

**Intergenicomic recombination and introgression breeding
in Longiflorum x Asiatic lilies**

Shujun Zhou

Promotor: Prof. dr. Richard G. F. Visser, Hoogleraar in de plantenveredeling,
Wageningen Universiteit

Co-promotor: Dr. ir. Jaap M. van Tuyl, senior wetenschappelijk onderzoeker,
Plant Research International

Promotiecommissie: Dr. J. H. de Jong (Wageningen Universiteit)

Prof. dr. ir. E. Jacobsen (Wageningen Universiteit)

Prof. dr. L. H. W. van der Plas (Wageningen Universiteit)

Dr. Ir. R. C. Snijder (Royal van Zanten Flowerbulbs BV,
Hillegom)

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1 General introduction

Origin of lily cultivars

Lily, belonging to the genus *Lilium* (Liliaceae), is one of the most important cut flowers worldwide. The Netherlands is the main bulb producer with the acreage of around 4500 ha for lily cultivation. Lily is in the fourth position of the top ten cut flowers in the Netherlands (www.vbn.nl). *Lilium* consists of about 80 species which are distributed over the mountainous regions of the northern hemisphere, mainly in Asia, North America and Europe (Comber 1949, De Jong 1974). Taxonomically, the species of *Lilium* are classified into seven sections, i.e., *Lilium*, *Martagon*, *Pseudolirium*, *Archelirion*, *Sinomartagon*, *Leucolirion* and *Oxypetala* (De Jong 1974). The wild species ($2n=2x=24$) within each section are more or less crossable and the hybrids are fertile (McRae 1990, Van Tuyl et al. 2002a, b). Among the four important lily cultivar groups, viz., Longiflorum, Asiatic, Oriental, and Longiflorum x Asiatic hybrids, the former three groups originated from hybridization within *Leucolirion*, *Sinomartagon* and *Archelirion* respectively (Table 1-1).

- a) **The Longiflorum hybrids** originate from intra- or interspecific hybridization of *L. longiflorum* Thunb. and *L. formosanum* Wallace of the *Leucolirion* section. About 150 cultivars were selected from these hybrids (Leslie 1982-2005). The Longiflorum cultivars possess white trumpet-shaped flowers with distinctive fragrance. They are easily cultivated year-round (McRae 1990).
- b) **The Asiatic hybrids** are obtained from intra- or interspecific crosses among at least 12 species of the *Sinomartagon* section. Their cultivation can be traced to the early 1800s in Japan (Shimizu 1987). The Mid-Century hybrids, which were produced during the 1930s and early 1940s at Oregon Bulb Farms, the United States, were a great milestone in Asiatic hybrids (McRae 1998). About 4000 cultivars were selected from these hybrids (Leslie 1982-2005). They possess a wide range of colors (orange, yellow, white, pink, red, purple and salmon) with early to late flowering (Woodcock & Stern, 1950). An

important feature is that some of the species of this section possess resistance to *Fusarium* and viruses (McRae 1998).

- c) **The Oriental hybrids** are derived from the hybridization of five species of Archelirion. They have been used since the early 1950s in lily breeding (McRae 1990). Around 2000 cultivars have been registered since 1990 (Leslie 1982-2005). Oriental cultivars possess pink, white or yellow flowers with a sweet fragrance, and most of them are resistant to *Botrytis* (McRae 1998).

Table 1-1. An overview of the wild species involved in the origins of their groups of cultivars, together with their main characteristics.

Cultivars	Sections	Species	Description of main traits
Longiflorum	Leucolirion	<i>L. longiflorum</i>	White trumpet-shaped flower, very fragrant; year-round forcing cultivation
		<i>L. formosanum</i>	Short stem; deliciously fragrant, red-purple trumpet flower
		<i>L. regale</i>	Horizontal white trumpet flower with a golden heart
		<i>L. nepalense</i>	Down-facing, flared and scented, pea-green flower with dark purple throat
Asiatic	Sinomartagon	<i>L. amabile</i>	Bright orange down-facing flower
		<i>L. bulbiferum</i>	Orange upright-facing flower
		<i>L. cernuum</i>	Early flowering; small, sugar-pink, Turk's-cap flower
		<i>L. concolor</i>	Small upright-facing, intense lacquer-red flower; thick and waxy tepals
		<i>L. dauricum</i>	Gold and vermilion, upright flowers; <i>Fusarium</i> resistant
		<i>L. davidii</i>	Orange flower with spots; virus tolerance
		<i>L. tigrinum</i>	Vigorous, strong stem; dark-purple-spotted orange Turk's-cap flower
		<i>L. lankongense</i>	Spicily fragrant, pink to violet with spots of violet-rose flower
		<i>L. leichtlinii</i>	Red-orange Turk's cap flower
		<i>L. maculatum</i>	Upfacing, maroon spotted apricot flowers
		<i>L. pumilum</i>	Sweetly scented, shiny-red, pendent, small Turk's-cap flowers.
Oriental	Archelirion	<i>L. alexandrae</i>	White-green horizontal trumpet flower
		<i>L. auratum</i>	Large flower, waxy leaves and tepals with few or no spot, sweet fragrance
		<i>L. nobilissimum</i>	Scented, pure white, and upright flowers, late flowering
		<i>L. rubellum</i>	Deliciously fragrant, wild-rose pink and slightly recurved trumpet flower
		<i>L. speciosum</i>	Recurved, spicily fragrant, pale pink to cerise with darker spots flower
		<i>L. henryi</i>	Orange spotted Turk's cap flower with dark red/black spots; virus resistance

Terminology

Usually, any hybrid between two species is called an “interspecific hybrid”. In this thesis, it is necessary to re-define the terminology “interspecific hybrids” so as to

distinguish the hybrids between the species within one section from those between the species of different sections. The Longiflorum, Asiatic or Oriental hybrids are not regarded as “interspecific hybrids” in this thesis, because these hybrids are fertile and their parental genome can not be discriminated with genomic in situ hybridization (GISH). Only the hybrids between species of different taxonomic sections or between the cultivars of the three different hybrid groups are called “interspecific hybrids”, because these hybrids are highly sterile and their parental genomes can be distinguished with genomic in situ hybridization.

The genomes of Leucolirion (Longiflorum hybrids or cultivars), Sinomartagon (Asiatic hybrids or cultivars) and Archelirion (Oriental hybrids or cultivars) are generally recognized as L (Longiflorum), A (Asiatic) and O (Oriental) genomes respectively. Therefore, the crosses between different genomes are usually expressed in an abbreviated way. For example, Longiflorum x Asiatic is written as “LL x AA” and the F1 progeny are indicated with “F1 LA hybrid”. Interploid crosses are also expressed in a similar way. For example, a cross between triploid and diploid is expressed with “3x-2x”. In any formula, the female parent is always at the left and the male parent on the right.

Introgression breeding

Introgression is the process in which some alien specific traits are transferred into cultivated crops. It plays an important role in the improvement of some of the major crops, such as wheat (Jiang et al. 1994, Wang et al. 1996), rice (Multani et al. 1994, Khush 2005), potato (Garriga-Calderé et al. 1999, Tek et al. 2004), *Alstroemeria* (Kamstra et al. 1999a, b, 2004), sugarcane (Ram et al 2001) and others (reviews, Van Tuyl et al. 2002a, Ramanna and Jacobsen 2003). The introgression of rye chromosome segments into wheat has revolutionized wheat production in some countries. In *Lilium*, because the species or cultivars, especially those of different sections, possess more valuable traits (Table 1-1), the main goals of modern lily breeding are to combine the three distinctive groups and realize introgression breeding. In order to combine some valuable horticultural

traits from different alien species into a cultivar, interspecific hybridization and recurrent backcrossing are required.

Interspecific hybrids

Production of interspecific hybrids is the first step for accomplishing introgression. Due to pre- and post-fertilization barriers that exist among *Lilium* species, usually, interspecific hybridization is difficult. Therefore, some special methods, such as cut-style pollination, embryo rescue and ovule culture techniques, are needed (Van Tuyl et al. 1991). Using these methods, many lily interspecific hybrids have successfully been made. For example, *L. longiflorum* (Leucolirion) x *L. monadelphum* (Lilium section), *L. longiflorum* x *L. martagon* (Martagon), *L. longiflorum* x Asiatic hybrids (Sinomartagon), *L. longiflorum* x *L. rubellum* (Archelirion), *L. longiflorum* x *L. canadense* (Pseudolirium) and Oriental hybrid x *L. pardalinum* (Pseudolirium) (Van Tuyl et al. 2000). Similar to other interspecific hybrids, lily interspecific hybrids are highly sterile (review, Van Tuyl et al. 2002a, Van Tuyl & Lim 2003). Chromosome doubling and $2n$ gametes have been used to restore the fertility of interspecific hybrids in many plant species (review, Van Tuyl et al. 2002a).

Chromosome doubling of F1 interspecific hybrids

Somatic chromosome doubling is also called “mitotic doubling” or “somatic doubling”. The method has been used for over 60 years since colchicine was first used to induce chromosome doubling (Blakeslee & Avery 1937). Currently, besides colchicine, oryzalin is also used for chromosome doubling to restore F1 hybrid fertility (Asano 1982b, Yabuya 1985, Eikelboom & Van Eijk 1990, Ishizaka 1994, Van Tuyl et al. 1992, Takamura et al. 2002). However, this technique contributes little to introgression breeding because it is hard to accomplish intergenomic recombination in the allopolyploids due to autosyndetic chromosome pairing during meiosis in somatically doubled allotetraploids (Ramanna & Jacobsen 2003). On the contrary, when sexual polyploidization is used for inducing polyploids, intergenomic recombination could occur during $2n$ gamete formation.

***2n* gametes formed in F1 interspecific hybrids**

2n gametes, i.e. “unreduced” gametes, possess the same chromosome numbers as somatic cells. The direct consequence of *2n* gamete formation is sexual polyploidization. This was recognized 70 years ago by Müntzing (1932). However, the importance of such polyploids was underestimated, because of the assumption that the occurrence of *2n* gametes in plants is rare and sporadic (Stebbins 1950). On the contrary, Harlan & De Wet (1975) proposed that nearly all plant species produce *2n* gametes and all natural polyploids of plant species have originated through *2n* gametes. This view has been widely accepted (Thompson & Lumaret 1992, Bretagnolle & Thompson 1995, Ramsey & Schemske 1998).

Cytological analyses of meiosis in those plants that produce *2n* gametes have shown that various types of meiotic abnormalities can lead to the formation of *2n* pollen or *2n* eggs (review, Ramanna & Jacobsen 2003). Generally, based on the particular meiotic stages at which nuclear restitution occurs, two main mechanisms of *2n* gamete formation are recognized. These are the first division restitution (FDR) and the second division restitution (SDR) (Mok & Peloquin 1975a, 1975b, Ramanna 1979). During normal process of meiosis, the homoeologous chromosomes are paired at metaphase I and disjoined normally at anaphase I. Following this, cytokinesis (CK I) occurs at telophase I and the second meiotic division proceeds in two separate cells—which is nothing but mitotic divisions. After a second cytokinesis (CK II) four haploid spores (*n*) are formed (Figure 1-1). Deviations can occur at any of these stages. For example, if meiosis proceeds normally up to telophase I (CK I) and does not complete the second division, the products of disjunction can reconstitute and give rise to SDR (Figure 1-1). On the other hand, if the homologous chromosomes do not pair, or partially pair and divide equationally before telophase I stage and give rise to a dyad, it can lead to FDR (Figure 1-1). Unlike the above mentioned abnormalities, i.e., SDR and FDR, some bivalents that are formed might disjoin normally, at the same time some univalents divide equationally leading to nuclear restitution. This is called IMR (Lim

et al. 2001b). From Figure 1-1, it is obvious that different mechanisms have different genetic consequences because of chromosome assortment and

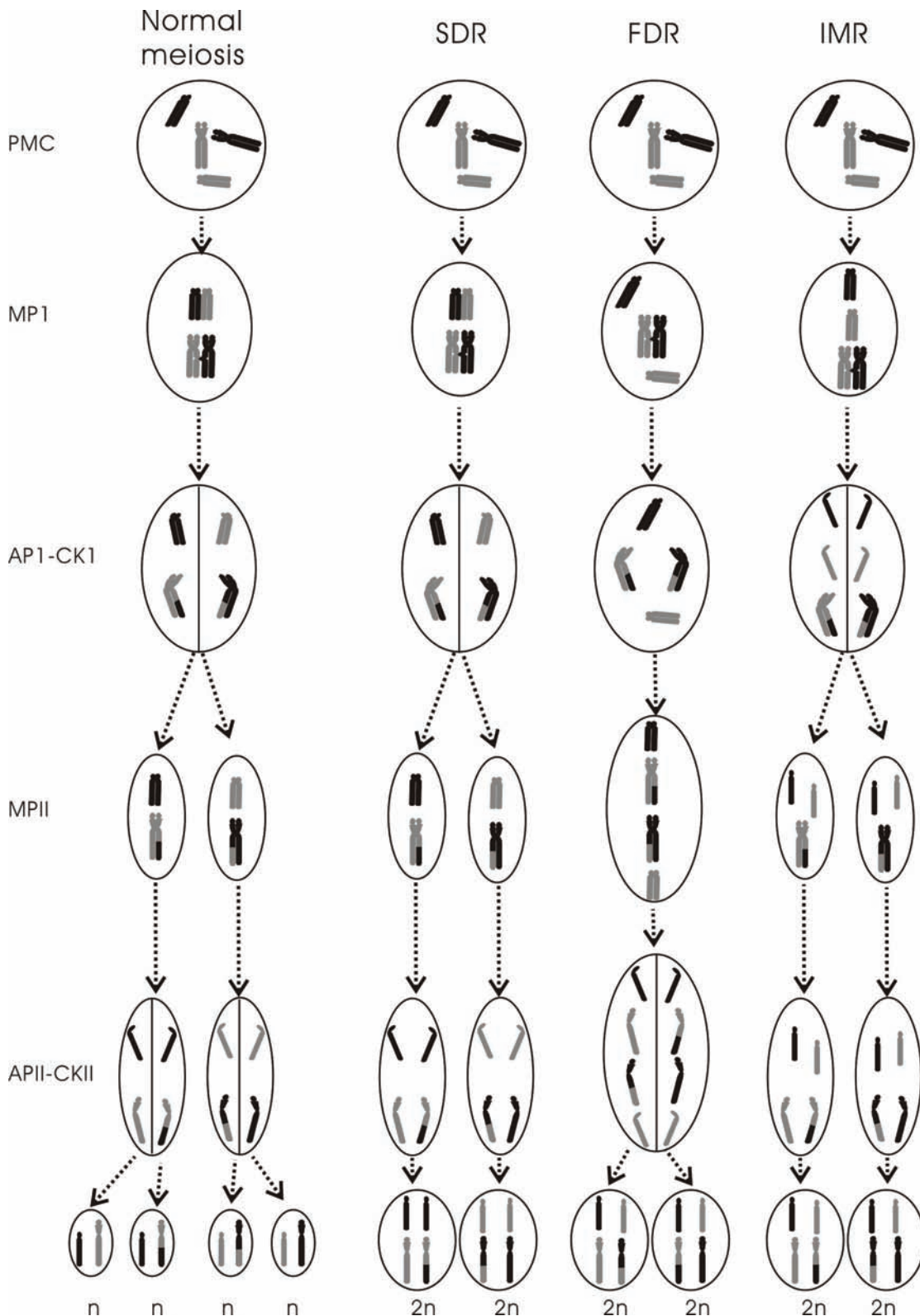


Figure 1-1. An illustration of mechanisms of $2n$ gamete formation in interspecific hybrids (only one chromosome assortment is diagrammed in all cases).

intergenomic recombination as has been demonstrated in *Alstroemeria* (Kamstra et al. 1999b, Ramanna et al. 2003), *Gasteria* x *Aloe* hybrids (Takahashi et al. 1997) and *Lilium* interspecific hybrids (Karlova et al. 1999, Lim et al 2001b, Barba-Gonzalez et al. 2005a, 2005b). Because intergenomic recombination in the sexual polyploids increases genetic variation, it has been proven that $2n$ gametes are valuable for crop improvement in many cases (reviews, Van Tuyl et al. 2002a, Ramanna & Jacobsen 2003).

The progenies of backcrosses

Interspecific hybrids usually combine both desirable and undesirable traits. In order to eliminate undesirable traits and keep desirable traits, recurrent backcrossing is necessary. When F1 interspecific hybrids produce functional $2n$ gametes, their BC1 progenies are usually triploid. Triploids are mostly sterile, but in several plant species the successful use of triploids as parents has been reported (reviews, Brandham 1982, Kuspira et al. 1986, Ramsey & Schemske 2002, Ramanna & Jacobsen 2003). In $2x-3x$ and reciprocal crosses, the progenies can be diploid or near diploid whereas, in $3x-4x$ and its reciprocal crosses, the progenies can be tetraploids or circa tetraploids (reviewed, Brandham 1982, Kuspira et al. 1986). Such interploid crosses have not only been made in autopolyploids, but also in allopolyploids (Lim et al 2003, Barba-Gonzalez 2005).

Genomic in situ hybridization and its relevance for cytogenetic research in *Lilium*

In situ hybridization was developed in 1960s (Pardue & Gall 1969). This is a crucial milestone in the history of chromosome research during the last 50 years (De Jong 2003). It has been extensively used in cytogenetics since the fluorescence was used to label the probes instead of isotopes in the 1980s (Leitch AR et al. 1994). Genomic in situ hybridization is a powerful modern cytogenetic technique. In this technique, usually, the total DNA of one parent of the hybrid is used as probe and labeled with digoxigenin or biotin and, the other

parental genomic DNA is used as a block. After hybridization on the chromosome preparations, the parental genomes of interspecific hybrids can be distinguished, such as in *Hordeum* (Schwarzacher et al. 1992), *Triticum* (Anamthawat-Jonsson et al. 1990), *Alstroemeria* (Kuipers et al. 1997), *Allium* (Khrustaleva et al. 1998), tomato (Ji et al. 2004), and so on. GISH is also used to analyze intergenomic recombination and monitor alien chromosomes in some backcross progenies, for example, in potato (+) tomato (Jacobsen et al. 1995), *Gasteria* x *Aloe* (Takahashi et al. 1997), *Allium* (Khrustaleva et al. 2000), *Brassica* (Wang et al. 2005), etc. This technique has also been successfully used in lily cytogenetic research. Since three genomes of *Lilium*, viz., Longiflorum (L), Asiatic (A) and Oriental (O) genome were recognized with GISH (Karlov et al. 1999), some F1 LA and OA hybrids, BC1 and BC2 progenies have been analyzed systematically (Lim et al. 2001b, 2003; Barba-Gonzalez et al. 2004, 2005a, 2005b, etc). These researches showed that the most homoeologous chromosomes of the investigated lily interspecific hybrids partly pair during their meiosis (Lim et al. 2001b, Barba-Gonzales et al. 2004, 2005a). Although nearly all of them are highly sterile, some of them could produce a low frequency of functional $2n$ gametes and gave rise to sexual triploids BC1 progenies. GISH analysis on some BC1 progenies showed that most of them resulted from FDR $2n$ gametes and less from IMR $2n$ gametes (Lim et al. 2001b, Barba-Gonzales et al. 2005a, 2005b). Many of these triploid BC1 progenies contained variable numbers of intergenomic recombinant chromosomes and segmental substitutions which can attain nulliplex condition (aaa). These substitutions are the main basis of genetic variation in sexual triploid BC1 progenies (Lim et al. 2001b, Barba-Gonzalez et al. 2005a, 2005b). Some of these allotriploid lilies could be used as parents to cross with diploid cultivars or allotetraploid lilies which had originated from somatic chromosome doubling of the F1 interspecific hybrids. In $2x-3x$ or reciprocal crosses of lilies, the progenies were predominantly near diploid and in $3x-4x$ crosses, the progenies were nearly tetraploid in OA hybrids but nearly pentaploid in LA hybrids (Lim et al. 2003, Barba-Gonzalez 2005). This phenomenon is similar to that observed in other

interploids crosses (review, Brandham 1982) except that 3x-4x cross generated near pentaploids in LA hybrids (Lim et al. 2003).

