use. The root tips were then incubated in a pectolytic enzyme mixture containing 1% (w/v) cellulose RS and pectolyase Y23 in 2 mM citrate buffer (pH 4.5) at 37°C for about 1 – 1.5 h depending on the size of the root tip. Squash preparations were made in a drop of 45% acetic acid and frozen in liquid nitrogen; the cover slips were removed by using a razor blade. Slides were dehydrated in absolute ethanol and air-dried.

3.4 DNA probe preparation

Genomic DNA from the Oriental cultivar “Sorbonne” and the Asiatic cultivar “Connecticut King” was isolated and respectively used as probe and block. For the GISH analysis the probe was boiled for 10 min to obtain a DNA size of 1 – 10 kb and the block DNA was boiled for 30 min. (100 bp – 500 bp). The probe was then labelled with fluorescein high prime by heating for 10 min at 70 °C and immediately chilled on ice for 5 min. Labelling was done according to the instructions of the manufacturer (Roche, Germany).

3.5 *In situ* hybridization

Slides were incubated at 37°C in RNase A with a concentration of 100 µg/ml for 1 h, and incubated in pepsin (5 µg/ml) for 10 min, followed by 4% paraformaldehyde for 10 min at room temperature. Between every step the slides were briefly washed in 2X SSC and finally dehydrated with 70%, 90% and absolute ethanol for 3 min in each and air-dried. Hybridization followed using a hybridization mixture consisting of 2X SSC, 50%

formamide, 10% dextransulphate, 0.25% SDS, 1.25 ng of probe and 0.5 µg of block DNA per slide. The hybridization mixture was heated at 70°C for 10 min and then placed on ice for at least 5 min. For each slide 40 µl hybridization mixture was used. The preparations were denatured at 80°C for 5 min and incubated overnight at 37°C in a humid chamber. Slides were washed 3X at room temperature in 2X SSC for 5 min and then washed in 0.1X SSC for 30 min at 42°C. Here after the slides were again washed for 3X in 2X SSC for 5 min each. Chromosomes were counterstained with 1 mg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector laboratories, Burlingame, CA) was added for its examination under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescent illumination, filter sets of DAPI and FITC. Images were captured by a Photometrics Sensys 1,305 x 1,024 pixel CCD camera and processed with Genus Image Analysis Workstation software (Applied Imaging Corporation). The DAPI images were sharpened with a 7x7 High Gauss spatial filter and pseudo-coloured in blue. The probe fluorescent was green pseudo-coloured. Optimal brightness and contrast were achieved by Adobe Photoshop image processing.

RESULTS

4.1 Ploidy levels of BC_1 and BC_2 progeny

All BC_1 and BC_2 plants have been analysed with GISH for their ploidy level, the number of chromosomes from O and A genomes and the number of recombinant chromosomes. Out of 14 BC_1 plants that were analysed, 13 triploid and 1 tetraploid was found (table 1). By GISH it was possible to identify the chromosomes of the parental genomes and also

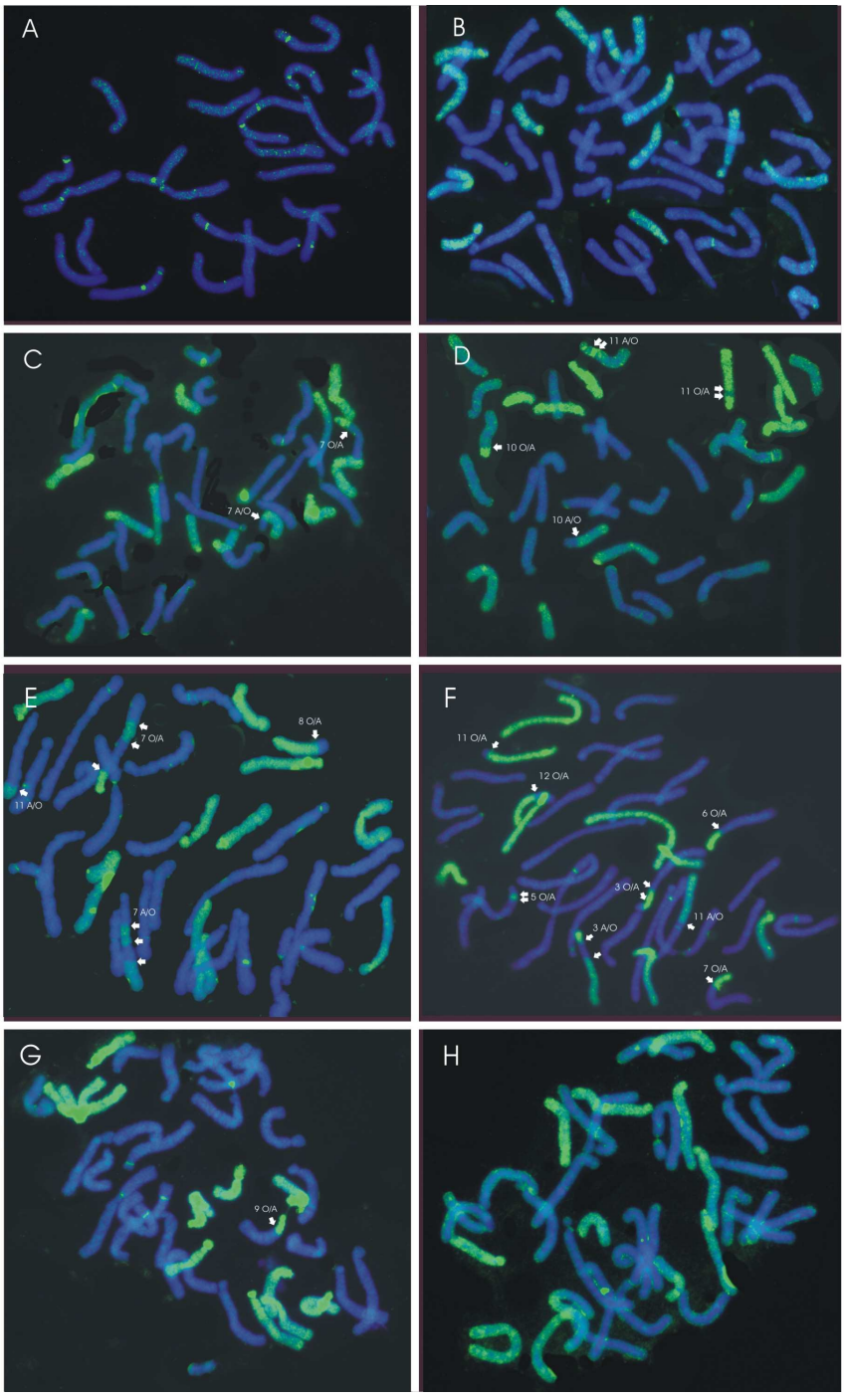

Fig. 6 GISH images from BC_1 progenies. Oriental DNA is detected with FITC signal (green) and the Asiatic DNA is counterstained with DAPI (blue). The arrows indicate recombinant segments and the recombinant chromosomes are mentioned either as O/A or A/O. (**a**) the diploid 022538-12-1 showing 24 A, (**b**) the tetraploid 022605-10 showing 12 O + 36 A, (**c**) the triploid 022605-11 showing 12 O + 24 A with 2 recombinant chromosomes, (**d**) the triploid 022605-16 showing 12 O + 12 A with 4 recombinant chromosomes, (**e**) the triploid 022605-30 showing 12 O + 25 A with 4 recombinant chromosomes, (**f**) the triploid 022605-35 showing 12 O + 24 A with 8 recombinant chromosomes, (**g**) the triploid 022605-37 showing 12 O + 24 A with 1 recombinant chromosome and (**h**) the triploid 044224-3 showing 12 O + 24 A.

the recombinant segments (fig. 6). Within the 13 triploids most genotypes contain 12 O and 24 A chromosomes, except for one remarkable genotype in where one extra Asiatic chromosome was present (022605-30). This is therefore called an aneuploid (3x+1). Also two triploid genotypes were found which seemed to have a lower number of A chromosomes (022605-41, 022605-44), but unfortunately the exact number of A chromosomes could not yet be determined and it could therefore not be said whether these are aneuploid triploids. Also one tetraploid (022605-10) has been found and originated due to a *2n* egg cell. This genotype contained 12 O and 36 A chromosomes as expected.

Table 1 The ploidy level, genomic composition and number of recombinant chromosomes of the progeny plants from 2x – 2x (*2n*) (BC_1) and 3x (*2n*) – 2x (BC_2) crosses analysed through GISH.

Cross	Genotype	Parents		Ploidy level	Genome composition		Number of recombinant chromosomes
		Female	Male		O ($^{O}/_{A}$)	A ($^{A}/_{O}$)	
	BC_1						
***2x - 2x** (2n)*							
AA x OA	022605-10	021040	951502-1	4x	12	36	0
AA x OA	022605-11	021040	951502-1	3x	12(1)	24(1)	2
AA x OA	022605-16	021040	951502-1	3x	12(2)	24(2)	4
AA x OA	022605-20	021040	951502-1	3x	12(4)	24(2)	6
AA x OA	022605-22	021040	951502-1	3x	12(1)	24(1)	2
AA x OA	022605-28	021040	951502-1	3x	12(1)	24	1
AA x OA	022605-30	021040	951502-1	3x+1	12(2)	25(2)	4
AA x OA	022605-35	021040	951502-1	3x	12(6)	24(2)	8
AA x OA	022605-37	021040	951502-1	3x	12(1)	24	1
AA x OA	022605-38	021040	951502-1	3x	12(1)	24(1)	2
AA x OA	022605-41	021040	951502-1	3x	12	22?	0
AA x OA	022605-44	021040	951502-1	3x	12(1)	22?(1)	2
AA x OA	044194-2	980072	969023-2	3x	12	24	0
AA x OA	044224-3	980072	969023-2	3x	12	24	0
	BC_2						
3x** (2n) - **2x							
AOA x AA	042616-2	022605-30	Pollyanna	2x+5	5(1)	24(2)	3
AOA x AA	042616-4	022605-30	Pollyanna	3x-3	8	25	0
AOA x AA	042620-3	022605-22	Pollyanna	3x-1	11(1)	24(1)	2
AOA x AA	042620-14	022605-22	Pollyanna	3x-1	11(2)	24(1)	3
AOA x AA	042622-6	022605-38	Pollyanna	3x-2	11	23	0

? = could not yet be confirmed

Also for the BC_2 progeny the ploidy level, the number of chromosomes from O and A genomes and the number of recombinant chromosomes has been determined. From the 5 BC_2 plants 4 near triploids (ranging from 3x-3 to 3x-1) and 1 near diploid (2x+5) has been found (table 1). Also the number of chromosomes from O and A genomes were counted. Out of 5 BC_2 plants 3 contained the original number of A chromosomes, in this case 24. The other two (042616-4 and 042622-6) contained respectively 25 and 23 A. For the number of O chromosomes none of the 5 BC_2 plants contained the complete set of O chromosomes (12). Genotype 042616-2 contained even only 5 O chromosomes and 8 O was found in 042616-4. Genotypes 042620-3, 042620-14 and 042622-6 only lost 1 O chromosome leaving 11. Loss of some of the O chromosomes was expected to happen.