Because lily interspecific hybrids have more potential as commercial cultivars, Plant Research International of Wageningen University and Research Centre and Dutch lily companies not only have obtained many F1 LA hybrids, but also selected many cultivars directly from their BC1 progenies within recent years. The LA materials of six Dutch lily breeding companies were used for this study. To investigate these materials with GISH would supply some valuable information for lily breeding which could be captured in the three following questions:

1. How does meiosis occur? Because some of the diploid F1 LA hybrids were successfully used as parents in order to select triploid cultivars in the BC1 progenies, it would be valuable to investigate meiosis in these hybrids.
2. What is the origin of $2n$ egg formation? Although some amount of information was available on the origin of $2n$ microspores, there was little information on the origin of $2n$ eggs and their genome compositions.
3. How can triploids resulting from $2n$ gametes be used in lily breeding? Since the use of $2n$ gametes in lilies inevitably leads to the production of allotriploid BC1 progenies, it was feasible to investigate how such triploids can be used in further breeding.

Scope of the thesis

The main aims of this thesis were to elucidate introgression of *Longiflorum* chromosomes into Asiatic cultivars by investigating microsporogenesis of F1 LA hybrids and the genome compositions of recurrent backcrossing progenies, and compare the lily hybrids created by Plant Research international and those supplied by the Dutch lily breeding companies. In Chapter 2, the diploid F1 LA hybrids, which were supplied by the Dutch lily breeding companies, were investigated for their microsporogenesis with conventional cytological methods and genomic in situ hybridization. Based on the results, intergenomic crossovers, which give rise to intergenomic recombination, were analyzed. The possibility of

formation of haploid, $2n$ and aneuploid gametes in F1 LA hybrids, and the potential of the mechanism of $2n$ gamete formation are discussed.

In Chapter 3, the genome compositions of the BC1 cultivars (LAA) produced by the Dutch lily breeding companies were analyzed with genomic in situ hybridization. Intergenomic recombination and mechanism of $2n$ egg formation are elucidated. The significance of $2n$ gametes for lily breeding is discussed.

In Chapter 4, the F1 LA hybrids, analyzed in Chapter 2, were used as parents to backcross with Asiatic cultivars. Some of the new BC1 progenies were analyzed with flow cytometry and GISH. The possibility of gamete formation which was expected based on the results in Chapter 2 was confirmed. The genome compositions of the BC1 cultivars and the new BC1 progenies are compared.

In Chapter 5, interploid crosses ($2x-3x$, $2x-4x$, $2x-5x$ and their reciprocal crosses) were made. Some progenies were analyzed with flow cytometry and GISH. The possibility of using allopolyploid lilies for introgression breeding is considered.

Chapter 6 gives a general discussion on the significance of the use of n and $2n$ gametes produced by LA hybrids for lily introgression breeding.

2 Analysis of crossovers during microsporogenesis in the F1 interspecific hybrids of Longiflorum x Asiatic (LA) lilies (*Lilium*)

Abstract

11 genotypes of F1 interspecific Longiflorum x Asiatic (LA) hybrids in *Lilium* were cytologically investigated with genomic in situ hybridization as well as traditional cytological methods. The chromosome associations among different F1 hybrids were quite variable ranging from 2.0 to 11.4 bivalents per pollen mother cell. Many crossover types, e.g., single crossovers (SC), three strand double (TSD), four strand double (FSD), and four strand triple (FST) crossovers between the non-sister chromatids in the tetrads of bivalents, were inferred by analyzing the disjoined bivalents at anaphase I of meiosis in some F1 LA hybrids. GISH results also clearly showed that, at anaphase I, all bivalents disjoined and most univalents divided, and both the disjoined bivalents (half-bivalents) and the divided univalents (sister chromatids) moved to the opposite poles. Based on the results of the meioses of the LA hybrids, it is concluded that these LA hybrids have possibilities to produce unreduced ($2n$), aneuploid or haploid (n) gametes.

Key words

Lilium, in situ hybridization, normal meiosis, abnormal meiosis, crossover, intergenomic recombination

Introduction

Crossover is an important event during meiosis because it causes meiotic recombination in the gametes. Numerous researches on crossover events have been reported in *Oryza sativa* (Harushima et al. 1998), *Zea mays* (Anderson et al. 2003), *Solanum tuberosum* (Jongedijk & Ramanna 1989), *Hordeum vulgare* (Pickering 1991), *Arabidopsis thaliana* (Copenhaver et al. 2004), and other plants (reviewed by Nilsson et al. 1993). In early researches, counting chiasmata at diakinesis or at metaphase I of meiosis with conventional staining was the main method for evaluating crossover events and recombination. This method is being replaced by molecular marker techniques through which genetic linkage maps can be made. However, in many cases, the number of crossovers from chiasmata counting is much lower than that calculated from molecular marker mapping in plants (Nilsson et al. 1993). With the technique of genomic in situ hybridization (GISH), which is a modern and more powerful cytogenetic tool, it has been possible to distinguish different genomes in interspecific hybrids (Schwarzacher et al. 1989, Anamthawat-Jonsson et al. 1990, Kuipers et al. 1997, Khrustaleva et al. 1998, Ji et al. 2004). It was also possible to observe homoeologous chromosome behavior at meiosis (Stevenson et al. 1998, Kamstra et al. 1999a, b), detect intergenomic recombinant chromosomes and determine the fate of alien chromosomes in their backcross progenies (Jacobsen et al. 1995, Takahashi et al. 1997, Khrustaleva et al. 2000, Wang et al. 2005). Finally, it has been proven possible to make an integrated recombination physical map of particular chromosomes (King et al. 2002, Khrustaleva et al. 2005). This technique has also been successfully used in *Lilium* to discriminate parental genomes of the F1 interspecific hybrids and detect intergenomic recombinant chromosomes in the BC1 and BC2 progenies (Karlova et al. 1999, Lim et al. 2000, 2001b, 2003, Barba-Gonzalez et al. 2004, 2005a, 2005b). From these extensive cytological analyses of the parental F1 hybrids and BC1 progenies in *Lilium* the following conclusions were derived: (i) in the F1 hybrids both first division restitution (FDR) as well as indeterminate meiotic restitution (IMR) can occur leading to $2n$ gamete formation; (ii) many BC1 progenies have intergenomic recombinant chromosomes

which are caused as a result of crossovers between homoeologous chromosomes during gamete formation in the F1 hybrids. However, how crossovers occur in the F1 interspecific hybrids in *Lilium* has not been studied.

In this chapter, 11 F1 hybrids were investigated for their homoeologous chromosome behaviour during microsporogenesis with conventional methods and the GISH technique. Crossover events were analyzed and possibilities of different types of gametes possibly produced by the F1 hybrids were discussed.

Materials and methods

Plant material

Two diploid lily cultivars ($2n=2x=24$), Asiatic 'Mont Blanc' and Longiflorum 'White Fox', and eleven diploid F1 LA hybrids ($2n=2x=24$) were used. These hybrids originated from crosses between Longiflorum cultivars (L-genome) and Asiatic cultivars (A-genome), which belong to the Leucolirion section and Sinomartagon section in the genus *Lilium*, respectively. All hybrids were supplied by three Dutch lily breeding companies, viz., De Jong Lelies BV, Testcentrum BV and Vletter & Den Haan BV. They were grown in greenhouse using standard growing conditions applicable for lily cultivation, and are being maintained at Plant Research International, Wageningen University & Research Center, the Netherlands.

Chromosome preparation with anthers

A 1 mm part of anther which was at metaphase I stage was cut and put on a slide. Under an anatomical microscope, the unnecessary debris of the anther wall were removed and the pollen mother cells were spread on the slide, 16 μ l 2% acetocarmin was swiftly added and gently mixed with pollen mother cells, then, covered with a square cover glass, pressed gently with the thumb and finally, the slide was examined with a light microscope. Good slides were sealed with nail polish around cover glass margin in order to observe and estimate the extent and difference of homoeologous chromosome pairing. The average of bivalents was the sum of bivalents of all observed pollen mother cells divided by the number of all observed pollen mother cells.

Genomic in situ hybridization (GISH)

Chromosome preparation with anthers for GISH was slightly different from that used for the conventional cytological method: 2% acetocarmin was replaced by 45% acetic acid, the slide was dipped in liquid nitrogen for about 20 seconds after squashing, then, the cover glass was removed with a razor blade as soon as the slide had been taken out of the liquid nitrogen, then, the slide was put in pure ethanol for 2 minutes, air dried, and finally, the slide was stored at -20°C until use. Probe and block preparation: Total genomic DNA was extracted from the Longiflorum 'White Fox' and the Asiatic "Mont Blanc" with CTAB method (Rogers & Bendich 1988). 'White Fox' DNA was sonicated to 1-10kb fragments and used as probe. 'Mont Blanc' DNA was autoclaved to 200-600bp fragments and used as block. The probe DNA was labeled with digoxigenin-11-dUTP by the nick translation method according to the manufacturer's instructions (Roche, Germany).

In situ hybridization mainly consisted of four parts, i.e., chromosome pre-treatment, hybridization, stringency washing and detection. For chromosome pre-treatment, the slides were treated with 100µg/mL RNase for one hour and 5µg/mL pepsin for 10 minutes at 37°C, then fixed in 4% paraformaldehyde for 10 minutes, after each of these treatments, the slides were washed with 2xSSC for 5 minutes three times, then, the slides were treated for three minutes with 70, 90 and 100% ethanol, respectively, finally, the slides were dried in air at least 30 minutes. For hybridization, the hybridization mix contained 50% formamide, 10% dextranulphate, 2xSSC, 0.25% SDS, 0.6-1.5ng/µL probe and 25-100ng/µL block DNA, in order to keep probe and block DNA denatured, the mix was cooled down with ice at least for 5 minutes as soon as it was treated at 70°C for 10 minutes; to each slide was added 40µL hybridization mix, a cover glass was added, and denatured at 80°C for 5 minutes, then kept in a 37°C humid box overnight. After hybridization, the slides were washed in 2xSSC for 15 minutes at room temperature, then, stringency washing was followed in 0.1xSSC at 42°C for 30 minutes. The probe labeled with dig-11-dUTP was detected with the digoxigenin detection system. The slide was counterstained with 2ng/µL DAPI. Fluorescence

microscopy was used to check the results and photographed with CCD camera attached to the microscope.

Results

At the onset of our experiments, all the diploid hybrids provided by the lily breeding companies were confirmed to be LA hybrids using two criteria: chromosome pairing and differential staining of chromosomes through GISH. The failure of chromosome pairing in variable degrees during metaphase I (Table 2-1), and the presence of 12 chromosomes each of L and A genomes (Figure 2-1a-c) confirmed that all the investigated plants were indeed F1 hybrids.

Table 2-1. Chromosome pairing at metaphase I of meioses in 10 F1 LA hybrids

Genotype	Number of cells observed	Number of bivalents	
		Range	Average
041501	25	9-12	10.7
041546	22	9-11	10.2
041549	20	5-12	8.9
041556	27	3-8	6.3
041557	19	2-11	7.3
041559	21	1-3	2
041560	26	10-12	11.4
041564	25	8-11	9.5
041565	27	6-9	7.2
041566	23	8-12	10.5

Chromosome associations

Chromosome associations were quite variable, not only among the 10 genotypes but also in different pollen mother cells of the same genotype (Table 2-1). Nevertheless, by comparing the average number of bivalents among genotypes (Table 2-1), as few as two bivalents per cell (041559) and as many as 11.4 bivalents per cell (041560) were observed. Thus, the variation in chromosome associations among different genotypes was quantitative rather than qualitative.

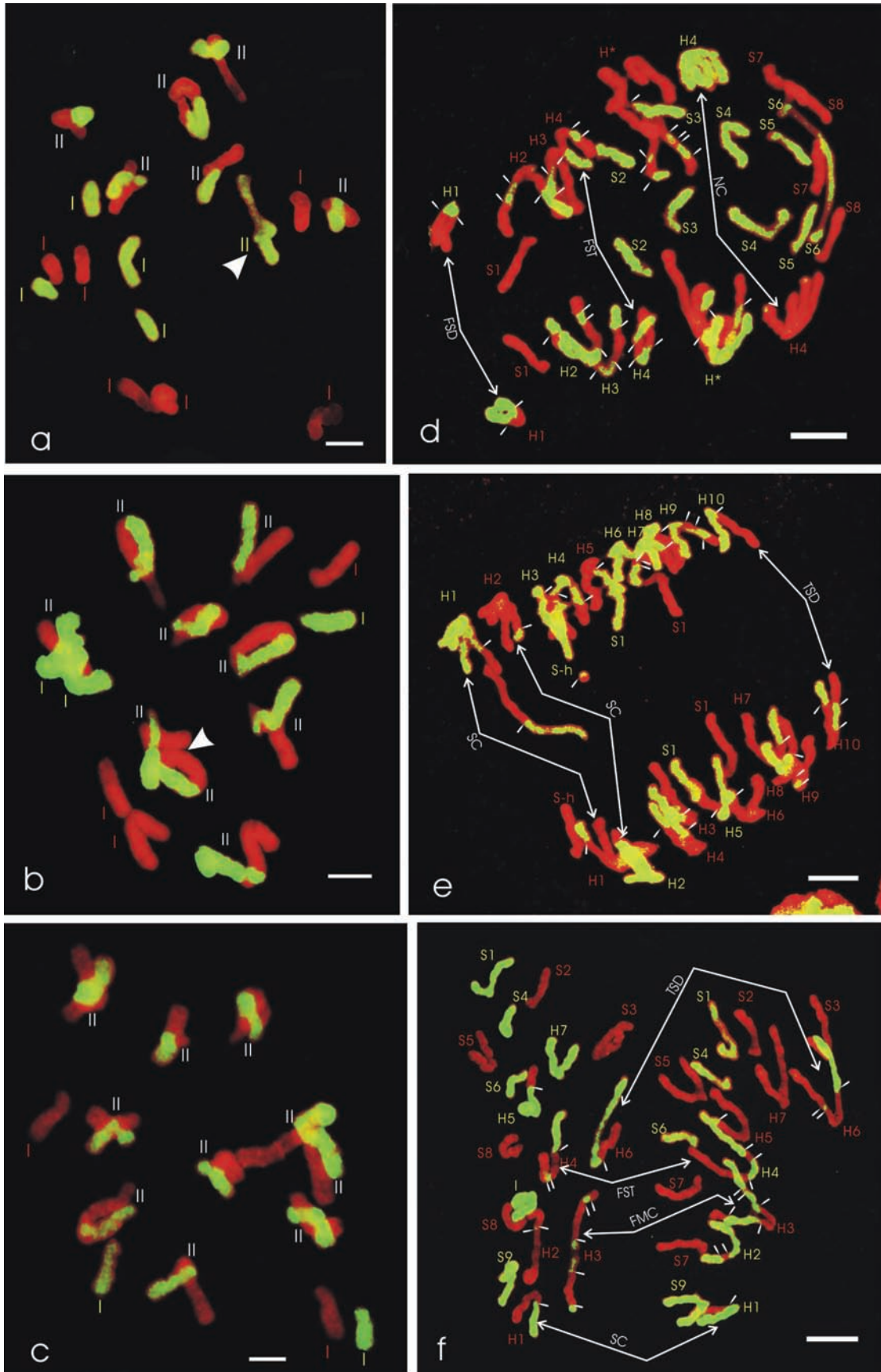


Figure 2-1. GISH results of F1 LA hybrids' abnormal meiosis: a, b & c are metaphase I of 041565, 041556 & 041557, respectively. "I" means univalent, "II" bivalent; d, e & f are anaphase I of 041546, 041546 & 041557, respectively. NC, SC, TSD, FSD, FST & FMC are the abbreviation of no crossover, single crossover, three strand double crossover, four strand double crossover, four strand triple crossover and four strand multiple crossover, respectively. More detailed explanations see text.

With very few exceptions, bivalents were formed involving the homoeologous chromosomes of L and A genomes (differentially coloured in Figure 2-1a-c). However, rarely, there were also instances of bivalents resulting from non-homologous association of two chromosomes of the same genome (Figure 2-1a, arrowhead). This was an indication for the presence of chromosomal interchanges within the genomes which might lead to multivalent formation in some cases (Figure 2-1b, arrowhead).

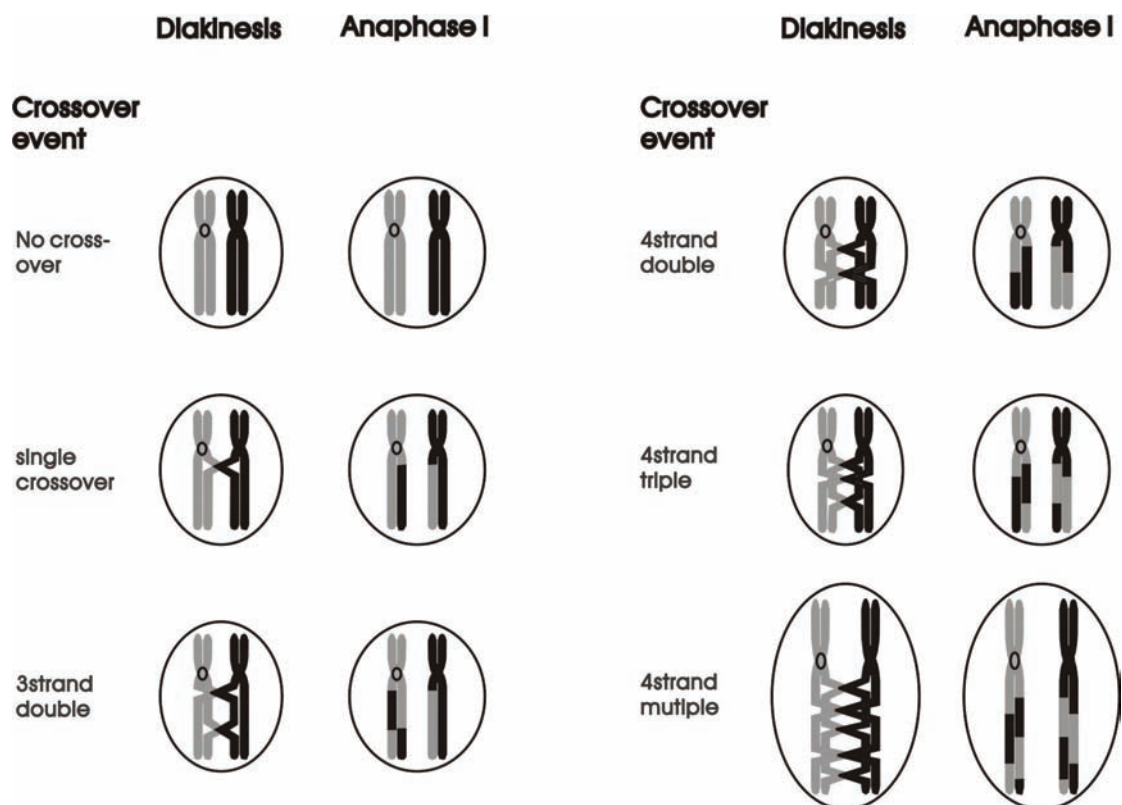


Figure 2-2. Crossover events interpreted from GISH results on anaphase I.