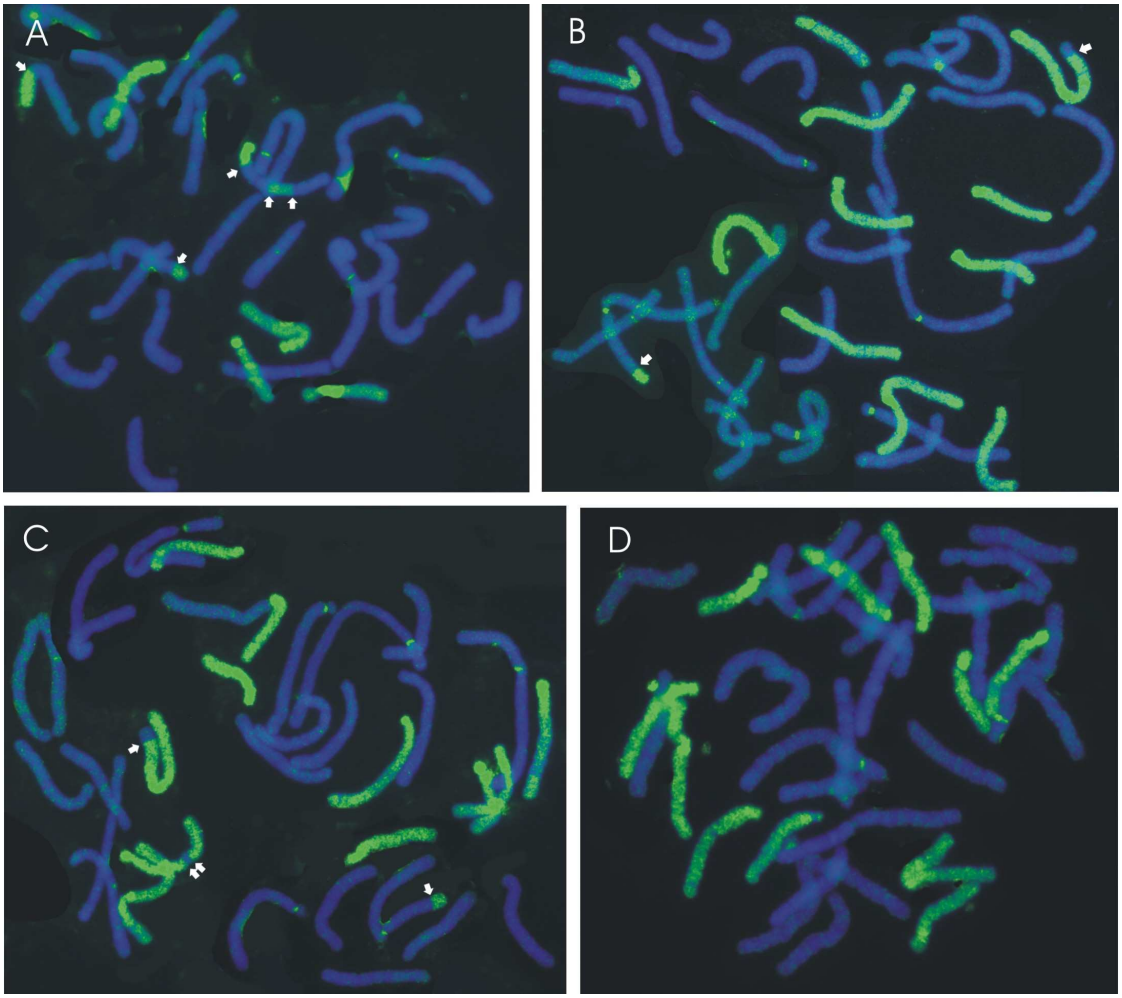

Fig. 7 GISH images from BC_2 progenies. Oriental DNA is detected with FITC signal (green) and the Asiatic DNA is counterstained with DAPI (blue). The arrows indicate recombinant segments. (**a**) the near diploid 042616-2 (2x+5) showing 5 O + 24 A and 3 recombinant chromosomes, (**b**) the near triploid (3x-1) 042620-3 showing 11 O + 24 A with 2 recombinant chromosomes, (**c**) the near triploid (3x-1) 042620-14 showing 11 O + 24 A with 3 recombinant chromosomes and (**d**) the near triploid (3x-2) 042622-6 showing 11 O + 23 A.

4.2 Homoeologous recombination

All 19 BC plants have been obtained by sexual polyploidization. From the 14 BC_1 plants, 10 plants (71%) possessed recombinant chromosomes (table 1) with a varying number from 1 to 8 recombinant chromosomes in different genotypes. A notable feature was found when 969023-2 was used as male parent (in genotype 044194-2 and 044224-3). This genotype was N_2O treated to induce the production of unreduced gametes and has not yet been analysed so far (Barba-Gonzalez, 2006). Here no recombinant chromosomes were found, but it should be noticed that this is only based on the analysis of 2 genotypes. When 021040 and 951502-1 (parents of other BC_1 progeny) were used the number of recombinant chromosomes in the progeny varied from 0 to 8. In most genotypes only one single crossover had occurred, except for 022605-16, 022605-30 and 022605-35. These have chromosomes with double or even more crossovers per chromosome (fig. 8). Also for BC_1 62.5% of the recombinant chromosomes are $^{O}/_{A}$ (= O chromosome with A introgression) and only 37.5% are $^{A}/_{O}$ (vice versa $^{O}/_{A}$). From the 5 BC_2 plants, 3 plants were found which have recombinant chromosomes (60%) varying from 2 to 3 (table 1). For the BC_2 plant no difference was found. Furthermore was noted that in general the larger chromosomes of the lily genome seemed to have fewer crossovers than the smaller ones. This was also already found in Barba-Gonzalez (2005b).