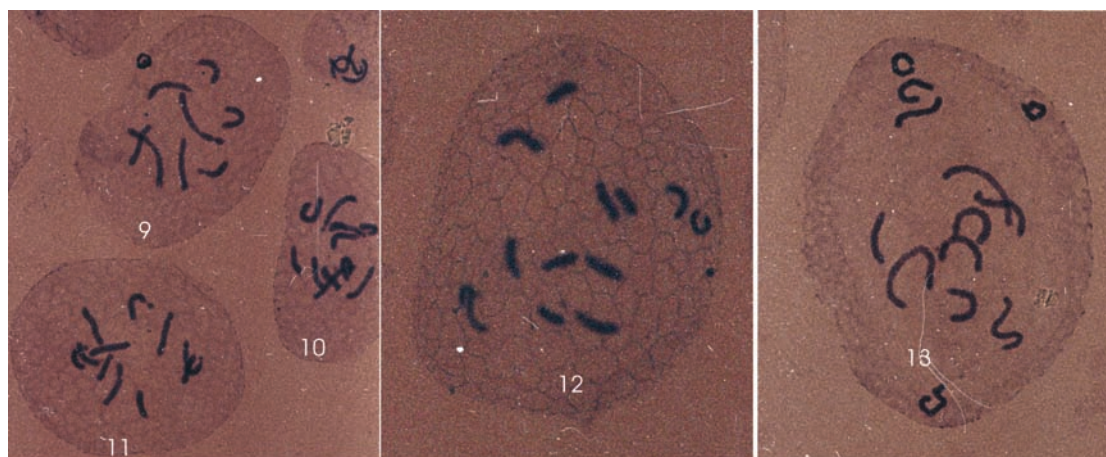


Figure 2-3. The variable numbers of chromosomes in different microspores caused by abnormal meiosis in F1 LA hybrid (041550).

Intergenomic crossover

Analysis of crossovers could be made at anaphase I when half-bivalents were formed, although the number or position of chiasmata could not be determined from the bivalents at metaphase I. When the bivalents disjoined normally, the two resulting half-bivalents retained their axes in the cell undisturbed. They are indicated by the same case letters (Figure 2-1d-f, red “H1”-yellow “H1”, red “H2”-yellow “H2”, etc.). Based on such pairs of half-bivalents, it was possible to determine the position and number of crossovers that had occurred between the non-sister chromatids of a tetrad in each bivalent. Examples of single crossover (SC), three strand double (TSD), four strand double (FSD), and four strand triple (FST) crossovers are illustrated in Figure 2-1d-f, and the interpretive drawings are shown in Figure 2-2. Apart from these, there were also instances in which normal disjunction of bivalents could occur even when chiasmata were not formed (Figure 2-1d, NC). In some cases, very few or no crossovers were present because few or no bivalents were formed in these pollen mother cells (PMCs) or genotypes. In other cases, however, 14 to 17 crossovers per meiosis were counted. For example, there were 32 breakpoints in the cell illustrated in Figure 2-1e, so 16 crossovers had occurred in this meiosis (Figure 2-1d-e, points). The high number of crossovers was, however, not evenly distributed among the bivalents. In some cases three or more crossovers were observed in half-bivalents whereas in others single crossovers appeared to be the norm.

Homoeologous chromosome behaviour

Clearly, in most cases, not only all bivalents disjoined but also most univalents divided as well (Figure 2-1d-f), and a notable feature was that both the disjoined bivalents and the divided univalents moved to opposite poles at anaphase I in most cases. As a consequence of this, two clear groups consisting of the divided bivalents and univalents were observed (Figure 2-1d-f).

Accordingly, in view of the occurrence of variable frequencies of univalents and bivalents in different PMCs within a plant and among genotypes, different types of chromosome movements were expected to occur during anaphase II. In most cases, probably, at anaphase II, the half-bivalents formed at anaphase I divided and moved to opposite poles, but the chromatids formed at anaphase I were distributed randomly to opposite poles. Thus, this would give rise to a considerable number of aneuploid microspores. This point was confirmed by counting chromosome number of microspores in 041550 which were indeed variable (Figure 2-3). This might be the reason that most F1 LA hybrids were highly sterile. In some other cases, if few bivalents were formed and disjoined early, the disjoined bivalents (half-bivalents) and all univalents directly entered meiosis II apparently without completing the first division. This resulted in FDR $2n$ gametes. If the bivalents disjoined and all univalents divided at the same time and both the disjoined bivalents (half-bivalents) and the divided univalents (sister chromatids) moved to opposite poles at anaphase I, then, the disjoined bivalents divided and formed a restitution nucleus rather than moving to opposite poles. This gave rise to IMR $2n$ gametes. Based on the behaviour of homoeologous chromosomes at anaphase I (Figure 2-1d-f), we concluded that LA hybrids had more potential possibilities to produce IMR pollen than FDR pollen. In those cases where all homoeologous chromosomes could pair in most pollen mother cells, haploid gametes could be formed due to normal meiosis. Based on the cytological analysis of the BC1 progenies involving these genotypes as parents, the occurrence of the three types of gametes have been confirmed (Chapters 3 & 4).

Discussion

The parental LA hybrids used in the present investigation are unique because they are selected by the lily breeders purely for the purpose of using them as parents to produce cultivars. The breeders had no knowledge of their chromosome pairing behaviour or restitution mechanisms.

The accurate detection of parental chromosomes through GISH during microsporogenesis has opened the possibilities not only to investigate restitution mechanisms (Lim et al. 2001), but also to elucidate the process of crossover between the non-sister chromatids in the tetrad of bivalents (Figure 2-1d-f & Figure 2-2). These aspects are especially helpful for explaining the types of functional $2n$ gametes which can be deduced in the BC1 progenies through karyotype analysis using GISH (Lim et al. 2001, Barba-Gonzalez et al. 2005b).

The detection of crossover events such as three strand double, four strand double, and four strand triple was confined in only a few organisms such as *Neurospora* and *Ustilago* which have ordered spore tetrads (Griffiths et al. 1996). In most cases, the crossover events were only inferred using multiple genetic markers. The present investigation shows that a direct visible evidence for crossover events can be obtained in lily interspecific hybrids through GISH analysis.

Unlike in previous studies where chromosome pairing was low in LA and OA hybrids (Lim et al. 2000, 2001b, Barba-Gonzalez et al. 2004), these F1 LA hybrids selected by the breeders show quantitative differences for chromosome pairing. The number of bivalents formed in different genotypes or different pollen mother cells in some particular genotypes varied from very few to 12 (Table 2-1). This suggested that most LA hybrids have abnormal meiosis and a few genotypes or pollen mother cells have also normal meiosis. This means the F1 LA hybrids have ability to produce $2n$ or aneuploid gametes by abnormal meiosis and produce n gametes by normal meiosis. In previous investigations, functional $2n$ gametes produced by F1 LA and OA hybrids in *Lilium* were found, and there were more BC1 progenies from FDR $2n$ pollen than IMR $2n$ pollen based on GISH analysis (Lim et al. 2001b; Barba-Gonzalez et al. 2005b). This apparently conflicts

with the present result. The main reason of this conflict might be that FDR $2n$ gametes are more viable than IMR $2n$ gametes or BC1 progenies from FDR $2n$ gametes are more vigorous than those from IMR $2n$ gametes because the chromosomal constitution of FDR $2n$ gametes is more balanced than that of IMR $2n$ gametes. Of course, the analyzed LA genotypes and the size of the analyzed BC1 populations also likely cause such conflicts. Although no aneuploid BC1 progeny was found in *Lilium*, many aneuploid progenies of $2x-3x$ crosses were recorded in *Lilium* (Lim et al. 2003, Barba-Gonzalez 2005) and other crops (Brandham 1982). Since LA hybrids produce a considerable number of aneuploid pollen, it is reasonable that some of them are likely to be viable and survived. If some aneuploid gametes are functional, aneuploid BC1 progenies are expected to occur. This will be confirmed in Chapters 3 and 4. Due to normal meiosis where all homoeologous chromosomes could pair, the haploid gametes generated by LA hybrids should contain more recombinant chromosomes as compared to $2n$ gametes. Hence, haploid gametes might very be useful for introgression breeding. Although meiosis in distant hybrids usually is abnormal because not all homoeologous chromosomes could form bivalents, the occurrence of haploid gametes in distant hybrids has been recorded in other plant genera, for example *Allium cepa* x *A. fistulosum* (Emsweller and Jones, 1945; Khrustaleva et al., 2005); *Alstroemeria aurea* x *A. inodora* (Kamstra et al. 1999a) and *Lycopersicon esculentum* x *Solanum lycopersicoides* (Chetelat et al. 1997). If a large number of F1 hybrids are screened for their pollen mother cells that have normal meiosis, it is expected that some interspecific hybrids which produce viable n gametes should be found.

3 Genome composition of triploid lily cultivars derived from sexual polyploidization of Longiflorum x Asiatic hybrids (*Lilium*)

Abstract

19 cultivars, which had originated from backcrosses between F1 LA (Longiflorum x Asiatic) hybrids ($2n=2x=24$) as female parents and Asiatic cultivars as male parents ($2n=2x=24$), were analyzed with genomic in situ hybridization (GISH). 17 of them were triploid ($2n=3x=36$), and two aneuploid ($2n=3x+1=37$). The triploid cultivars had resulted from the functional $2n$ eggs produced by their female parents (F1 hybrids) because first division restitution (FDR) occurred in their meiosis during megasporogenesis. Similarly, the aneuploid cultivars had originated from viable $2n+1$ eggs. The extra chromosome in cultivar 041555 or 041572 resulted from one univalent or one half-bivalent which might have lagged behind when the sister chromatids of the other univalents and half-bivalents were segregating during the FDR process in their LA hybrid parents, respectively. The result that 15 cultivars possessed recombinant chromosomes and four did not have recombinant chromosomes showed that intergenomic recombination might play an important role during the selection of the cultivars directly from BC1 progenies. That five cultivars of the 15 recombinant cultivars only had reciprocal recombinant chromosomes and 10 cultivars had non reciprocal recombinant chromosomes indicates that the latter is of more importance. Because, of the 10 non reciprocal recombinant cultivars, nine cultivars contained substitution for recombinant segments, it also indicates that such substitutions could be an important source for the genetic variation of the sexual triploid BC1 progenies. In such cases there was a potential for the expression of the recessive genes of the backcross parent in a nulliplex (aaa) condition. Genetic variation resulting from

such nulliplex loci might have played a role in the selection of some of the cultivars.

Key words

Lilium, in situ hybridization (GISH), unreduced gametes, intergenomic recombination, First division restitution, indeterminate meiotic restitution

Introduction

Interspecific hybrids and polyploids are two common features of some economically important ornamental crops (Van Tuyl et al. 2002a). Lily, one of the most important cut flowers in the Netherlands, belongs to the genus *Lilium* of the family Liliaceae. In the genus, three groups of lily cultivars, i.e. Longiflorum (L-genome), Asiatic (A-genome) and Oriental (O-genome), have been recognized with genomic in situ hybridization (Karlova et al. 1999). It is desirable to combine or introgress some horticultural traits from different genomes into one cultivar in lily breeding. However, it is very difficult to obtain F1 interspecific hybrids in *Lilium*. With cut style pollination and embryo rescue techniques, it has been possible to make crosses between different lily species from different sections. Similar to other interspecific hybrids, nearly all the F1 LA (Longiflorum x Asiatic) and OA (Oriental x Asiatic) hybrids are sterile. In order to overcome the sterility, chromosome doubling was used in lily breeding (Van Tuyl 1989, Van Tuyl et al. 1992). However, chromosome doubling could not contribute much to introgression plant breeding, because such amphidiploid hybrids usually produce identical 2x-gametes (Ramanna & Jacobsen 2003; Van Tuyl & Lim 2004). On the other hand, 2n gametes have been shown to be more valuable for polyploidization and introgression breeding in some cases, e.g., *Alstroemeria* (Ramanna 1992, Ramanna et al 2003). Some F1 interspecific hybrids in *Lilium* could produce functional 2n pollen to some extent and 2n pollen has been used for introgression lily breeding (Van Tuyl et al. 1989, 2000, 2002). Intensive cytological analyses on

some F1 hybrids and their sexual triploid BC1 progenies revealed that, the functional $2n$ pollen in lily F1 distant hybrids results from first division restitution (FDR) and indeterminate meiotic restitution (IMR), and intergenomic recombination occurs during meiosis of their microsporogenesis (Lim et al. 2001b; Barba-Gonzalez et al 2004, 2005a, 2005b). However, little attention was paid to female gametes produced by F1 LA hybrids or OA hybrids, although such $2n$ gametes have been used in lily breeding by Dutch lily breeding companies in recent years. In order to fill this knowledge gap, 19 cultivars, obtained from Dutch breeding companies, were analyzed with genomic in situ hybridization. Because all cultivars were selected from the first generation of backcrosses between diploid F1 hybrids ($2n=2x=24$) as female parents and Asiatic cultivars ($2n=2x=24$) as male, the karyotypes of the cultivars were analyzed through GISH so that the composition of $2n$ eggs could be assessed, and the usefulness of $2n$ gametes discussed.

Materials and method

Plant materials

All 19 cultivars used in this experiment were supplied by two Dutch lily breeding companies: Royal Van Zanten BV and Vletter & Den Haan BV. They originated from backcrosses in which F1 LA hybrids ($2n=2x=24$) were used as female and Asiatic cultivars ($2n=2x=24$) as male. They were grown in the greenhouse of Plant Research International, Wageningen University, The Netherlands, under standard growing conditions.

Chromosome preparation with root tips

When the young roots growing on stems were about 2cm long, 5 root tips in each case were pretreated in 0.7mM cycloheximide for 6 hours at 4°C, then, transferred into a fixative which was composed of one part acetic acid and three part ethanol in volume. After the root tips were fixed at 4°C for two days, the fixative was discarded and 80% ethanol was added in place of fixative, after which the root tips were stored at -20°C until use.

The root tips were washed for 10 minutes each three times with citrate buffer, then, treated with a pectolytic enzyme mix containing 1% (w/v) pectolyase Y23 and 1% (w/v) cellulase RS for about 75 minutes until the root tips became soft for squashing. One softened root tip was put on a slide and, under an anatomical microscope, the root cap and other unnecessary parts were removed and the meristem part was left on the slide, 16µl 45% acetic acid was immediately added and mixed gently with a needle, then, covered with a square cover glass and squashed gently with the thumb. The slide was dipped in liquid nitrogen for about 20 seconds after squashing, after which the cover glass was removed with a blade as soon as the slide had been taken out of the nitrogen. Then, the slide was put in pure ethanol for 2 minutes and, finally, the slide was air dried and stored at -20°C until use.

Genomic in situ hybridization (GISH)

Probe and block preparation: Total genomic DNA was extracted from the Longiflorum cultivar 'White Fox' and the Asiatic cultivar 'Mont Blanc' with the CTAB method (Rogers & Bendich 1988). 'White Fox' DNA was sonicated to 1-10kb fragments and used as probe. 'Mont Blanc' DNA was autoclaved to 200-600bp fragments and used as block. The probe DNA was labeled with digoxigenin-11-dUTP by nick translation method according to the manufacturer's instruction (Roche, Germany).

The in situ hybridization procedure consisted of four parts, i.e., chromosome pre-treatment, hybridization, stringent washing and detection. For chromosome pre-treatment, the slides were treated with 100µg/mL RNase for one hour and 5µg/mL pepsin for 10 minutes at 37°C, then, fixed in 4% paraformaldehyde for 10 minutes. After each of these treatments, the slides were washed with 2xSSC for 5 minutes three times, then, the slides were treated for three minutes with 70, 90 and 100% ethanol respectively and, finally, the slides were dried in air at least for 30 minutes. For hybridization, the hybridization mix contained 50% formamide, 10% dextran sulphate, 2xSSC, 0.25% SDS, 0.6-1.5ng/µL probe and 25-100ng/µL block DNA. In order to keep the probe and block DNA denatured, the mix was

cooled down with ice at least for 5 minutes as soon as it was treated at 70°C for 10 minutes; 40µL mix was added to each slide, the slide was covered with a cover glass and denatured at 80°C for 5 minutes, then kept in a 37°C humid box overnight. After hybridization, the slides were washed in 2xSSC for 15 minutes at room temperature, then, stringent washing was followed in 0.1xSSC at 42°C for 30 minutes. The probe labeled with dig-11-dUTP was detected with the digoxigenin detection system. The slide was counterstained with 2ng/µL DAPI. Fluorescence microscopy was used to check the results and photographs were taken with a CCD camera attached to the microscope.

Chromosome classification and karyotyping

Based on the GISH results and according to the rule used by Stewart (1947) and Lim et al (2001a), chromosome classification and karyotypes were made.

Results

Chromosome numbers and genome composition of the cultivars

Representative GISH chromosome images of four cultivars, 041553, 041572, 041574 and 041580, are shown in Figure 3-1. Based on GISH results, the chromosome number and the genomic composition of all 19 cultivars are summarized in Table 3-1, and their karyotypes are shown in Figure 3-2. All cultivars consisted of both L- and A-genomes. 17 BC1 cultivars were triploid ($2n=3x=36$) and two aneuploid ($2n=3x+1=37$) (Table 3-1). All triploid cultivars consisted of 24 Asiatic chromosomes and 12 Longiflorum chromosomes (in the case of recombinant chromosomes, only the centromeres were taken into account). In view of their chromosomal composition, all of them had resulted from FDR $2n$ eggs produced by the F1 hybrids. Of the two aneuploid cultivars, which had 37 chromosomes, one had an extra Longiflorum chromosome and the other had an extra Asiatic chromosome. Both of them resulted from $2n+1$ egg produced by the F1 hybrids. In these cases, the extra chromosome in 041555 or 041572 originated from one univalent or one half-bivalent which might have lagged behind when the sister chromatids of the other univalents and half-bivalents

were segregating during the FDR process of their LA hybrids parents, after which $2n+1$ gametes were formed (Figure 3-2f & i: °; Figure 4-5: aneuploid).

Intergenomic recombination and crossover events

Four cultivars did not have any recombinant chromosome, they are represented with a common karyotype (Figure 3-2a). As to the karyotypes of the other 15 cultivars which had recombinant chromosomes, only the set (s) of chromosomes that contained substitution of segments or addition of chromosomes are shown (Figure 3-2b-p). For each set of chromosomes, the pair of chromosomes received from the LA parent is shown at the left and the single chromosome of the backcross parent, i.e., Asiatic cultivar, is shown on the right in each case (Figure 3-2).

For 15 cultivars, which contained recombinant chromosomes, in each genotype 1-3 pairs of homoeologous chromosomes were involved (Figure 3-2 b-p & Table 3-2). When chromosome 1 or 2 (metacentric chromosomes) was the recombinant one, the breakpoint occurred in the long or short arm, whereas when chromosomes 3 to 12 (acro- or sub-acrocentric chromosomes) were the recombinant ones with the exception of chromosome 8 of 041583 (Figure 3-2p), the breakpoints occurred only on the long arms, and all recombinations were either distal or proximal. Their karyotypes showed that most of the recombinant chromosomes probably resulted from a single crossover at pachytene stage during megasporogenesis of the F1 hybrids. When a single crossover occurs within a pair of homoeologous chromosomes (bivalent), the non-sister chromatids originating from the bivalent in its FDR $2n$ gametes would be non recombinant (L,A) and reciprocal recombinant (L/A, A/L), or two non reciprocal recombinant (L/A,A and L,A/L). Apart from the L,A type, which is indistinguishable from the non-crossover FDR $2n$ gametes, three other types, namely L/A ,A/L (Figure 3-2: °), L/A,A (Figure 3-2: *) and L,A/L (Figure 3-2: •) were found in these cultivars. The numbers of these different types were 10, 9 and 8 respectively (Table 3-2).

Table 3-1. The genome composition of 19 LAA cultivars derived from "LA x AA" (the number of recombinant chromosomes is in brackets).

Cultivars	Chromosome number	Genome composition	
		L (L/A)	A (A/L)
041551	36	12(1)	24(1)
041552	36	12(2)	24(2)
041553	36	12(1)	24(2)
041554	36	12(1)	24
041555	37	12(1)	25
041568	36	12	24
041569	36	12(1)	24(1)
041571	36	12(2)	24(2)
041572	37	13(2)	24(1)
041573	36	12(1)	24(1)
041574	36	12(1)	24(1)
041575	36	12(1)	24(1)
041576	36	12	24
041578	36	12(1)	24
041579	36	12	24
041580	36	12(3)	24(2)
041581	36	12	24(3)
041582	36	12	24
041583	36	12(1)	24(1)

*Abbreviation: L = Longiflorum chromosome; A = Asiatic chromosome

A/L = Asiatic chromosome whose segment was substituted with Longiflorum fragment.

L/A = Longiflorum chromosome whose segment was substituted with Asiatic fragment.

Table 3-2. Status of recombinant chromosomes in 15 LAA cultivars (the classification number of chromosomes is given in brackets).

Cultivars	No of recombinant chromosomes	No of paired homoeologous chromosomes	Reciprocal product present* L/A; A/L	Non reciprocal products	
				L/A	A/L
041551	2	1	1/1(8)	0	0
041552	4	3	1/1(10)	1(5)	1(11)
041553	3	3	0/0	1(12)	2(8,9)
041554	1	1	0/0	1(5)	0
041555*	1	1	0/0	1(5)	0
041569	2	1	1/1(2)	0	0
041571	4	3	1/1(1)	1(8)	1(10)
041572*	3	2	1/1(8)	1(12)	0
041573	2	1	1/1(9)	0	0
041574	2	1	1/1(9)	0	0
041575	2	1	1/1(12)	0	0
041578	1	1	0/0	1(6)	0
041580	5	3	2/2(7,11)	1(8)	0
041581	3	3	0/0	0	3(1,8,10)
041583	2	2	0/0	1(1)	1(8)
Total	37	27	10	9	8

* indicates the aneuploid cultivar which resulted from FDR gamete with an extra chromosome

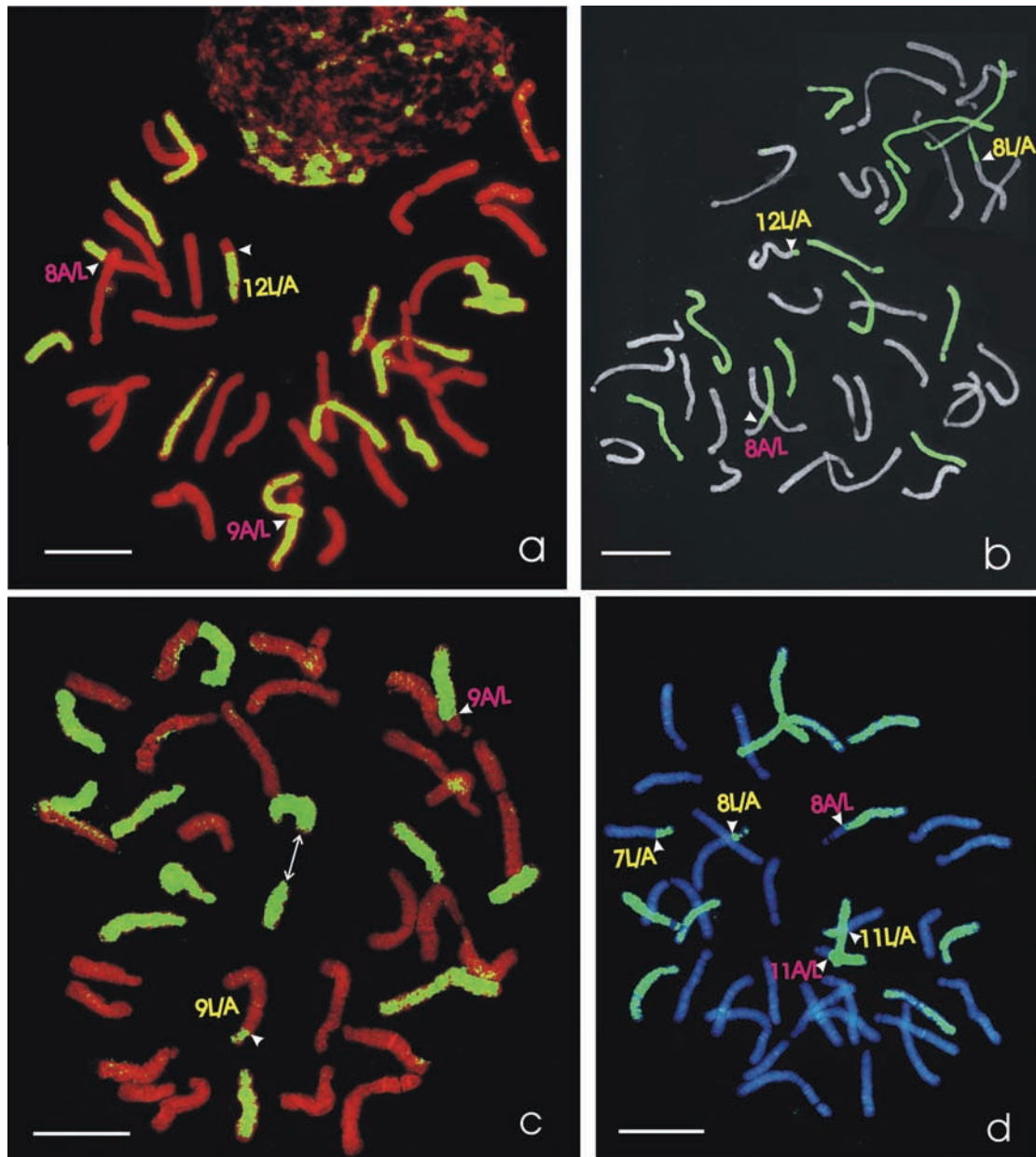
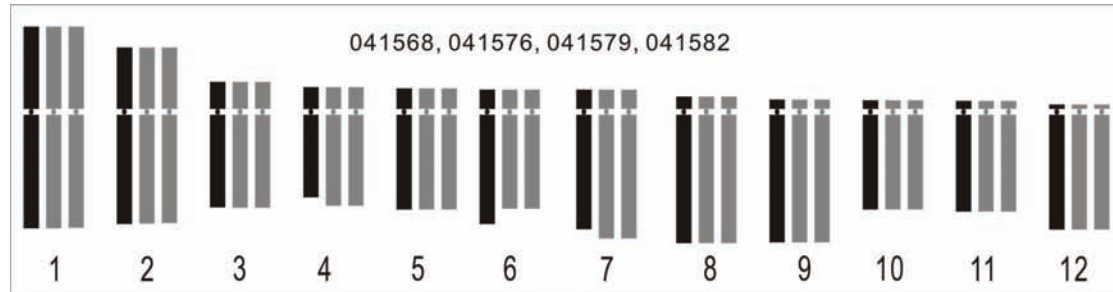


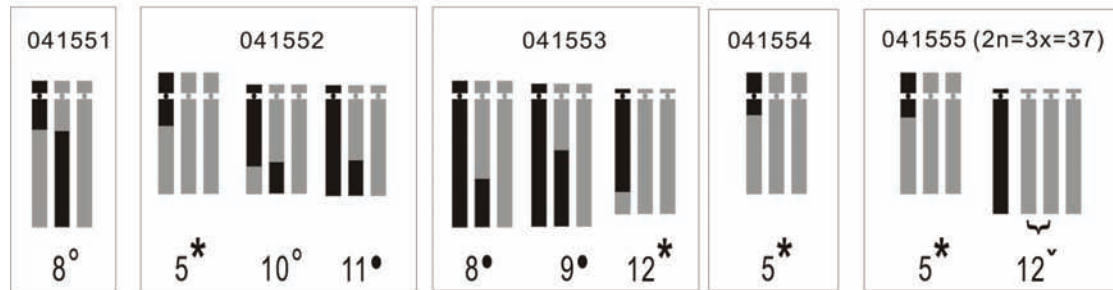
Figure 3-1. Genomic in situ hybridization on BC1 cultivars. Yellow or green chromosomes belong to L-genome, red, purple or grey chromosomes belong to A-genome. The recombinant chromosomes and their breakpoints were indicated. Bar =10 μ m. a. 041553, b. 041572, c. 041574 (the double arrow indicates two arms of one chromosome), and d. 041580.

◆—————▶
Figure 3-2. Karyotypes of 19 cultivars derived from crosses between diploid F1 LA hybrids and Asiatic cultivars. Black and grey bars represent Longiflorum and Asiatic chromosomes, respectively.

In those triploid BC1 progenies that possessed non reciprocal products of $L/A, A+A$, the latter being the chromosome of the backcross parent, there was a substitution for the distal recombinant segment (Figure 3-2: *). In these cases,



a



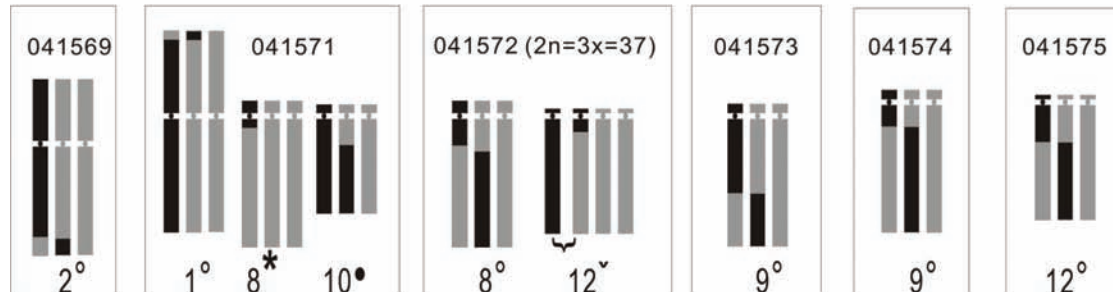
b

c

d

e

f



g

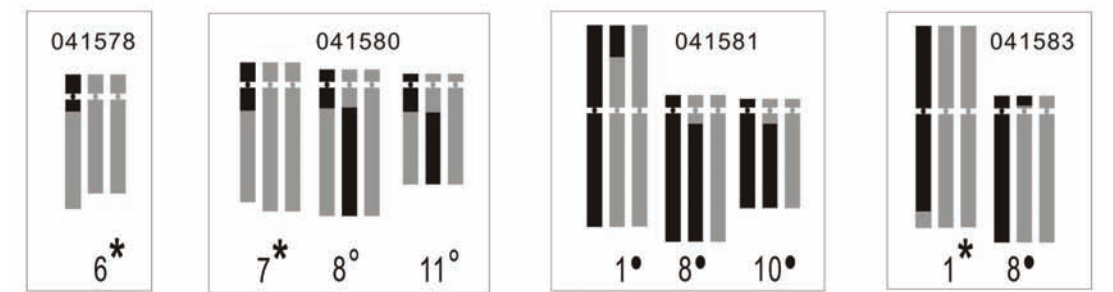
h

i

j

k

l



m

n

o

p

- ° = reciprocal recombination
- = nonreciprocal recombination and no substitution formed
- * = nonreciprocal recombination and substitution formed
- ˘ = chromosomes originated from sister chromatids indicated with "˘"

nulliplex condition (aaa) might be formed and play an important role in genetic variation of such BC1 progenies. On the contrary, such substitutions did not occur when the $\underline{L,A/L}+A$ combination was present.

According to the genome composition of the cultivars, reciprocal recombination could hardly change the genetic information unless the breakpoint(s) would have occurred in a functional gene. The consequence of this kind of recombination should be almost the same as that of non recombination. However, non reciprocal recombinations could cause genetic variations in BC1 progenies, especially, when substitution occurred for the recombinant segment. Among the 10 cultivars which contained non reciprocal recombinants, there were 9 cultivars in which substitution for the recombinant segment was formed. These results imply that this type of non reciprocal recombinants might have played an important role for cultivar selection.

Discussion

Mitotic polyploidization by chromosome doubling contributes little to introgression breeding, however, meiotic polyploidization induced by $2n$ gametes opens a promising way in plant polyploid and introgression breeding (Ramanna & Jacobsen 2003, Van Tuyl and Lim 2003). The previous researches reported that lily F1 interspecific hybrids produce functional unreduced male gametes which cause genetic variation of ALA and AOA BC1 progenies, respectively (Lim 2000, Barba-Gonzalez et al. 2005b). This point is confirmed by lily polyploid breeding with unreduced female gametes in the present investigation. The present research showed that F1 LA hybrids could produce functional unreduced female gametes and most cultivars contained recombinant chromosomes. All of these researches confirmed that $2n$ gametes are very valuable to lily polyploid breeding. Clearly, this is mainly because the genetic variation of $2n$ gametes caused by intergenomic recombination increases drastically the chances for selecting new cultivars directly from BC1 population.

Besides intergenomic recombination, the mechanism of $2n$ gamete formation is another important aspect for sexual polyploidization. The previous researches on

BC1 progenies revealed that the mechanisms of $2n$ pollen formed in lily F1 interspecific hybrids are FDR and IMR (Lim et al. 2001b, Barba-Gonzalez et al. 2004, 2005a, 2005b). Numerous examples of FDR $2n$ gametes produced by F1 interspecific hybrids have been reported in *Aegilops squarrosa* × *Triticum durum* (Sasakuma & Kihara 1981), emmer wheat × *A. squarrosa* (Fukuda & Sakamoto 1992), durum wheat × *A. squarrosa* and rye × *A. squarrosa* (Xu & Dong 1992, Xu & Joppa 1995), wheat × barley (Islam & Shepherd 1980), *Alstroemeria* interspecific hybrids (Ramanna et al. 2003), and others (Van Tuyl & Lim 2003). In present research, all triploid cultivars resulted from FDR $2n$ eggs. Even the two aneuploid cultivars resulted from FDR female gametes with an extra chromosome according to their genome composition. It is reasonable to assume that the FDR gametes are more vigorous than IMR gametes, because the chromosomal composition of FDR gametes is more balanced than those of IMR gametes. For $2n$ pollen, the investigation into microsporogenesis of F1 LA hybrids confirmed this point (Chapter 2).

Two aneuploid cultivars were found in the present investigation which means that some aneuploid eggs produced by F1 LA hybrids could survive in BC1 progenies. Although LAs have the possibility to produce large amount aneuploid pollen (Chapter 2), no aneuploid BC1 ALA or AOA progenies were found in the previous studies (Lim et al. 2001b, Barba-Gonzalez et al. 2004, 2005a, 2005b). Since many cultivars in some vegetative propagated crops, e.g. *Hyacinthus* (Van Scheepen 1991) and *Narcissus* (Brandham 1992), are aneuploid, it is expected that more and more aneuploid lily cultivars would be selected when interspecific hybrids in *Lilium* could produce functional aneuploid gametes.

In the mean time, the role of the backcross parent for the variation observed in BC1 progenies of crosses between LA hybrids and Asiatic cultivars could not be neglected. Out of 19 cultivars, in the present investigation, four cultivars did not have any recombinant chromosomes, and five cultivars only had reciprocal recombinant chromosomes. Why could so many of these kinds of BC1 progenies be selected as cultivars although their genome compositions look the same? Firstly, they originated from different cross combinations. Different Dutch lily

breeding companies used different Longiflorum and Asiatic cultivars to obtain different F1 LA hybrids, so, variation must have existed among different F1 LA hybrids, although their karyotypes look the same. Secondly, the fact that Longiflorum and Asiatic cultivars are very heterozygous should be considered. Their genetic backgrounds are very complex, especially, Asiatic cultivars originate from crosses among at least 12 wild species within the Sinomartagon section in the genus *Lilium*. So, there would be two factors which could cause variation within a BC1 LA population: one factor is the variation of $2n$ gametes formed in F1 LA hybrids; the other is chromosome assortment or recombination during n gamete formation in the backcrossing parent 'Asiatic cultivar'. The variation caused by $2n$ gametes produced by F1 LA hybrids can be directly detected by the GISH technique. However, it is also expected that chromosome assortment or recombination during haploid gamete formation of Asiatic cultivar could result in variation within its BC1 population, although the variation could not be detected at chromosome level with GISH. When no recombination had occurred in $2n$ gametes, the chromosome assortment or recombination during n gamete formation in the backcrossing parent would be the only factor which caused variation within one BC1 population.

Indeed, chromosome doubling plays hardly a role in introgression breeding. However, it is important for polyploid breeding, and if F1 hybrid could not produce some amount of $2n$ gametes, chromosome doubling is necessary to overcome F1 sterility for polyploid breeding. This technique has successfully been used for polyploidy breeding in numerous ornamental crops, e.g., *Iris* (Eikelboom & Van Eijk 1990), *Nerine*, *Lilium*, *Tagetes*, etc (Van Tuyl and Lim 2003).

Some examples of n gametes produced by F1 interspecific hybrids have been observed in *Allium cepa* x *A. fistulosum* (Emsweller & Jones 1945, Khrustaleva et al. 2000), *Alstroemeria aurea* x *A. inodora* (Kamstra et al. 1999a). Since fertile haploid gametes with homoeologous recombination are ideal for introgression breeding without increasing the ploidy level of the following generation, it would be valuable for introgression breeding in lilies if any such kind of F1 LA interspecific hybrids could be found (Chapter 4).

4 Investigations into the crossability of diploid Longiflorum x Asiatic hybrids of lilies (*Lilium*) with Asiatic cultivars and the obtained ploidy levels of the BC1 progenies

Abstract

Fertility and crossability of 30 F1 hybrids obtained from crosses between Longiflorum and Asiatic groups of lilies (LA hybrids) were tested and compared. Their BC1 progenies were analyzed using flow cytometry and genomic in situ hybridization. Although almost all LA hybrids were sterile, generally, most LA hybrids had better female fertility than male fertility. Genome compositions of some of the BC1 progenies analyzed with GISH showed that LA hybrids produced not only $2n$ or circa $2n$ gametes, but also n gametes as well. Different LA hybrids produced different kinds of gametes. Most of the LA hybrids produced only functional $2n$ gametes which resulted from first division restitution (FDR) and indeterminate meiotic restitution (IMR) during micro- or mega-sporogenesis of LA hybrids. However, five genotypes produced functional n gametes that resulted from normal meiosis of LA hybrids. This is the first time to find that LA hybrids produce functional n gametes. Both n and $2n$ gametes are expected to play different roles in lily breeding. The former could be more useful for introgression breeding while the later will be more useful for breeding polyploid cultivars.