4.3 Chromosome constitution and intergenomic recombination

Both BC_1 and BC_2 plants have been analysed for the restitution method that has occurred based upon the GISH results. Also karyotypes have been made (fig. 8). In here the A chromosome obtained from the female parent is shown at the left side and the O and A chromosomes obtained from the OA hybrid used as male parent are shown in the middle and the right side of the karyotype.

BC_1

According to the GISH results, all the BC_1 plants have been obtained through FDR mechanism. This is based upon the fact that almost all plants have received full complement of 12 O + 24 A. All plants have received 12 individual O + A chromosomes from the *2n* gametes of the OA hybrids. This was found in all BC_1 plants except for genotypes 022605-10 (4x) and 022605-30 (3x+1). For genotype 022605-10 (4x), 12 O

+ 36 A have been found, where it received 12 O + 12 A from the OA hybrid, but also the full 24 A from the diploid Asiatic cultivar due to a *2n* egg cell. Because of the unaltered chromosome number, also a FDR must have happened. Genotype 022605-30 (3x+1) also originated as a result of FDR. In here, 1 bivalent probably lagged behind and did not separate normally during anaphase II (fig. 4) resulting in a 2n+1 and 2n-1 gamete. In this case the 2n+1 gamete fused with the haploid Asiatic gamete resulting in a 3x+1 plant.

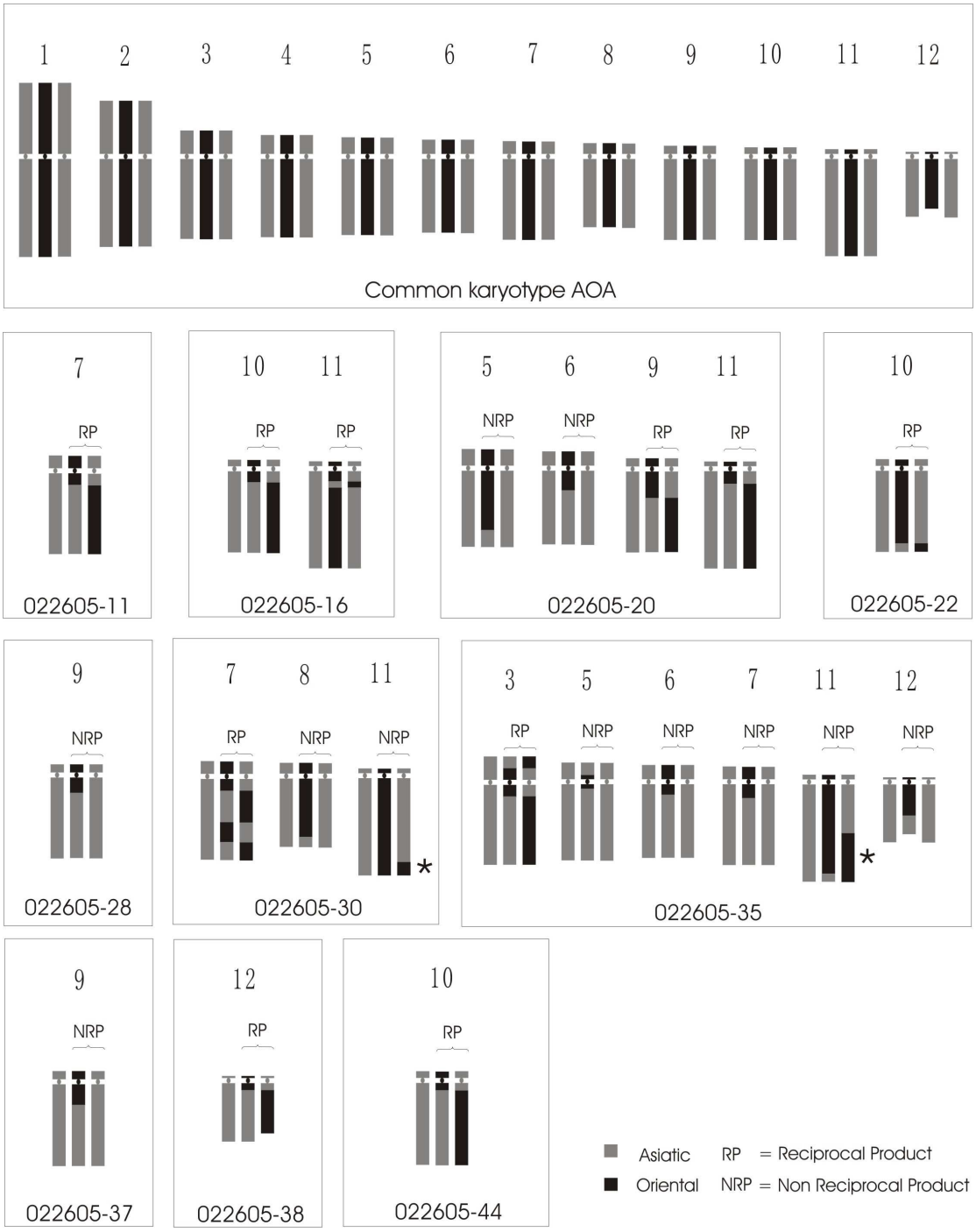

Fig. 8 Common karyotype of BC_1 (AOA) and 10 BC_1 progeny plants showing recombinant chromosomes.

For genotype 022605-41 and 022605-44 it was unfortunately not yet possible to determine the number of Asiatic chromosomes and it could therefore not be said which restitutional mechanism has occurred.

Another confirmation for FDR is that with a single crossover and FDR being the mechanism the non-sister chromatids may consist of reciprocal products ($^{O}/_{A}$; $^{A}/_{O}$), or non-reciprocal products (O, $^{A}/_{O}$ or A, $^{O}/_{A}$). This is shown in fig. 3. When IMR would have occurred, reciprocal recombination products can not be found. In 8 out of 10 BC_1 plants with recombinant chromosomes 1 or more of these reciprocal recombination products (marked as RP in fig. 8) were found and have therefore been obtained through FDR. Other cases have a combination of A, $^{O}/_{A}$.

Furthermore, in most of the BC_1 plants the recombinant chromosomes had single crossovers, but there were also cases of double crossovers (022605-16 and 022605-35) and even triple crossovers (022605-30) (fig. 8). Most likely these were two strand crossovers, but this can not be confirmed from the analysis of somatic chromosomes alone.

A complete list of the 022538 and 022605 population analysed so far (also by Barba-Gonzalez) is given in Appendix I.

BC_2

Also all BC_2 plants have originated through sexual polyploidization and therefore recombinant chromosomes were expected to be found (Barba-Gonazalez, 2005c (in press in Euphytica)). As said before, from 5 BC_2 plants 3 possess recombinant chromosomes, which most likely have occurred through multivalent chromosome pairing. But because this is not actually observed, bivalent pairing can also not be discarded. In most cases only single crossover events happened except for one chromosome in 042616-2 and one in 042620-14.

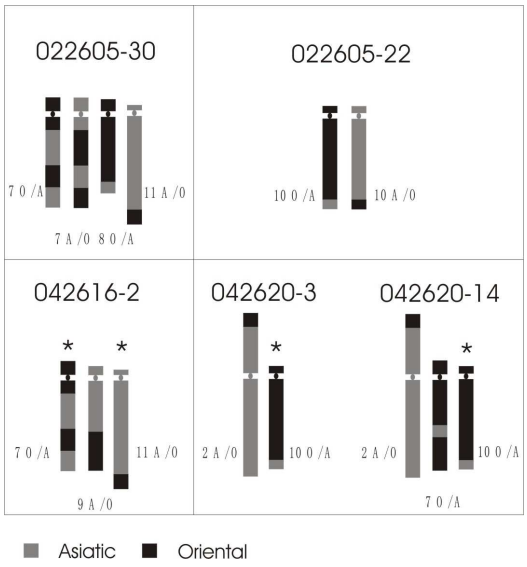

Fig. 9 Karyotype of BC_2 progeny plants showing recombinant chromosomes with corresponding female parent. Asterisks (*) show transmitted chromosomes, without further recombination, from BC_1 to BC_2.

4.4 Transmission of recombinant chromosomes

As said before, the O and A chromosomes were identified and recombinant chromosomes, either $^{O}/_{A}$ or $^{A}/_{O}$, were determined through GISH. Based on this, it was determined which of the recombinant chromosomes from the BC_1 were transmitted to their corresponding BC_2 progeny. From the BC_1, three parents were used to obtain the BC_2, viz. 022605-22, -30 and -38. Both 022605-22 and 022605-38 contain 2 recombinant chromosomes each whereas 022605-30 contains 4 recombinant chromosomes (fig. 9). It was found that in all analysed BC_2 plants at least 1 recombinant chromosome has been received from the AOA parent (fig. 9) except for 042616-4 and 042622-6 which do not have any recombinant chromosomes at all. From 022605-22, chromosome 10 $^{O}/_{A}$ has been transmitted to both its progenies (042620-3 and -14) and also three new recombinant chromosomes have been found in the progeny. From 022605-30 chromosomes 7 $^{O}/_{A}$ and 11 $^{A}/_{O}$ have been transmitted to one of its progeny (042616-2) and also one new recombinant chromosome was found. Second analysed progeny (042616-4) did not have any recombinant chromosomes at all. Also from 022605-38 none of the recombinant chromosomes was transmitted to its progeny, because it did not possess any recombinant chromosomes at all.

For a better estimation of the transmission of recombinant chromosomes from the BC_1 to the BC_2, analysis with FISH using the 45S rDNA signal has to been done. Also more progeny need to be analysed to make a good estimation of the segregation of the chromosomes.

DISCUSSION

In the past, much research has been done on the functioning of *2n* gametes and many obtained hybrids have been analysed by GISH. These *2n* gametes are found to be advantageous for several reasons. Not only sterility is overcome, but also intergenomic recombination can be accomplished and also so called "permanent hybrids", as is the case in allotetraploids produced by mitotically doubled hybrids, are circumvented. A notable feature is that genetic variation can already be obtained in the BC_1 progenies, because substitutions can be obtained for the recombinant segments (Barba-Gonzalez *et al.*, 2005b). For example, as shown in Fig.8, whenever the non-reciprocal product of recombination (NRP) of $^{0}/_{A}$ is present along with the A chromosome of the backcross parent, substitutions for the recombinant segments occur. In such cases the recessive loci of the backcross parent have the potential to express their phenotypes already at the BC_1 generation. After the ground-breaking LA hybrid work of Asano, van Tuyl, Lim and others, also the research of OA hybrids has proven to be very successful. Not only for commercial breeding purposes, but also for the understanding of genetic mechanisms. In this work the research of OA hybrids has been extended and is discussed below.