Key words

Lilium, $2n$ gametes, n gametes, intergenomic recombination, first division restitution (FDR), indeterminate meiotic restitution (IMR)

Introduction

Modern lily cultivars mainly consist of three groups: Longiflorum, Asiatic and Oriental. Taxonomically, each group originates from crosses between varieties, cultivars or species within one section, i.e., *Leucolirion*, *Sinomartagon* and *Archelirion*, respectively (De Jong 1974, McRae 1998). These three groups have been confirmed to possess different genomes by genomic in situ hybridization (Karlova et al. 1999), and are named L-, A- and O-genomes, respectively. It is very difficult to obtain interspecific F1 hybrids in *Lilium*, however, crosses between different groups have been successful with the cut style pollination followed by embryo rescue techniques (Van Tuyl et al. 1991; Asano 1978, 1982a, b; Asano & Myodo 1980). Similar to most other interspecific hybrids, F1 distant interspecific hybrids in *Lilium* are highly sterile. In order to overcome their sterility, mitotic doubling and meiotic doubling have been used in lily breeding (Van Tuyl 1989, Van Tuyl & Lim 2003). Because an amphidiploid induced by mitotic doubling produces identical $2x$ gametes due to homologous pairing during meiosis, i.e., without intergenomic recombination, this technique is mainly useful for polyploidization breeding. On the contrary, meiotic chromosome doubling produces genetically variable $2n$ gametes as a result of intergenomic recombination, which are highly valuable for polyploidization as well as for introgression breeding (Ramanna & Jacobsen 2003). The previous researches with GISH analysis of triploid BC1 progenies indicated that they result from functional $2n$ pollen produced by the F1 hybrids. The mechanisms of $2n$ pollen formation were shown to be first division restitution (FDR) and indeterminate meiotic restitution (IMR) (Lim et al. 2001, 2003; Barba-Gonzalez et al. 2005a, 2005b). A recent investigation with GISH on 19 BC1 cultivars unraveled that these cultivars resulted from functional FDR $2n$ and $2n+1$ eggs (Chapter 3). But, no BC1 progenies which resulted from aneuploid pollen, IMR $2n$ egg or haploid gametes have been found with GISH analysis so far. Since a recent investigation on microsporogenesis of F1 hybrids between Longiflorum hybrids and Asiatic hybrids (LA) obtained by Dutch lily breeding companies showed that most LA hybrids have abnormal meiosis and some LA hybrids or some pollen mother cells have normal meiosis (Chapter 2), it was

expected that it would be possible for some of these LA hybrids to produce a very small amount of viable aneuploid pollen and haploid gametes. With a hope to find LA hybrids which can produce some functional haploid gametes, 30 LA hybrids, which were used as parents by the Dutch lily breeding companies to produce triploid lily cultivars (Chapter 3) and some of which were investigated for their microsporogenesis with GISH and conventional cytological methods (Chapter 2), were used in present investigation. Their fertility, crossability and their BC1 progenies were evaluated.

Material and methods

Plant material

30 diploid LA hybrids ($2n=2x=24$), used as parents to produce cultivars, were supplied by four Dutch lily breeding companies, De Jong Lelies BV, Testcentrum BV, Vletter & Den Haan BV and World Breeding BV (Table 4-1). For the purpose of backcrossing with the above genotypes, three diploid Asiatic cultivars ($2n=2x=24$), 'Mont Blanc', 'Pollyanna' or 'Vivaldi' were used. They were grown using standard growing conditions applicable for lily cultivation at Plant Research International, Wageningen University & Research Center, the Netherlands.

Pollen germination test

One day before anthesis, the anthers were collected and stored in an exsiccator for pollen germination. The media used for pollen germination contained 100g/L sucrose, 5g/L bacteriological agar, 20mg/L boric acid and 200mg/L calcium nitrate. When anthers were open, a small amount of pollen grains was placed on this media at 25°C for 5-20 hours. The percentage of pollen germination was estimated using an anatomical microscope.

Backcross procedure

All the LA hybrids were used as female parents to backcross with 'Mont Blanc', 'Pollyanna' or 'Vivaldi', and their progenies are called LAAs. However, when the LA hybrids were used as male parents in their backcrosses with Asiatic cultivars, only

those which had a little male fertility according to pollen germination test were used and their progenies are named ALAs.

Embryo rescue

About 4-10 weeks after pollination, the maturation of fruits was checked. The fruits were harvested for embryo rescue when they were soft or yellow. The surface of harvested fruit was sterilized with 80% ethanol for a few seconds in a laminar air flow cabinet. Then, cut it open and its ovules were put in a sterilized Petri-dish. The swollen ovules, i.e. those of which embryo or embryo sacs developed, were selected for in vitro culture. After their coats were carefully removed under an anatomic microscope, they were put on the medium (pH=5.8) consisting of 2.2g MS, 60g sucrose and 4g gelrite per liter. After this, they were stored in a dark chamber at 25°C.

Evaluation of fertilities and crossabilities of LA hybrids

Male fertility was estimated by the percentage of their pollen that germinated (Table 4-1). Female fertility was expressed by the percentage of embryos and swollen embryo sacs obtained by embryo rescue (Table 4-1). It was calculated as the number of the embryos and swollen sacs obtained by embryo rescue divided by the number of the ovules used in embryo rescue x 100. The percentage of germinated embryos or sacs was calculated as the number of the germinated embryos and embryo sacs divided by the number of embryos and embryo sacs obtained by embryo rescue x 100. The percentage of germinated embryos or embryo sacs represents the viabilities of the embryos or swollen sacs. The crossability was used to indicate the rate of success of a particular cross. It was calculated with the formula: the number of the seedlings divided by the number of ovules used for embryo rescue x 100.

Flow cytometry

Flow cytometry was used to evaluate the ploidy levels of the BC1 progenies. When the embryos or embryo sacs germinated and grew until 4-5 leaves sprouted, one

leaf, scale or root was collected from each seedling for testing its ploidy level as described by Van Tuyl & Boon (1997).

Genomic in situ hybridization (GISH)

Chromosome preparation was made according to the description of Zhou et al. (2003).

Probe and block preparation: Total genomic DNA was extracted from *L. longiflorum* 'White Fox' and Asiatic cultivar 'Mont Blanc' with the CTAB method (Rogers & Bendich 1988). 'White Fox' DNA was sonicated to 1-10kb fragments and used as probe. 'Mont Blanc' DNA was autoclaved to 200-600bp fragments and used as block. The probe DNA was labeled with digoxigenin-11-dUTP by the nick translation method according to the manufacturer's instruction (Roche, Germany). In situ hybridization mainly consisted of four parts, i.e., chromosome pre-treatment, hybridization, stringency washing and detection. For chromosome pre-treatment, the slides were treated with 100µg/mL RNase for one hour and 5 µg/mL pepsin for 10 minutes, then fixed in 4% paraformaldehyde for 10 minutes, after each of these treatments, the slides were washed with 2xSSC for 5 minutes each three times, then, the slides were treated for three minutes with 70, 90 and 100% ethanol, respectively. Finally, the slides were dried in air at least for 30 minutes. For hybridization, the hybridization mix contained 50% formamide, 10% dextranulphate, 2xSSC, 0.25% SDS, 0.6-1.5ng/µL probe and 25-100ng/µL block DNA, in order to keep probe and block DNA denatured, the mix was cooled down with ice at least for 5 minutes as soon as it was treated at 70°C for 10 minutes; 40µL mix was added to each slide, the slide was covered with a cover glass, and denatured at 80°C for 5 minutes, then kept in a 37°C humid box overnight. After hybridization, the slides were washed in 2xSSC for 15 minutes at room temperature, then, stringency washing was followed in 0.1xSSC at 42°C for 30 minutes. The probe labeled with dig-11-dUTP was detected with the digoxigenin detection system. The slide was counterstained with 2ng/µL DAPI. Fluorescence microscopy was used to check the results and photographed with a CCD camera attached to the microscope.

Results

The male and female fertilities of LA hybrids

The male fertilities and female fertilities of LA hybrids are shown in Table 4-1. According to their pollen germination, 24 of the 30 LA hybrids were completely male sterile. If these male sterile LA hybrids were used as male parents to backcross with any Asiatic cultivar, it was expected that no BC1 progeny could be obtained. In order to evaluate their female fertilities, all LA hybrids were used as female parent in backcrosses with the Asiatic cultivar 'Pollyanna'. Surprisingly, only four genotypes (041518, 041519, 041522 and 041566) aborted, the others contained more or less swollen embryo sacs (Table 4-1). Clearly, the female fertilities of the LA hybrids were in general much better than their male fertilities, although the reverse was also observed in genotypes 041501, 041519 and 041502 (Table 4-1).

Table 4-1. The comparison between male and female fertilities of 30 diploid LA F1 hybrids

Genotype	Pollen germination percentage	Swollen embryo sacs percentage*
041501	5-10	0.3
041502	20-30	14.5
041511	0	0.35
041512	0	0.7
041514	0	0.75
041517	0	0.9
041518	0	0
041519	20-30	0
041521	0	0.5
041522	0	0
041523	0	0.35
041543	0	1.1
041544	0-1	8.75
041545	0	1
041546	0	7
041547	0	3.5
041548	0	5.5
041549	0	2.65
041550	0	12.5
041556	0	7.75
041557	1-5	4.25
041558	0	43.35
041559	0	12.5
041560	0	6.65
041562	0	15
041563	0	5.5
041564	1-2	3.35
041565	0	4.75
041566	0	0
041567	0	2

* The data were based on the results of "LA x Pollyanna"

The results of backcrosses between LA hybrids and Asiatic cultivars

That the LA hybrids were selected as male parents to backcross with Asiatic cultivars was based on their pollen germination results (Table 4-1). So, only six LA hybrids were selected as male parents to backcross with 'Pollyanna', 'Mont Blanc' or 'Vivaldi' (Table 4-2). 12 combinations of this kind of backcross (AA x LA) were made by pollination (Table 4-2). Four combinations somewhat developed, others aborted according to the result of embryo rescue (Table 4-2: Em & Es). Even in the four successful combinations, most pollinated flowers could not develop well because, on average, there were 1~7.7 swollen embryo sacs or embryos obtained from each harvested fruit (Table 4-2: Em & Es per fruit). Their crossabilities varied from 0.5 to 1.5% (Table 4-2: Crossability). In total, 42 embryos or embryo sacs were obtained by embryo rescue, and 29 of them germinated. Finally, 21 of them survived and developed into plants.

Table 4-2. The results of crosses using F1 LA hybrids as male and Asiatic cultivars as female*.

LA ♂	AA ♀	Code of crosses	Fruits	Em & Es	Em & Es per fruit	Em & Es germinated	Plants	Germinated (%)	Crossability (%)
041501	Pollyanna	044594	9	0	0	0	0		
	Mont Blanc	044600	2	0	0	0	0		
041502	Pollyanna	044592	6	0	0	0	0		
	Mont Blanc	044601	3	23	7.7	15	9	65.22	1.5
	Vivaldi	044638	3	12	4	8	6	66.67	1
041519	Pollyanna	044586	2	0	0	0	0		
	Mont Blanc	044595	3	5	1.7	4	4	80	0.67
041544	Pollyanna	044596	4	0	0	0	0		
041557	Pollyanna	044598	4	0	0	0	0		
	Mont Blanc	044602	2	2	1	2	2	100	0.5
041564	Pollyanna	044560	2	0	0	0	0		
	Mont Blanc	044559	2	0	0	0	0		
Total			42	42		29	21		
Average								69.05	0.25

* LA=F1 LA hybrids AA=Asiatic cultivars Em & Es=embryo(s) and embryo sac(s)

* Crossability is calculated as the number of plants divided by 200x the number of fruits, because there is about 200 ovules per fruit in lilies

All 30 LA hybrids were used as female parents to backcross with Asiatic cultivars (Table 4-3). 46 combinations of this kind of backcross (LA x AA) were made (Table 4-3). After embryo rescue, 12 combinations completely aborted because there were no embryos or swollen embryo sacs obtained. The other 34 combinations were apparently successful (Table 4-3). Most of their fruits developed well on the

basis of their appearance. However, inside the fruits they were quite different because the number of swollen embryo sacs or embryos in each fruit varied from 0.6 to 86.7 (Table 4-3). Their crossabilities varied from 0.05 to 8.33% (Table 4-3). In total, 543 embryos or embryo sacs were obtained by embryo rescue, and 91 of them germinated, of which 30 survived and developed into plants. When the results between LA x AA and AA x LA were compared, obviously, the viability of developed embryo sacs or embryos from AA x LA was much higher than that from LA x AA, because, on average, 69.05% of the embryo sacs or embryos from AA x LA could germinate, whereas, only 16.76% of those from LA x AA germinated (Tables 4-2 & 4-3). It is unclear why many more swollen embryo sacs of LA x AA than those of AA x LA could not germinate and develop into plants. Although the average crossability of AA x LA (0.25%) is almost two times higher than that of LA x AA (0.11%), we can not conclude that the average crossability of AA x LA is better than that of LA x AA, because the data of AA x LA is based on only six LA hybrids which have some extent of male fertility based on the pollen germination test. Because of absence of pollen germination it was expected that no progenies could be obtained if the other 24 male sterile LA hybrids would have been used as male parents. So, theoretically, the average crossability of AA x LA is 0.05% when all 30 F1 hybrids are taken into account, making the average crossability of LA x AA better than that of AA x LA. This means that it was more likely to get progenies when LA hybrids were used as female parents. However, if only the combinations in which male fertile F1 LA hybrids were used as male parents to backcross with Asiatic cultivars are taken into account, the percentage of germinated embryo sacs, and crossability of AA x LA are higher than those of LA x AA, respectively. This indicates, that it was easier to get plants from AA x LA than from LA x AA when LA had a relatively good male fertility. As to a particular LA genotype, its crossability, whether of LA x AA or of AA x LA, always varied depending on its backcrossing parent, i.e. Asiatic cultivars. According to the present results, in AA x LA backcrosses, 'Mont Blanc' as female parent is better than 'Pollyanna' (Table 4-2); however, as male parent in some cases of LA x AA, 'Pollyanna' is better and in others, 'Mont Blanc' is better (Table 4-3).

Table 4-3. The result of crosses using F1 LA hybrids as female and Asiatic cultivars as male* .

LA ♀	AA ♂	Code of crosses	Fruits	Em & Es	Em & Es per fruit	Em & Es germinated	Plants	Germinated (%)	Crossability (%)
041501	Pollyanna	044582	5	3	0.6	1	0	33.33	
	Mont Blanc	044629	7	0	0	0	0		
041502	Pollyanna	044583	1	29	29	3	0	10.34	
	Mont Blanc	044630	11	19	1.7	3	1	15.78	0.05
041511	Pollyanna	044546	3	2	0.7	0	0		
041512	Pollyanna	044584	5	7	1.4	1	0	14.29	
041514	Pollyanna	044585	2	3	1.5	2	0	66.67	
041517	Pollyanna	044545	4	7	1.75	0	0		
041518	Pollyanna	044627	2	0	0	0	0		
041519	Pollyanna	044586	2	0	0	0	0		
041521	Pollyanna	044588	5	5	1	0	0		
041522	Pollyanna	044589	3	0	0	0	0		
041523	Pollyanna	044628	3	2	0.7	0	0		
041543	Pollyanna	044518	6	13	2.2	4	1	30.77	0.08
	Mont Blanc	044511	3	20	6.7	9	2	45	0.33
041544	Pollyanna	044543	2	35	17.5	2	1	5.71	0.25
	Mont Blanc	044613	2	0	0	0	0		
041545	Pollyanna	044610	6	12	2	0	0		
	Mont Blanc	044615	3	0	0	0	0		
041546	Pollyanna	044568	1	14	14	1	0	7.14	
	Mont Blanc	044616	7	0	0	0	0		
041547	Pollyanna	044542	3	21	7	4	0	19.05	
	Mont Blanc	044617	8	4	0.5	0	0		
041548	Pollyanna	044567	2	22	11	4	1	18.18	0.25
	Mont Blanc	044618	2	1	0.5	1	0	100	
041549	Pollyanna	044541	3	16	5.3	3	2	18.75	0.33
	Mont Blanc	044619	7	0	0	0	0		
041550	Pollyanna	044581	1	25	25	6	2	24	1.00
	Mont Blanc	044620	2	0	0	0	0		
041556	Pollyanna	044520	2	31	15.5	2	0	6.45	
	Mont Blanc	044525	1	8	8	2	1	25	0.50
041557	Pollyanna	044540	2	17	8.5	4	0	23.52	
	Mont Blanc	044571	1	10	10	4	4	40	2.00
041558	Pollyanna	044539	0.3	26	86.7	8	5	30.77	8.33
	Mont Blanc	044512	1	25	25	4	1	16	0.50
041559	Pollyanna	044611	1	25	25	2	1	8	0.50
041560	Pollyanna	044538	3	40	13.3	9	4	22.5	0.67
	Mont Blanc	044621	1	0	0	0	0		
041562	Pollyanna	044535	1	30	30	3	1	10	0.50
041563	Pollyanna	044536	1	11	11	1	1	9.09	0.50
	Mont Blanc	044513	1	9	9	0	0		
041564	Pollyanna	044537	3	20	6.7	3	1	15	0.17
041565	Pollyanna	044624	2	19	9.5	5	1	26.31	0.25
041566	Mont Blanc	044612	1	0	0	0	0		
041567	Pollyanna	044565	3	12	4	0	0		
	Mont Blanc	044623	1	0	0	0	0		
Total			136.3	543		91	30		
Average								16.76	0.11

* The abbreviations and the calculation for crossability are the same as in Table 4-2

Table 4-4. The results of BC1 progenies' ploidy levels using flow cytometry.

Types of backcross	Code of crosses	Parents		Plants tested with flow cytometry	No. of Plants with ploidy level	
		Female	Male		2x	3x
AA x LA	044595	Pollyanna	041519	3	1	2
	044601	Mont Blanc	041502	9		9
	044602	Mont Blanc	041557	2	2	
	044638	Vivaldi	041502	6		6
LA x AA	044511	041543	Mont Blanc	2	2	
	044518	041543	Pollyanna	1		1
	044525	041556	Mont Blanc	1		1
	044535	041562	Pollyanna	1		1
	044536	041563	Pollyanna	1	1	
	044541	041549	Pollyanna	1		1
	044611	041559	Pollyanna	1		1
	044624	041565	Pollyanna	1		1
	044512	041558	Mont Blanc	1		1
	044538	041560	Pollyanna	4	4	
	044539	041558	Pollyanna	5		5
	044567	041548	Pollyanna	1		1
	044571	041557	Mont Blanc	4		4
	044630	041502	Mont Blanc	1		1
	Total				45	10

Ploidy levels of BC1 progenies based on flow cytometry

Out of the 51 new BC1 progenies, 45 were tested using flow cytometry for their ploidy level (Table 4-4). 10 of them were diploid, and 35 were triploid. Out of 20 ALA BC1 progenies, only three were diploid. However, among 25 LAA progenies, seven were diploid. Therefore, a few LA genotypes produced functional haploid gametes. According to this result, it was found that 041543, 041560 and 041563 produced only functional n eggs; 041519 produced n and $2n$ pollen; and 041557 produced functional n pollen and $2n$ eggs.

Chromosome numbers and genome compositions of BC1 progenies

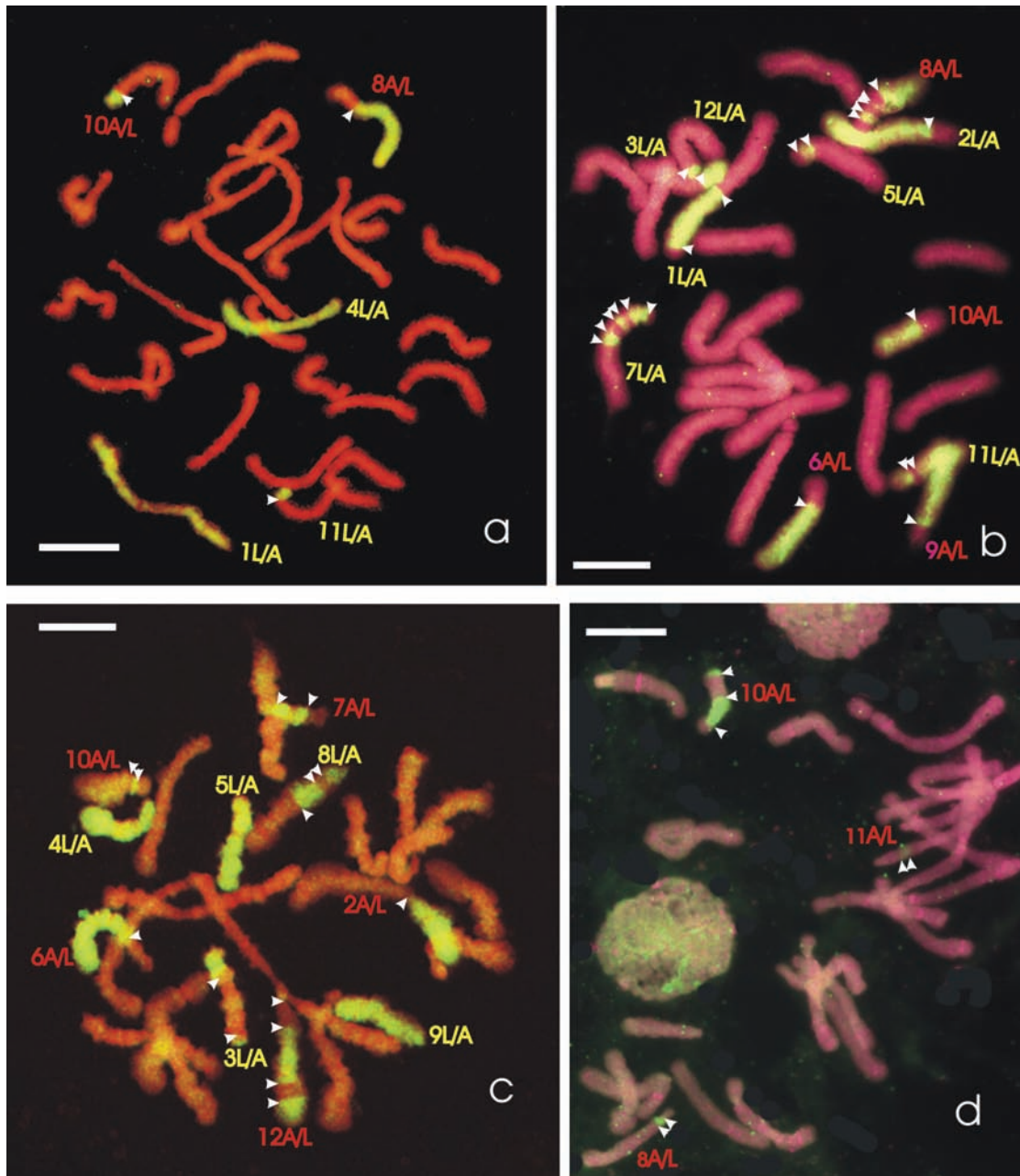
Five diploid and 18 triploid progenies were analyzed with GISH technique. The representative GISH results of four diploid BC1 progenies (044511-1, 044538-1, 044538-4 and 044602-2) and four triploids (044539-1, 044601-2, 044601-3, and 044638-3) are shown in Figures 4-1 and 4-2, respectively. Based on the GISH

results, the genome compositions of all the 23 BC1 progenies are summarized in Table 4-5, and the karyotypes of diploid and triploid BC1 progenies are shown in Figure 4-3 and Figure 4-4, respectively. GISH results clearly showed that all the BC1 progenies were composed of L-genome and A-genome. Five diploid BC1 progenies ($2n=2x=24$) resulted from n gametes produced by LA hybrids. This confirmed that normal meiosis had occurred in the LA hybrids (Figure 4-5: haploid). Out of 18 triploid BC1 progenies, 14 were euploid, four were aneuploid. 10 of the 14 euploids ($2n=3x=36$) resulted from FDR $2n$ gametes because each of them contained 12 L-genome chromosomes and 24 A-genome chromosomes (Table 4-5 and Figure 4-5: FDR), and four of them resulted from IMR $2n$ gametes because each of them contained 10 L-genome chromosomes and 26 A-genome chromosomes (Table 4-5 and Figure 4-5: IMR). Three of the four aneuploids had 35 chromosomes ($2n=3x-1$), and one of them had 37 ($2n=3x+1$), the addition or missing chromosomes in the aneuploid progenies were caused by abnormal FDR process during which one univalent or half-bivalent had lagged from others when the sister chromatids were segregating (Table 4-5 and Figure 4-5: Aneuploid).

Intergenomic recombination and crossover events

According to the GISH results on five diploid BC1 progenies (Figures 4-1 and 4-3), they had 3 to 11 recombinant chromosomes, and they contained 1~7 L-genome chromosomes (Figure 4-3 & Table 4-5). The number of breakpoints (1~6) on each recombinant chromosome implied that different crossover events must have occurred in the paired homoeologous chromosomes during meiosis of LA hybrids, e.g. chromosome 7 of 044538-1 had six breakpoints and, therefore, multiple crossover events must have occurred in the paired homoeologous chromosomes. However, that the chromosomes which also originated from haploid gametes of LA hybrids did not have any breakpoint does not mean that no crossovers had occurred in their paired homoeologous chromosomes. In Figure 4-5 (haploid), when a single crossover occurs in a pair of homoeologous chromosomes (bivalent), the possibilities of both recombinant haploid gamete and non-recombinant haploid gamete are 50%, respectively. In fact, if one sister chromatid

is not involved, regardless of how many crossovers had occurred in the paired homoeologous chromosomes, the possible occurrence of non-recombinant haploid gametes is 25%. In BC1 progenies the surviving chromosomes originated from haploid gametes of LA hybrids were the consequence of sister chromatid segregation and assortment during meiosis of LA hybrids and, probably, they were related to their backcrossing parents.



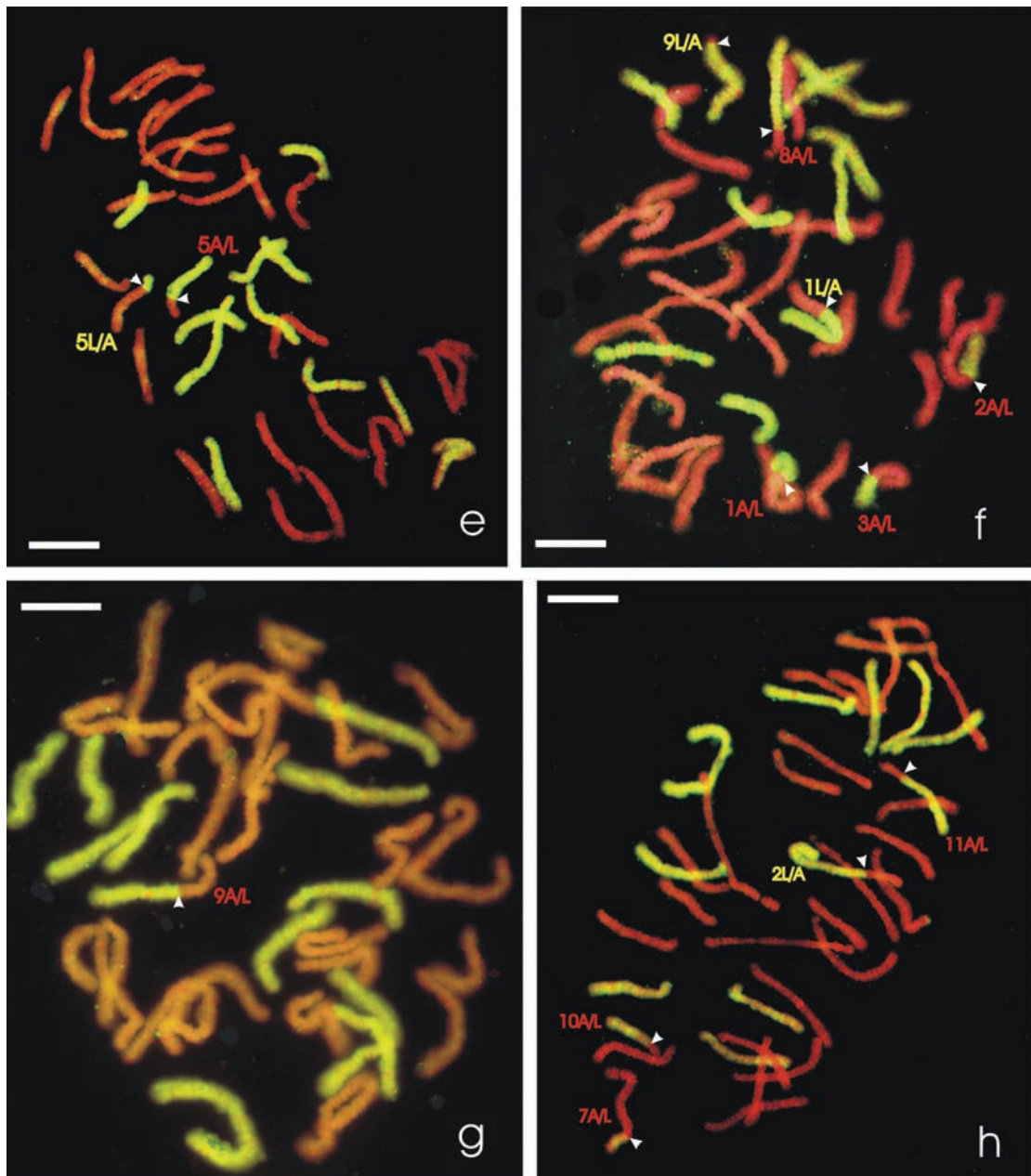


Figure 4-2. Genomic in situ hybridization in triploid and aneuploid BC1 progenies. Yellow or green chromosomes belong to L-genome, Red or pink chromosomes belong to A-genome. The recombinant chromosomes and their breakpoints were indicated. e. 044539-1, f. 044601-2, g. 044601-3, h. 044638-3. Bar =10 μ m.

←
Figure 4-1. Genomic in situ hybridization in diploid BC1 progenies. Yellow or green chromosomes belong to L-genome, Red or pink chromosomes belong to A-genome. The recombinant chromosomes and their breakpoints were indicated. a. 044511-1, b. 044538-1, c. 044538-4, and d. 044602-2. Bar =10 μ m

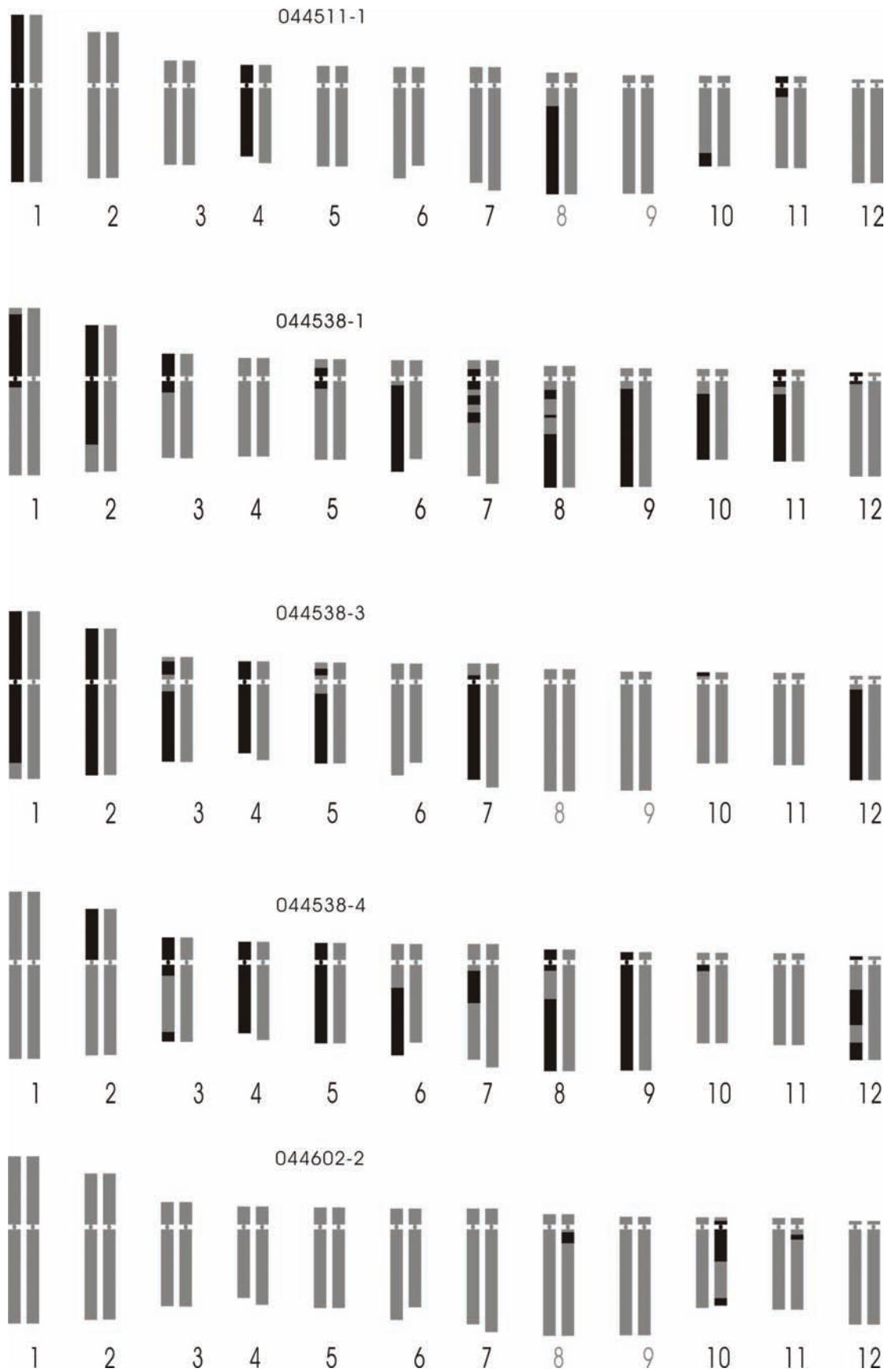
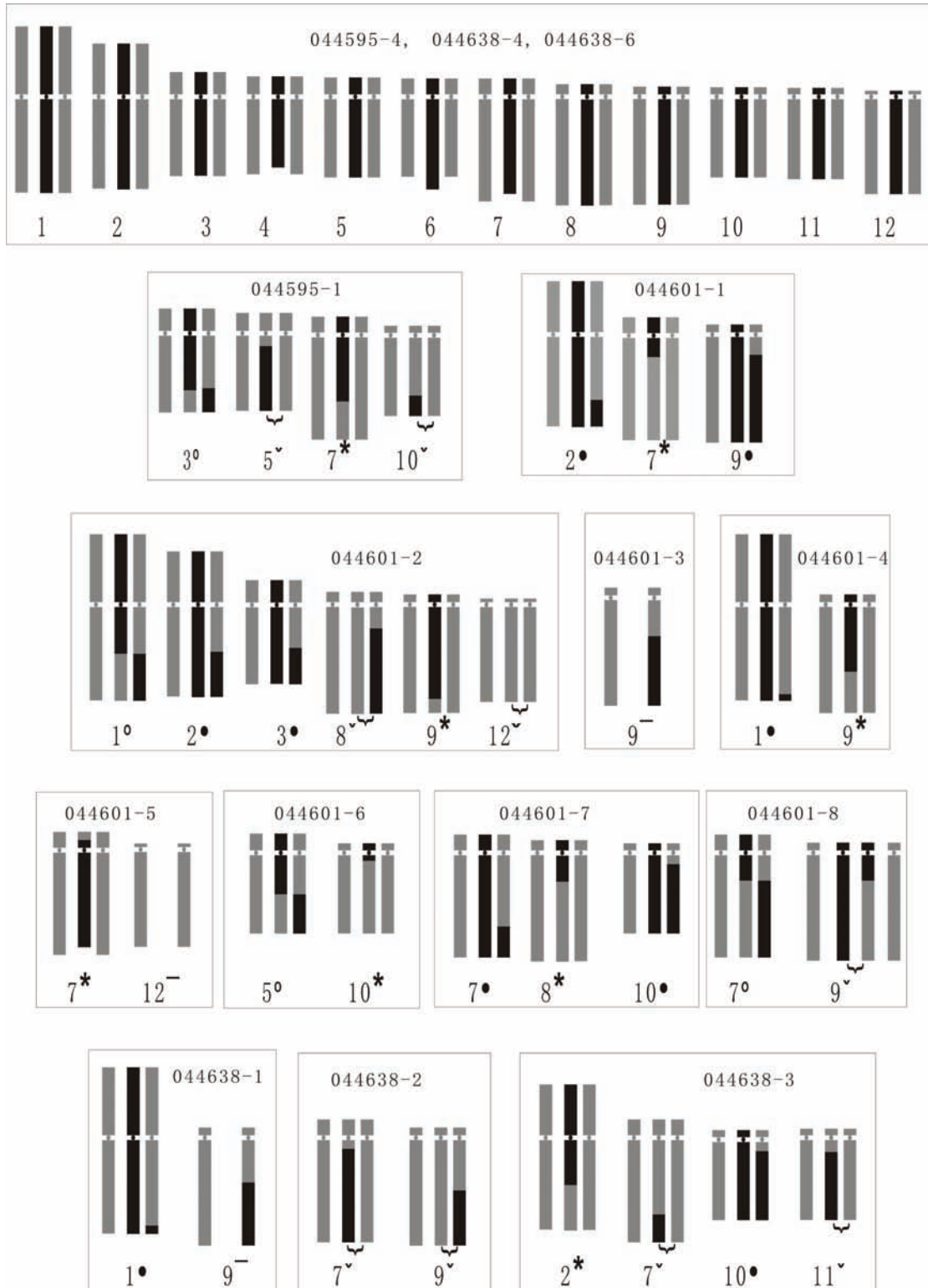
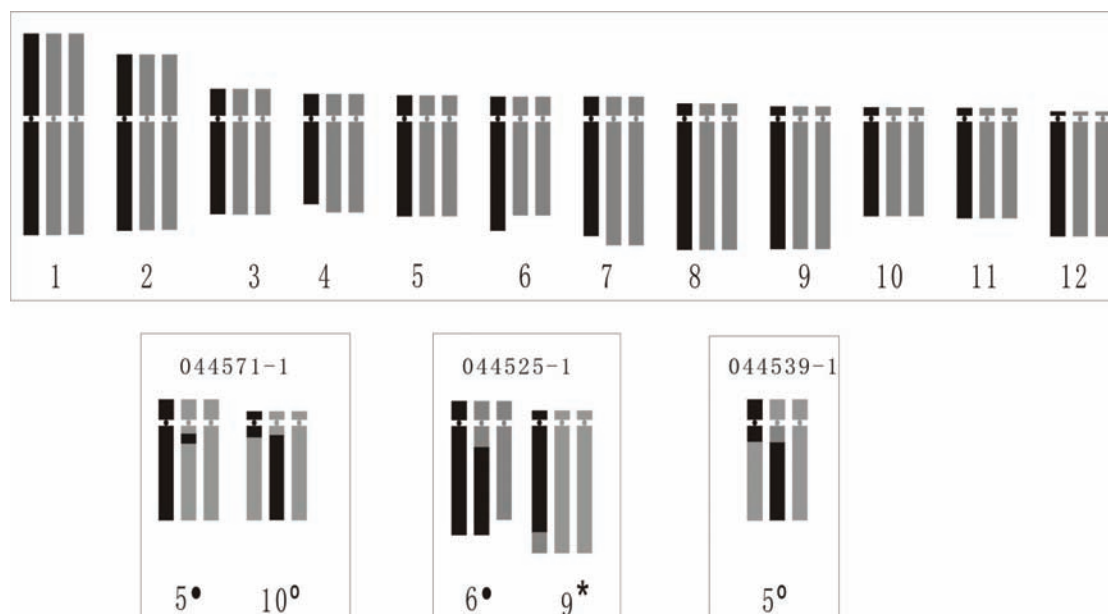


Figure 4-3. The karyotypes of five diploid BC1 progenies. Grey and black bars represent Asiatic and Longiflorum chromosomes, respectively.