In the first part of this thesis AOA progenies have been analysed and conclusions have been drawn about intergenomic recombination and chromosome assortment. Looking at this intergenomic recombination in the AOA progenies a clear difference is found when the two OA hybrids 951502-1 and 969023-2 were used as male parent, donating a *2n* gamete. In the progeny, 71% of the BC_1 plants contain recombinant chromosomes with 951502-1 as a parent, whereas no recombination was found when 969023-2 was used as a parent. Unfortunately only two progenies have been analysed of 969023-2. Therefore conclusions can not yet be drawn about the functioning of 969023-2 as a parent and even more important, what the effect is of the N_2O treatments on the occurrence of recombination in progenies. In Barba-Gonzalez *et al.* (2006) it is already shown that recombination does occur in progenies obtained from N_2O treated parents and that N_2O does not induce chromosome doubling in pre-meiotic stages. Because of this it is expected that recombination will be found when more progeny is analysed. Another possibility for the difference in the occurrence of intergenomic recombination might be a genotypic variation. This is shown in Barba-Gonzalez *et al.* (2005b), where a

distinction is made between 951502-1 and 952400-1, both used as male parents. Here 79.1% of the BC_1 progeny contained recombinant chromosomes when 951502-1 was used as parent and only 35.7% when 952400-1 was used. Also genotype 952400-1 gives much fewer *2n* gametes then genotype 951502-1. This makes 951502-1 in this context, a better crossing parent than 952400-1. To be able to draw conclusions about the effect of the N_2O treatment on the occurrence of recombination in the progeny and the usefulness of 969023-2 as a parent, more progenies have to be analysed.

Besides creating more genetic variation, recombination between the different genomes can also be beneficial for molecular marker research. For making molecular maps the recombination between the linked loci is calculated and map distances are expressed as centimorgans. Unfortunately such maps do not represent where exactly the loci are physically located on the respective chromosome. To accomplish this, it is necessary to combine the molecular or genetic maps with the physical features of the chromosomes. For example, it is necessary to determine whether the loci are on the long or shortarm, how far away from the centromere or the secondary constriction or whether they are at the proximal or distal positions of the centromeres. Because crossover sites (or breakpoints) can be made visible with GISH as well as the centromeres in such a physical map, it becomes easy to determine whether the distance between a centromere and breakpoint is very close or very far away. This means, the larger the number of independent breakpoints are known for a particular chromosome, the more accurately the markers can be assigned to the chromosome regions and the more accurate the integrated map will be. As pointed out in chapter 1.3, the 022605 population is especially chosen and the number of crossover sites (breakpoints) can therefore be of great importance. Especially chromosome 11 is for example potentially useful, because several breakpoints have already been determined in six different genotypes.

As was shown earlier, we did find intergenomic recombination, but most likely no chromosome assortment has occurred. In FDR this was not expected to happen, because as a rule the sister chromatids move to the opposite poles in the equational division of the nucleus at meiosis. In the case of IMR this is different, because some of the half-bivalents disjoin and the two sister chromatids are included in one of the two restitution nuclei. This was however not observed in the results, but has been reported in similar progenies (Barba-Gonzalez *et al.*, 2005b). Only in one case (022605-30), where an aneuploid (3x+1)

was found chromosome assortment might be possible, but is not expected. In this case a bivalent probably lagged behind and did not separate normally during anaphase II, leaving a 2n+1 and 2n-1 gamete.

It was remarkable to find that all BC_1 plants originated through FDR, most containing crossovers. As was mentioned earlier both reciprocal products $^{O}/_{A}$ and $^{A}/_{O}$ and non-reciprocal products, O, $^{A}/_{O}$ or A, $^{O}/_{A}$ can be found. Most important feature here is the identification of $^{O}/_{A}$ chromosomes. This is important, because this can lead to substitution of chromosome segments, as was already pointed out above and can facilitate the expression of the recessive loci of the BC parent in nulliplex condition; in this case the Asiatic genome (aaa). For genetic variation this is very important. This is observed in genotypes 022605-20, -22, -28, -30, -35 and -37. In 022605-35 this was even found in 4 chromosome pairs. This type of genetic variation appeared to be important in producing interspecific polyploid hybrids cultivars of lily involving *Longiflorum* x Asiatic parents (Van Tuyl, unpublished results).

In view of what has been achieved in the case of *Longiflorum* x Asiatic hybrid cultivars, as pointed above, it is attractive to produce similar triploid cultivars involving Oriental x Asiatic lilies. In that case, it is imperative that the *2n* gamete producing OA hybrid has to be backcrossed to the Oriental hybrids (OO). This obviously gives rise to triploid BC_1 progenies in which substitutions for the recombinant segments of O genome can be achieved. This means, the wealth of desirable horticultural characteristics for the Oriental hybrids become available for breeding. In addition, the chromosome pair 11 in both 022605-30 and 022605-35 deserves a mention. In this case, an extra part of the Oriental genome is introgressed into the AOA hybrid (marked with asterisks in fig. 8). Such genotypes might open the possibilities for the introduction of chromosome specific traits into other genotypes, because these chromosomes can be more readily identified in the progenies.