(1) The karyotypes of 15 ALA BC1 progenies (only the chromosome sets with recombination are shown in 12 cases)



(2) The karyotypes of three LAA BC1 progenies

Figure 4-4. The karyotypes of 18 BC1 progenies derived from crosses between diploid LAs and Asiatic cultivars. Black and grey bars represent Longiflorum and Asiatic chromosomes, respectively.

° = reciprocal recombination

• = non reciprocal recombination and no substitution formed

* = non reciprocal recombination and substitution formed

~ = chromosomes originated from sister chromatids indicated with “{”

- = a chromosome missing.

Of the 15 triploid ALA progenies, three progenies (044595-4, 044638-4 and 044638-6) that did not have any recombinant chromosome, are represented by a common karyotype (Figure 4-4(1)). The other 12 progenies (044595-1, 044601-1~8, 044638-1~3) are shown with their set(s) of chromosomes which contained recombination or addition or missing chromosomes (Figure 4-4(1)). All three LAAs contained recombinant chromosomes. Similarly, only the set(s) of chromosomes that contained recombinant chromosome(s) are shown in Figure 4-4(2). In ALA progenies, for each set of chromosomes, the pair of chromosomes received from the LA parent is shown on the right and the single chromosome of the backcross parent, i.e., Asiatic cultivar, is shown at the left in each case; and vice versa in LAA progenies. Except that chromosome 5 of 044571-1 (LAA) at least resulted from two crossovers, most other recombinant chromosomes probably resulted

from a single crossover. In total, 6 $\underline{L/A,A/L}$ (Figure 4-4: ° reciprocal recombination), 11 $\underline{L,A/L}$ (Figure 4-4: • non reciprocal recombination and no substitution formed) and 9 $\underline{L/A,A}$ (Figure 4-4: * non reciprocal recombination and substitution formed) were found in 15 recombinant triploid and circa triploid BC1 progenies. These intergenomic recombinations were the main reason for the observed $2n$ gamete variation.

Table 4-5. The chromosome numbers and genome compositions of BC1 progenies and origin of gamete type.

Genotypes of BC1 progeny	Parents		Chromosome number	Genome composition		Origin of gamete type
	♀	♂		L ($^L/A$)	A ($^A/L$)	
044511-1	LA	AA	24	3(1)	21(2)	n
044538-1	LA	AA	24	7(7)	17(4)	n
044538-3	LA	AA	24	4(2)	20(4)	n
044538-4	LA	AA	24	5(2)	19(5)	n
044602-2	AA	LA	24	1(1)	23(2)	n
044525-1	LA	AA	36	12(1)	24(1)	FDR-2n
044539-1	LA	AA	36	12(1)	24(1)	FDR-2n
044571-1	LA	AA	36	12(1)	24(2)	FDR-2n
044595-1	AA	LA	36	10(2)	26(3)	IMR-2n
044595-4	AA	LA	36	12(0)	24(0)	FDR-2n
044601-1	AA	LA	36	12(1)	24(2)	FDR-2n
044601-2	AA	LA	36	10(2)	26(4)	IMR-2n
044601-3	AA	LA	35	11(0)	24(1)	2n-1
044601-4	AA	LA	36	12(1)	24(1)	FDR-2n
044601-5	AA	LA	35	11(1)	24(0)	2n-1
044601-6	AA	LA	36	12(2)	24(1)	FDR-2n
044601-7	AA	LA	36	12(0)	24(3)	FDR-2n
044601-8	AA	LA	37	13(2)	24(1)	2n+1
044638-1	AA	LA	35	11(1)	24(1)	2n-1
044638-2	AA	LA	36	10(0)	26(2)	IMR-2n
044638-3	AA	LA	36	10(1)	26(3)	IMR-2n
044638-4	AA	LA	36	12(0)	24(0)	FDR-2n
044638-6	AA	LA	36	12(0)	24(0)	FDR-2n

L ($^L/A$) = The number of Longiflorum chromosomes (the number of Longiflorum chromosomes which contained Asiatic chromosome segments), and vice versa to A ($^A/L$).

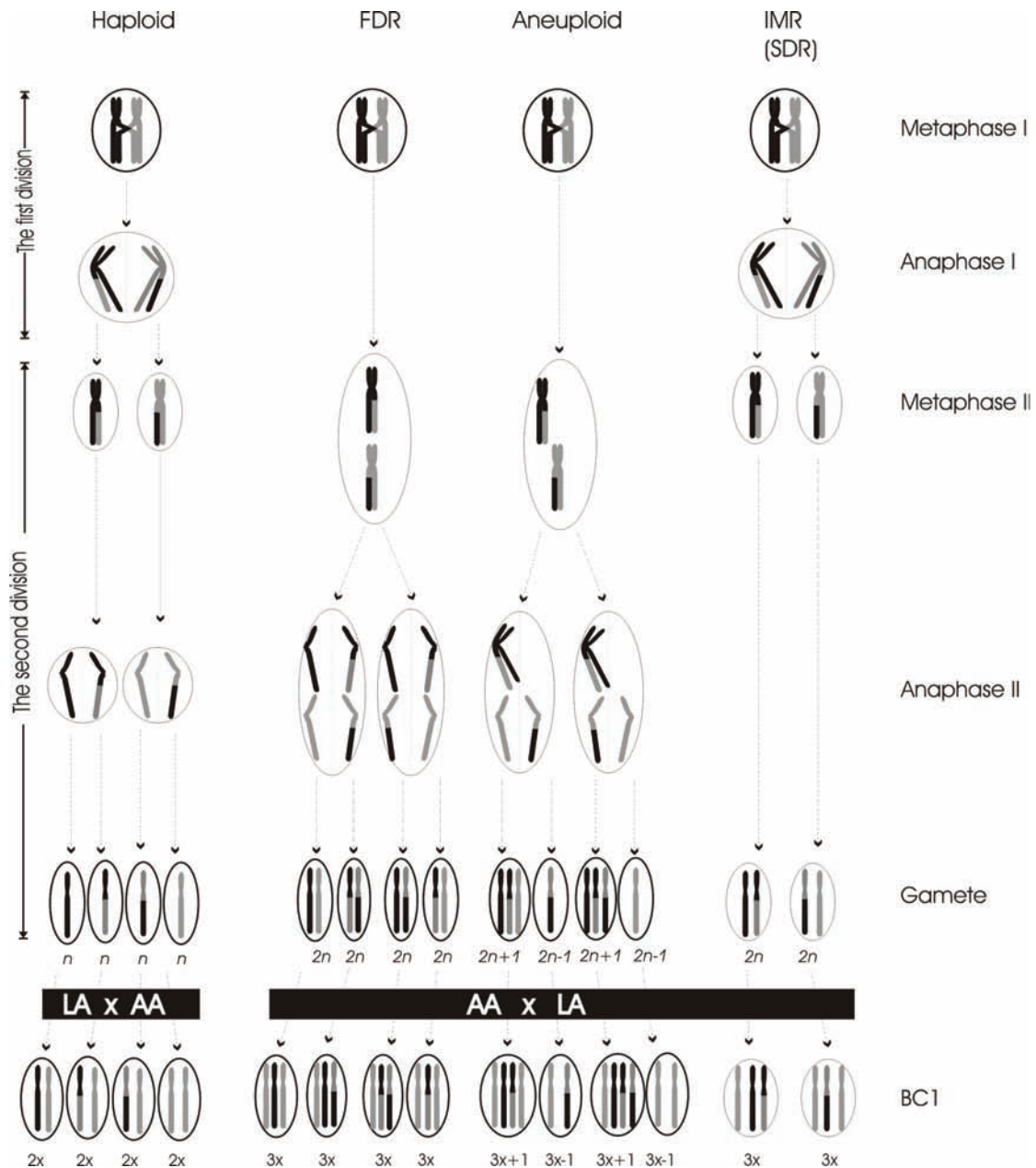


Figure 4-5. The possible consequences of a pair of homologous chromosomes with a single crossover in haploid, FDR $2n$, aneuploid and IMR $2n$ gamete formation after meiosis of LA hybrids, and their expression in BC1 progenies when Asiatic cultivars were used as backcrossing parents.

Discussion

The fertilities of the LA hybrids which were used as parents to produce lily cultivars from BC1 progenies by the Dutch lily breeding companies were variable. Most of them are highly male sterile, however, a few LA hybrids had some amount of male fertility as was evident from their pollen germination. This result is almost the same as was previously reported in LA and OA lily hybrids (Van Tuyl et al.

2002a, Lim et al. 2001b, Barba-Gonzalez et al. 2004), and similar to most other distant interspecific hybrids, such as *Alstroemeria aurea* x *A. caryophyllaea*, *Iris hollandica* x *I. tingitana*, *Tagetes erecta* x *T. patula*, and so on (reviewed in Van Tuyl & Lim 2003). With regard to their female fertilities, most of the LA hybrids selected by the Dutch lily breeding companies are not as completely sterile as their male sterility. Probably, this is the reason why most of cultivars selected directly from BC1 progenies by Dutch breeders originated from LA x AA crosses (Chapter 3). Based on an investigation on microsporogenesis it was evident that these LA hybrids have the possibility to produce not only $2n$ or aneuploid gametes but also haploid gametes, because abnormal and normal meiosis in different LA hybrids or different pollen mother cells of the same plant were observed (Chapter 2). In previous research, functional $2n$ pollen produced by OA hybrids (Barba-Gonzalez et al. 2005a, 2005b), and viable $2n$ pollen (Lim et al. 2001), $2n$ eggs and aneuploid eggs (Chapter 3) produced by LA hybrids were found. However, no functional haploid gametes or aneuploid pollen were found. In addition to $2n$ gametes, functional haploid gametes and aneuploid pollen produced by LA hybrids were found in the present investigation. Three triploid LAA progenies resulting from FDR $2n$ eggs are similar to those found in LAA cultivars (Chapter 3) except that aneuploid LAA progeny was not found in the present research. Obviously, the triploid LAA progenies analyzed in this research were very limited. With regard to 15 triploid ALA progenies, they are similar to the previous observation in ALA and AOA progenies except for four aneuploids found in the present research. As to the intergenomic recombination in the triploid or circa triploid BC1 progenies, it is similar to that observed in LAA cultivars (Chapter 3) except that chromosome 5 of 044571-1 contained an interstitial recombination. Theoretically, such a small interstitial recombination is very important for introgression of specific alien traits. The most interesting aspect is that diploid BC1 progenies were found in the present investigation. Similar phenomena were found in *Allium cepa* x *A. fistulosum* (Emsweller & Jones 1945, Khrustaleva et al. 2000) and *Alstroemeria aurea* x *A. inodora* (Kamstra et al. 1999a), but never found in *Lilium* before,

except for some close interspecific hybrids which originated from crosses within one section (Van Tuyl et al. 1989).

The diploid BC1 progenies could be more useful than triploids in introgression breeding of lily. This is because most of the diploid BC1 progenies have more recombinant chromosomes, especially, some of them contained more interstitial recombinations. And, the diploid BC1 progenies might probably be more fertile than triploid BC1 progenies.

Although fertile n gametes with homoeologous recombination are possibly ideal for introgression breeding without increasing the ploidy level of the following generation, the role of $2n$ gametes could not be replaced by n gametes. When F1 LA hybrids could only produce fertile $2n$ gametes, $2n$ gametes are the best choice for introgression and polyploidization breeding. Moreover, polyploid cultivars have some advantages over their diploid forms. In many cases, such as *Narcissus* (Brandham 1986), triploid and tetraploid cultivars have successfully replaced diploid forms. Brandham (1993) pointed out that different crops have different optimal ploidy level of selective success or horticultural fitness. Indeed, in vegetatively propagated ornamentals like *Alstroemeria* and lily, the triploid ploidy level is preferred over diploid (Van Tuyl & Lim 2003). This means that in some crops polyploidization is very important in breeding some crops.

In order to realize and accelerate introgression and polyploidization at the same time, it would be ideal that n gametes and $2n$ gametes could be produced by any F1 interspecific hybrid. This is almost impossible. Nevertheless, we found that O41519 and O41557 produced functional n and $2n$ gametes. This phenomenon looks similar to what happened in a fertile diploid plant (Van Tuyl et al. 1989). However, it is not laborious to discriminate diploid and triploid of their BC1 progenies, because all lily interspecific hybrids are highly sterile and then, their BC1 progenies are usually very limited.

Concluding it can be said that n gametes and $2n$ gametes play different roles in lily breeding. The former would seem more useful for introgression breeding, the later more useful for breeding polyploid forms.

5 Analysis of progeny derived from crossing Longiflorum x Asiatic lilies (*Lilium*) of different ploidy level and its significance

Abstract

Interploid crosses among lily diploid Asiatic cultivars 'Mont Blanc', 'Pollyanna' and 'Vivaldi' (AA: $2n=2x=24$), Longiflorum cultivar 'White Fox' (LL: $2n=2x=24$), allotriploids (ALA or LAA: $2n=3x=36$), allotetraploids (LALA: $2n=4x=48$) and allopolyploids (ALALA: $2n\approx 5x\approx 60$) and intraploid crosses among allotetraploids were made in the present research. Using hand pollination and embryo rescue, 11 seedlings of $2x-3x$ and nine of $3x-2x$ (BC2) were obtained from eight and nine fruits respectively. Similarly, 125 seedlings of $2x-4x$ and two of $4x-2x$ were obtained from 18 and 17 fruits respectively; and 114 seedlings of $2x-5x$ (BC3) were obtained from 47 fruits. No plants were obtained from 20 fruits of $5x-2x$ and 15 fruits of $4x-4x$ crosses. Because *Lilium* has tetrasporic eight-nucleate embryo sacs (Fritillaria type), the diploid, triploid, tetraploid, and pentaploid lilies produce tetraploid, hexaploid, octaploid and decaploid secondary nucleus in their embryo sacs respectively. Considering the results of lily crosses, it was suggested that tetraploid secondary nucleus might be ideal for lily endosperm development, hexaploid acceptable, but octaploid or decaploid might not be ideal for endosperm development due to its higher DNA content. Eight seedlings of $2x-3x$, seven of $3x-2x$ (BC2) and 37 of $2x-5x$ (BC3) were analyzed with flow cytometry for their ploidy levels. Most of the BC2 progenies were diploid, and all BC3 were triploid. Five diploid BC2 and seven triploid BC3 progenies were analyzed for their chromosome constitution with genomic in situ hybridization. Some Longiflorum chromosomes or their segments remained in the BC2 progenies. This implied that some specific Longiflorum traits could be introgressed into an Asiatic cultivar. Most of the BC3 progenies were pseudoeuploid that possessed euploid

chromosome number ($2n=3x=36$) but the parental genomes were aneuploid. This anomalous situation was the result of chromosome substitutions, whereas apart from this, only one aneuploid ($2n=3x+1=37$) was present. Both the pseudoeuploids and the aneuploids might contribute to genetic variation and could be potentially useful for breeding.

Key words

Lilium, interploid cross, intraploid cross, tetrasporic eight-nucleate embryo sac, secondary nucleus, endosperm, genomic in situ hybridization (GISH).

Introduction

Polyploidization and introgression are the two most important aspects in plant breeding. Only F1 hybrids are not enough for breeding in most cases because F1 hybrids usually combine desirable and undesirable traits of their parents. This means, further backcrossing is inevitable. One important bottleneck is that most of the diploid lily interspecific hybrids ($2n=2x=24$), similar to other distant hybrids, are sterile. Mitotic doubling and meiotic doubling are two ways to restore their fertilities, and both of them have been used for lily polyploid breeding (Van Tuyl 1989, Van Tuyl et al. 2002b). Such allopolyploids, regardless of their origin whether from mitotic or sexual polyploidization, usually may contain two different ones of the three lily genomes, viz., L- A- and O-genomes of Longiflorum, Asiatic and Oriental hybrid lilies respectively (Karlova et al 1999, Lim et al 2001b, Barba-Gonzalez et al 2005a, 2005b, Chapters 3 & 4). In order to introgress some specific traits into a cultivar, interploid crossing is necessary. Many interploid crosses in several plant species have been reported, as for examples in azalea (Saka et al. 2006), *Lotus* (Beuselinck 2003), potato (Carputo and Barone 2005), and others (reviews, Brandham 1982, Kuspira et al. 1986, Ramsey & Schemske

2002, Ramanna & Jacobsen 2003). One of the relevant features of these crosses is that autotriploids have been successfully used in crossing with diploids despite the former being odd-polyploids that are expected to be sterile (Brandham 1982, Kuspira et al. 1986). This was explained from the fact that in autotriploids the homologous chromosomes can pair normally and produce fairly balanced gametes that are fertile. Thus, in reciprocal crosses between diploids and autotriploids, the progeny were predominantly diploid or near diploid, but in reciprocal crosses between triploid and tetraploids they are tetraploid or nearly so. In order to explain the reason for failure and success of autopolyploids of many species, the so-called '1.5 rule' representing the ratio of the ploidy levels of endosperm/embryo was used (Brandham 1982). It should be mentioned that all plant species reviewed in the above instances have monosporic eight-nucleate embryo sacs (Polygonum type). By the contrast, Lily has tetrasporic eight-nucleate embryo sacs (Fritillaria type). Unlike autotriploids, there are instances in which allotriploids have also been successfully used in interploid crosses (Ramanna and Jacobsen 2003). Similar to autotriploids, allotriploids also have a tendency to produce progenies depending on the type of crosses. For example in 2x-3x or reciprocal crosses of lilies, the progenies were predominantly near diploid and in 3x-4x crosses, the progeny were nearly tetraploid (Lim et al. 2003, Barba-Gonzalez 2005). Because the female gametophyte of lilies originates from the "tetrasporic eight-nucleate embryo sacs" type, an important feature of the embryo sacs is that the secondary nucleus invariably possesses a ploidy level that is twice the chromosome complement of the sporophyte. As a result, the ratio between embryo/endosperm is expected to be 2.5, which is quite different from that of monosporic eight nucleate embryo sacs. In this investigation, interploid and intraploid crosses have been made involving allopolyploids derived from crossing Longiflorum x Asiatic lilies. These include, along with the diploid parents, allotriploids, allotetraploids (LALA) derived from doubling the chromosome numbers of the F1 LA hybrids and allopentaploids derived from crossing the aforementioned allotriploid (ALA) with allotetraploid (LALA). With a view to explore the prospects of utilizing different types of allopolyploids in introgression breeding in

lilies, the following three aspects were investigated: i) crossability of genotypes with different ploidy levels as estimated from the progeny production, ii) the genome composition of the progenies resulting from the above crosses especially the transmission of recombinant chromosomes if any, and iii) the types of embryo/endosperm ratios of ploidy levels that might result from different types of crosses. The relevance of these results for introgression breeding in lily is discussed.