For BC_2 progenies, unfortunately, only few results were obtained. This was due to a low occurrence of metaphase stages in the root tips used for chromosome preparations. The reason for this is not known. From the results which were obtained all BC_2 plants were found to be aneuploids and all varied in the number of O chromosomes. This was expected (in a range from x+1 to 3x-1), because of univalent pairing in the AOA parents

where the A genomes paired regularly, but univalents from O genome were disturbed or moved randomly to different poles and segregated randomly during meiosis. In these aneuploids, finding a near diploid with recombinant chromosomes is of special interest. This is because of the possibility that near diploid progenies, obtained from a 3x – 2x cross, might produce haploid gametes, eliminating all O chromosomes, but retaining some Oriental recombinant segments. These can then be more easily used for further breeding purposes and still retain introgression. In these results, 042616-2 is therefore interesting because it is a near diploid (2x+5), which contains 2 $^{A}/_{O}$ chromosomes. Most important feature in these near diploids is though that they result from aneuploid gametes that are still functional. Looking at the intergenomic recombination in all three analysed BC_2 progenies, in total 50% of the recombinant chromosomes were directly transmitted by the parents. Other 50% are newly obtained recombinations. Most remarkable might be the finding of recombination in two of the larger chromosomes (chromosome 2 in 042620-3 and -14). This is in contrast in to what was found by Barba-Gonzalez (2005b) where the larger chromosomes had fewer recombinations compared to the smaller chromosomes. Remark though, is that in Barba-Gonzalez BC_1 is considered and in this case BC_2. But there might be a difference between which chromosomes recombine in BC_1 and BC_2.

As shown, with *in situ* hybridization techniques already a lot is achieved and this will in the future probably even become more. It can give a lot of information about the origin and genetic background of many hybrids and these techniques can all be used in further breeding of new cultivars.

ACKNOWLEDGEMENTS

At the end of this thesis I have also come to the end of my study Plant Sciences at Wageningen University. Doing this thesis I have learned a lot and it has developed me further and extended my knowledge on many aspects. Also the techniques I have learned here at PRI will hopefully be of great use for me in my future work.

Therefore I would especially like to thank Dr. Ir. Jaap M. van Tuyl for giving me this opportunity and guiding me carefully. It has been a great pleasure to work with you and your group. Also I would like to thank Dr. M.S. Ramanna, who helped me greatly with difficult questions about the genetics of lily, sexual polyploidization and also how to write a scientific report. Thank you. Then Shujun Zhou, who taught me how to do the GISH work, helped me to analyse my material and he showed me how to draw critical conclusions. I know I disturbed you many times while you were writing your own PhD thesis, but it helped me a lot and I hope you can finish soon yourself. Also I would like to thank Rodrigo Barba-Gonzalez for critically reviewing my report. And then last but not least, Nadeem Khan, who helped me analyse the material and helped me to do lab work whenever I couldn't find the time or when I was busy writing. I hope your coming PhD project will be a success!

Thank you all very much and I wish you good luck in the future.

Martin Beers

LITERATURE

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

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

Asano, Y., Studies on crosses between distantly related species of Lilies. VI. Pollen-tube growth in interspecific crosses on *Lilium longiflorum*. J. Jpn. Soc. Hortic. Sci. 49: 392-396. 1980.

Asano, Y., Chromosome association and pollen fertility in some interspecific hybrids of *lilium*. Euphytica 31: 121 – 128. 1982.

Barba-Gonzalez, R., Lokker, A.C., Lim, K-B., Ramanna, M.S. and Van Tuyl, J.M., 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. 2004.

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

Barba-Gonzalez, R., Ramanna, M.S., Visser, R.G.F. and Van Tuyl. J.M., 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. 2005b.

Barba-Gonzalez, R., Van Silfhout, A.A., Visser, R.G.F., Ramanna, M.S. and Van Tuyl, J.M., Utilization of allotriploid BC_1 progenies of Oriental x Asiatic lilies (*Lilium*) in introgression breeding – an assessment based on GISH analysis. In press in Euphytica. 2005c.

Barba-Gonzalez, R., Miller, C.T., Ramanna, M.S. and Van Tuyl, J.M., Nitrous oxide (N_2O) induces *2n* gametes in sterile F_1 hybrids between Oriental x Asiatic lily (*Lilium*) hybrids and leads to intergenomic recombination. Euphytica (*Published online DOI: 10.1007/s10681-005-9032-0*). 2006.

Bingham, E.T., McCoy, T.J., Cultivated alfalfa at the diploid level: origin, reproductive stability and yield of seed and forgage. Crop Sci. 19: 97-101. 1979.

Bretagnolle, F., Thompson, J.D., Tansley Review No. 78 Gametes with somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol. 129: 1-22. 1995.

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

Lim, K-B., Karlov, G.I.., Khrustaleva L.I.,, De Jong, J.H. and Van Tuyl, J.M., Introgression of Interspecific Hybrids of Lily using Genomic In Situ Hybridization (GISH). Acta Hortic 508: 105-111. 1998.

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

Lim, K-B., Van Tuyl, J.M., Identification of parental chromosomes and the detection of ribosomal DNA sequences in interspecific hybrids of *Lilium* revealed by multicolor *in situ* hybridization. Acta. Hort. 570: 403-408. 2002.

Lim, K-B, Barba-Gonzalez, R., Zhou, S., Ramanna, M.S. and Van Tuyl, J.M. Meiotic Polyploidization with Homoeologous Recombination Induced by Caffeine Treatment in Interspecific Lily Hybrids. Korean J of Genetics. 27 (3): 219-226. 2005.

MacRae, E.A., American lily hybridising – an historical review: In Lilies and related plants, supplement 1990. P 5th Int. Lily Conf., London, July 1989. Roy. Hort. Soc-Lily. pp 29-40. 1990.

MacRae, E.A., Lilies: a guide for growers and collectors. Timber press. Portland, Oregon. 1998.

Mendiburu, A.O., Peloquin, S.J., High yielding tetraploids from 4x-2x and 2x-2x mating. Amer. Potato J. 48: 300-301. 1971.

Myodo, H., Successful setting and culture of hybrid embryo's between remote species of the genus *Lilium*. Lily Yearbook North Am. Lily Soc. 28: 13-26. 1975.

Plant Research International, Innovative molecular breeding techniques for resistance breeding in lily, Project proposal, Wageningen, 2005.

Ramanna, M.S., First division restitution gametes trough fertile desynaptic mutants of potato. Euphytica 32: 337-350. 1983

Ramanna, M.S. and Jacobsen, E., Relevance of sexual polyploidization for crop improvement – A review. Euphytica 133: 3 – 18, 2003.

Ramanna, M.S., Kuipers, A.G.J., Jacobsen, E., Occurrence of numerically unreduced (*2n*) gametes in *Alstroemeria* interspecific hybrids and their significance for sexual polyploidization. Euphytica 133: 95-106. 2003.

Rhee, H.K., Improvement of breeding efficiency in interspecific hybridization of lilies. 2002.

Schwarzacher, T., Heslop-Harrison, J.S., Reprobing of preparations in: Kingston F (ed), Practical *in situ* hybridization. Bios Scientific Publishers Limited. Oxford, UK. pp 110. 2000.

Skiebe, K., Die bedeutung von unreduzierten gameten für die polyploidezüchtung bei der Fliededprimel (*Primula melacoides* Franchet.). Züchter 28: 353-359. 1958.

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

Van Tuyl, J.M., De Vries, J.N., Bino, R.J., Kwakkenbos, T.A.M., Identification of *2n*-pollen producing interspecific hybrids of *Lilium* using flow cytometry. Cytologia 54: 737-745. 1989.

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

Van Tuyl, J.M., Chung, M-Y., Chung, J-D. and Lim, K-B., Introgression studies using GISH in interspecific *Lilium* hybrids of *L. longiflorum x Asiatic*, *L. longiflorum x L. rebellum and L. auratum x L. henryi*. North Am Lily yearbook 2002.

Veronesi, F., Mariani, A., Bingham, E.T., Unreduced gametes in diploid *Medicago* and their importance in alfalfa breeding. Theor. Appl. Genet. 72: 17-41. (1986).

Used internet sites:

- The *Lilium* information page (www.liliumbreeding.nl)

APPENDICES

Appendix I

BC_1 populations 022538 and 022605 analysed so far (also by Barba-Gonzalez).

Cross	Genotype	Parents		Ploidy level	Genome composition		Number of recombinant chromosomes
		Female	Male		O ($^{O}/_{A}$)	A ($^{A}/_{O}$)	
AA x OA	022538-1	Amarone	951502-1	3x	12(4)	24(3)	7
AA x OA	022538-3	Amarone	951502-1	3x	12(4)	24(2)	6
AA x OA	022538-5	Amarone	951502-1	3x	12(3)	24(2)	5
AA x OA	022538-7	Amarone	951502-1	3x	13(3)	23(3)	6
AA x OA	022538-8	Amarone	951502-1	3x	12(2)	24(2)	4
AA x OA	022538-9	Amarone	951502-1	3x	13(2)	23(2)	4
AA x OA	022538-14	Amarone	951502-1	3x	12(3)	24(2)	5
AA x OA	022538-15	Amarone	951502-1	3x	11(1)	25(3)	4
AA x OA	022605-1	Amarone	951502-1	3x	12(1)	24(1)	2
AA x OA	022605-3	Amarone	951502-1	4x	12(4)	36(3)	7
AA x OA	022605-7	Amarone	951502-1	3x	12(3)	24(3)	6
AA x OA	022605-8	Amarone	951502-1	3x	12(2)	24(1)	3
AA x OA	022605-9	Amarone	951502-1	3x	12(4)	24(3)	7
AA x OA	022605-10	Amarone	951502-1	4x	12	36	0
AA x OA	022605-11	Amarone	951502-1	3x	12(1)	24(1)	2
AA x OA	022605-13	Amarone	951502-1	4x	11(1)	37(1)	2
AA x OA	022605-15	Amarone	951502-1	4x	12	36	0
AA x OA	022605-16	Amarone	951502-1	3x	12(2)	24(2)	4
AA x OA	022605-20	Amarone	951502-1	3x	12(4)	24(2)	6
AA x OA	022605-22	Amarone	951502-1	3x	12(1)	24(1)	2
AA x OA	022605-28	Amarone	951502-1	3x	12(1)	24	1
AA x OA	022605-30	Amarone	951502-1	3x+1	12(2)	25(2)	4
AA x OA	022605-35	Amarone	951502-1	3x	12(6)	24(2)	8
AA x OA	022605-37	Amarone	951502-1	3x	12(1)	24	1
AA x OA	022605-38	Amarone	951502-1	3x	12(1)	24(1)	2
AA x OA	022605-40	Amarone	951502-1	3x	12(2)	24(2)	4
AA x OA	022605-41	Amarone	951502-1	3x	12	**22?**	0
AA x OA	022605-42	Amarone	951502-1	3x	12(1)	24(3)	4
AA x OA	022605-44	Amarone	951502-1	3x	12(1)	**22?(1)**	2