Materials and methods

Plant materials

The materials used in this research are listed in Table 5-1. Three of them are diploid Asiatic cultivars ($2n=2x=24$); one diploid Longiflorum cultivar ($2n=2x=24$); three allotriploid cultivars ($2n=3x=36$) which resulted from sexual polyploidization and supplied by World Breeding BV, Royal Van Zanten BV and Vletter & Den Haan BV, respectively; seven allotetraploids (LALA: $2n=4x=48$) which were from chromosome doubling of F1 LA hybrids and supplied by World Breeding BV; and three near allopolyploids (ALALA: $2n\approx 5x\approx 60$) which are BC2 progenies of the cross between allotriploid (ALA) and allotetraploid (LALA) (Lim et al. 2003). The allotetraploid (LALA) was derived from somatic chromosome doubling but the BC2 progeny resulting from this cross possessed variable numbers of recombinant chromosomes (Lim et al. 2003). All the materials were grown in the greenhouse using standard growing conditions for lily cultivation at Plant Research International, Wageningen University and Research Centre, the Netherlands.

Pollen germination, pollination and embryo rescue

The method of pollen germination was the same as described in Chapter 4. The crosses were made by hand pollination and followed by embryo rescue. One day before anthesis, the anthers were removed if the plants were used as female parents or the anthers were stored in exsiccator for pollination if the plants were used as male parents. When the flower was fully open, pollen was placed on its

stigma and encapsulated with aluminum foil to seclude from other pollen contamination. About 4-10 weeks after pollination, the maturation of fruits was checked. The fruits were harvested for embryo rescue when they were soft or yellow. The surface of harvested fruit was sterilized with 80% ethanol for a few seconds in a laminar air flow cabinet. Then, cut it open and its ovules were put in a sterilized Petri-dish. The swollen ovules, i.e. those of which embryo or embryo sacs developed, were selected for in vitro culture. After their coats were carefully removed under an anatomic microscope, they were placed on the medium (pH=5.8) consisting of 2.2g MS, 60g sucrose and 4g gelrite per liter. After this, they were stored in a dark chamber at 25°C.

Flowcytometric measurement

The flowcytometric measurement was performed according to the method described by Van Tuyl and Boon (1997).

Chromosome preparation and in situ hybridization

The method of chromosome preparation and in situ hybridization was the same as described in Chapter 4.

Results

Interploid crosses

In order to avoid unnecessary pollination work, as a first step, pollen germination was tested in vitro for all genotypes that were used for crossing (Table 5-1). Considering their pollen germination, usually those genotypes whose percentage of pollen germination was higher than 1% were selected as male parents, while all female parents were utilized regardless of their pollen germination. The results of the crosses are shown in (Table 5-2).

Table 5-1. The materials used and their pollen germination.

Accession	Ploidy level ($x=12$)	Origin	Genome composition	Pollen germination*
Mont Blanc	2x	Asiatic cv.	AA	++++
Pollyanna	2x	Asiatic cv.	AA	++++
Vivaldi	2x	Asiatic cv.	AA	++++
White Fox	2x	Longiflorum cv.	LL	++++
041513	3x	BC1 cv.	LAA	+
041552	3x	BC1 cv.	LAA	±
041584	3x	BC1 cv.	ALA	±
041515	4x	Amphiploid	LALA	±
041524	4x	Amphiploid	LALA	+++
041525	4x	Amphiploid	LALA	+++
041527	4x	Amphiploid	LALA	+++
041530	4x	Amphiploid	LALA	++
041531	4x	Amphiploid	LALA	+++
041532	4x	Amphiploid	LALA	++
997118-5	5x	BC2 progeny	ALALA	++
997118-8	5x	BC2 progeny	ALALA	++
997118-12	5x	BC2 progeny	ALALA	++

*One, two, three and four plus (+) represent that the percentage of their pollen germination was usually around 1-5, 5-50, 50-80, and 80-100% respectively.

"±" indicated that the genotype was highly sterile. The percentage of pollen germination was less than 1%.

Both 2x-3x and 3x-2x crosses were successful. About 4-10 weeks after pollination, most fruits of 2x-3x did not develop well. On average, 2.25 (18/8) swollen ovules per fruit were obtained, and 61% (11/18) germinated and grew up into plants. On the contrary, most fruits of 3x-2x apparently developed well. On average, 14.56 (131/9) swollen ovules per fruit were obtained, but only 6.87% (9/131) germinated and grew up into plants. In total, 11 descendants of 2x-3x and nine of 3x-2x were obtained from pollinated eight and nine flowers respectively. This showed that allotriploid lilies could be used not only as male parents when they had relative good male fertility, but also as female parents regardless of their pollen germination.

Most combinations of 2x-4x crosses were very successful except for 044549 and 044550. In total, 18 fruits were rescued by embryo rescue, 195 developed ovules were obtained, 125 plants were obtained. The two failed crosses (044549 and 044550) were expected because their male parent '041515' was highly

Table 5-2. The results of interploidy crosses.

Crosses	Parents		Expected genome composition	No of fruits rescued	No of swollen ovules	No of Plants
	Female ♀	Male ♂				
2x-3x (BC2)						
044529	Pollyanna	041513	A LAA	4	9	6
044530	Mont Blanc	041513	A LAA	4	9	5
Total				8	18	11
3x-2x(BC2)						
054517	041584	Mont Blanc	ALA A	4	80	3
044519	041513	Pollyanna	LAA A	2	14	3
044524	041513	Mont Blanc	LAA A	2	7	1
044634	041552	Pollyanna	LAA A	1	30	2
Total				9	131	9
2x-4x						
044549	Mont Blanc	041515	ALA	2	0	0
044550	Pollyanna	041515	ALA	2	0	0
054561	Vivaldi	041524	ALA	1	28	12
054562	Vivaldi	041525	ALA	1	29	18
044522	Mont Blanc	041527	ALA	1	40	32
044523	Pollyanna	041527	ALA	7	19	13
044553	Mont Blanc	041530	ALA	1	13	9
054563	Vivaldi	041530	ALA	1	21	15
054564	Vivaldi	041531	ALA	1	15	9
054565	Vivaldi	041532	ALA	1	30	17
Total				18	195	125
4x-2x						
044544	041515	Pollyanna	LAA	2	3	1
044570	041524	Pollyanna	LAA	3	0	0
044569	041525	Pollyanna	LAA	4	9	0
044516	041527	Pollyanna	LAA	2	14	1
044526	041530	Pollyanna	LAA	3	7	0
044590	041531	Pollyanna	LAA	1	1	0
044591	041532	Pollyanna	LAA	2	0	0
Total				17	34	2
2x-5x(BC3)						
044501	White Fox	997118-5	L ALALA	6	5	1
044502	White Fox	997118-8	L ALALA	6	17	6
044504	Pollyanna	997118-5	A ALALA	10	13	4
044505	Pollyanna	997118-8	A ALALA	10	5	4
044506	Pollyanna	997118-12	A ALALA	10	85	61
044507	Mont Blanc	997118-5	A ALALA	2	14	14
044508	Mont Blanc	997118-8	A ALALA	1	13	10
044509	Mont Blanc	997118-12	A ALALA	2	29	14
Total				47	181	114

sterile (Table 5-1). However, most crosses of 4x-2x were not successful. Seven combinations of this type were made and 17 fruits were rescued. 34 ovules were obtained, and only two plants developed from genotypes 044544 and 044516. No plant of 4x-4x crosses was obtained from 15 pollinated flowers. The result was contrary to the expectation, because these tetraploids had originated from chromosome doubling of the F1 LA hybrids and they had good fertility based on their pollen germination. Differences occurred also between 2x-5x and its reciprocal crosses. 114 plants were obtained from 47 fruits of 2x-5x crosses (BC2), whereas no progeny of 5x-2x was obtained from 20 pollinated flowers.

Ploidy level and genome composition of BC2 and BC3 progenies

Eight progenies of 2x-3x and seven of its reciprocal crosses were tested for ploidy level with flow cytometry. 14 of them were diploid and one hexaploid (Table 5-3). This shows that the allotriploids produced functional haploid gametes. However, there was no reasonable explanation for the occurrence of the hexaploid BC2 progeny. The chromosome numbers of five diploid BC2 progenies were confirmed with genomic in situ hybridization. The representative GISH results are shown in Figure 5-1 and their genome compositions are summarized in Table 5-4. Their recombinant chromosomes are diagrammed in Figure 5-3 (1). As to their genome composition, only one recombinant Longiflorum chromosome remained in 044529-2 ($2n=2x=24$) (Figure 5-1a), one segment of Longiflorum chromosome in 044530-1 ($2n=2x=24$) (Figure 5-1c), one whole Longiflorum chromosome and two recombinant segments of Longiflorum chromosomes in 044634-1 ($2n=2x+1=25$) (Figure 5-1d), and no Longiflorum chromosome in 044529-3 ($2n=2x=24$) (Figure 5-1b) and 044529-4 ($2n=2x=24$). It was obvious that only a few Longiflorum chromosomes or segments remained in these BC2 progenies. This implied that introgression of some specific Longiflorum traits into an Asiatic cultivar could be realized in some BC2 progenies.

37 progenies of 2x-5x were tested using flow cytometry for their ploidy levels. All of them were triploid (Table 5-3). This showed that the allopolyploids produced functional 2x pollen. Seven of them were confirmed with genomic in situ

hybridization. The representative GISH results are shown in Figure 5-2 and their genome compositions are summarized in Table 5-4. Six of them were triploid ($2n=3x=36$) and one hypertriploid ($2n=3x+1=37$). Their recombinant chromosomes are represented in Figure 5-3 (2). With respect to their genome composition, they contained 9-12 Longiflorum chromosomes and, correspondingly, 15-12 Asiatic chromosomes contributed by male gametes produced by their allopolyploid parents (Table 5-4). Among the seven BC3 progenies analyzed by GISH, only 044504-3 did not contain any recombinant chromosome, other six had 2-6 recombinant chromosomes in each genotype. Different genotypes had different recombinations. For example, 044507-2, 044507-5 and 044507-6 originated from the same cross 'Mont Blanc x 997118-5' (Table 5-2), but the breakpoints of their recombinant chromosomes were different at positions or numbers (Figure 5-3(2)).

Table 5-3. The ploidy levels of some interplod crosses evaluated by DNA measurement with flow cytometry.

Interplod cross	No. of plants measured by flow cytometry	No. of plants with ploidy level		
		2x	3x	others
2x-3x (BC2)				
044529	3	3		
044530	5	5		
Total	8	8		
3x-2x(BC2)				
044519	3	3		
044524	1	1		
044634	3	2		1(6x)
Total	7	6		1
2x-5x(BC3)				
044501	1		1	
044502	6		6	
044504	4		4	
044507	12		12	
044508	4		4	
044509	10		10	
Total	37		37	

Table 5-4. Genome composition of BC2 and BC3 progenies based on GISH results

Code	genotype	Chromosome number	Chromosomes contributed by			
			female gamete		male gamete	
			L (^L / _A)	A (^A / _L)	L (^L / _A)	A (^A / _L)
BC2						
044529-2	A LAA	24		12	1(1)	11
044529-3	A LAA	24		12	0	12
044529-4	A LAA	24		12	0	12
044530-1	A LAA	24		12	0	12(1)
044634-1	LAA A	25	1	12(2)		12
BC3						
044501-1	L ALALA	37	12		11(1)	14(1)
044502-2	L ALALA	36	12		11(2)	13(4)
044504-3	A ALALA	36		12	9	15
044506-4	A ALALA	36		12	9(2)	15(1)
044507-2	A ALALA	36		12	12(3)	12(1)
044507-5	A ALALA	36		12	9(1)	15(3)
044507-6	A ALALA	36		12	11(3)	13(1)

L (^L/_A) = The number of Longiflorum chromosomes (including Longiflorum chromosomes that contained Asiatic chromosome segments), and vice versa to A (^A/_L).

Table 5-5. The expected ploidy levels of embryo and endosperm of lily intra- & interploid crosses (The ploidy levels of secondary nucleus are in brackets).

Types	Examples	Endosperm	Secondary nucleus	Embryo	Remarks	References
2x-2x	AA x AA	5x (4x)		2x	++++	McRae 1998 etc.
	AA x LA	6x (4x)		3x	+	Chapter 4
	LA x AA	5x (4x)		3x	+	Chapter 5
2x-3x	AA x LAA	5x* (4x)		2x*	+	Chapter 5
	AA x ALA	5x* (4x)		2x*	+	Lim 2000
	AA x AOA	5x* (4x)		2x*	+	Barba-Gonzalez 2005
2x-4x	AA x LALA	6x (4x)		3x	++	Chapter 5
	LL x LRLR	6x (4x)		3x	++	Lim et al 2000
2x-5x	AA x ALALA	6x (4x)		3x	+	Chapter 5
	LL x ALALA	6x (4x)		3x	+	Chapter 5
3x-2x	ALA x AA	7x (6x)		2x*	+	Lim et al 2003
	LAA x AA	7x (6x)		2x	+	Chapter 5
	AOA x AA	7x (6x)		2x*	+	Barba-Gonzalez 2005
3x-4x	ALA x AAAA	8x (6x)		5x+2*	+	Lim et al 2003
	ALA x LALA	8x (6x)		5x+1*	+	Lim et al 2003
	AOA x OAOA	8x (6x)		4x*	+	Barba-Gonzalez 2005
4x-2x	LALA x AA	9x (8x)		3x	±	Chapter 5
4x-4x	LALA x LALA	10x (8x)		4x	±	Chapter 5
5x-2x	ALALA x AA	11x (10x)		3x*	±	Chapter 5

The asterisk (*) indicated the endosperm or embryo usually was aneuploid.

The plus (+) meant that the cross was successful if the male parent had a little fertility.

The more successful crosses are indicated by a higher number of plus (+) signs.

The plus/minus (±) represented that the cross was hardly successful even if both male and female parent had good fertility.

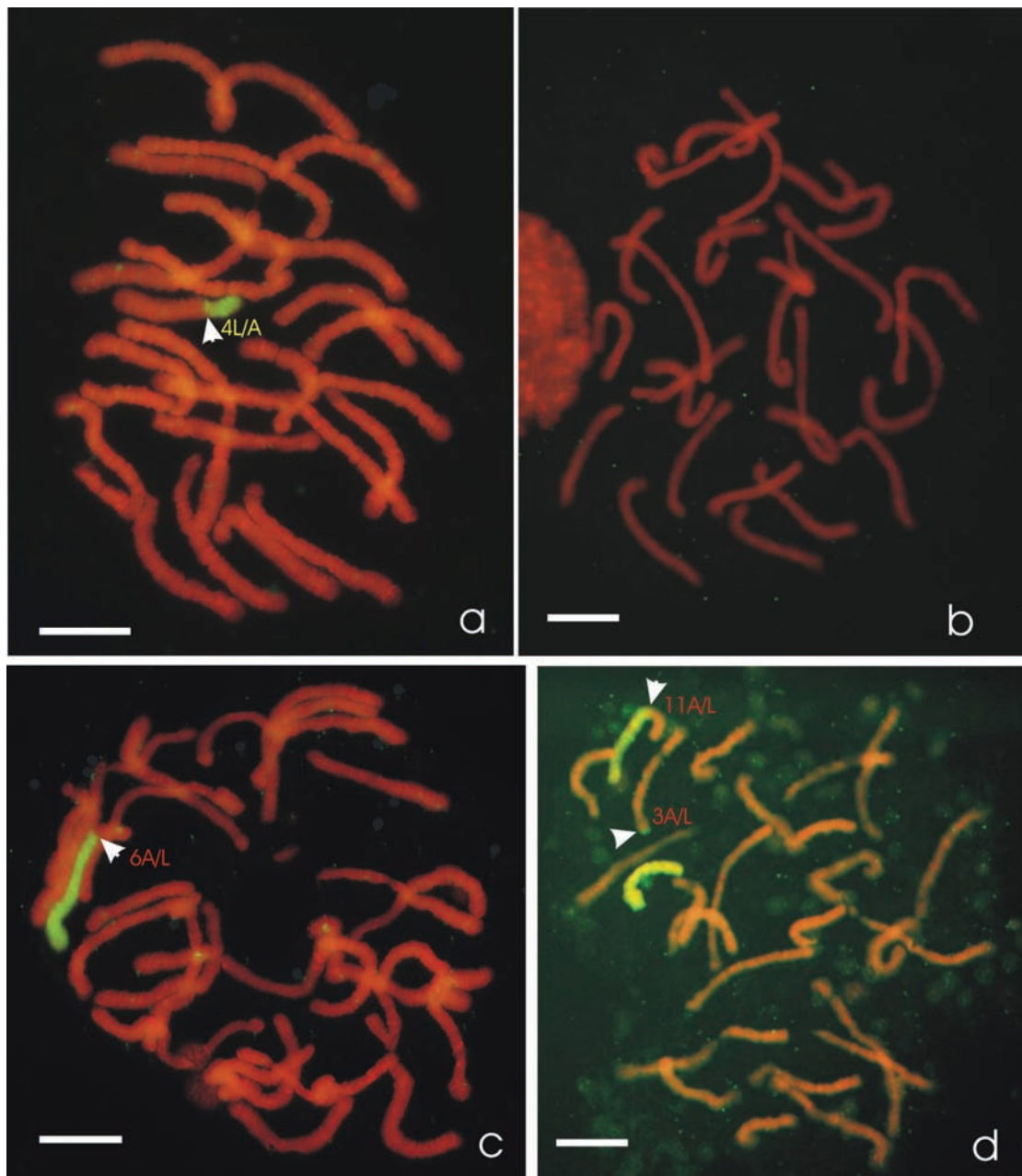


Figure 5-1. GISH results of BC2 progenies. In all cases, yellow green chromosomes or segments belong to Longiflorum genome (L) and red chromosomes or segments belong to Asiatic genome (A), because Longiflorum genomic DNA was labeled with digoxigenin and detected with FITC system, and Asiatic DNA was used as block DNA. The recombinant chromosomes were indicated with a number and L/A or A/L. The number indicates which chromosome is recombinant. L/A or A/L represents Longiflorum or Asiatic recombinant chromosome, respectively.

a. 044529-2 ($2n=2x=24$), b. 044529-3 ($2n=2x=24$), c. 044530-1 ($2n=2x=24$), and d. 044634-1 ($2n=2x+1=25$). Bar = $10\mu\text{m}$.

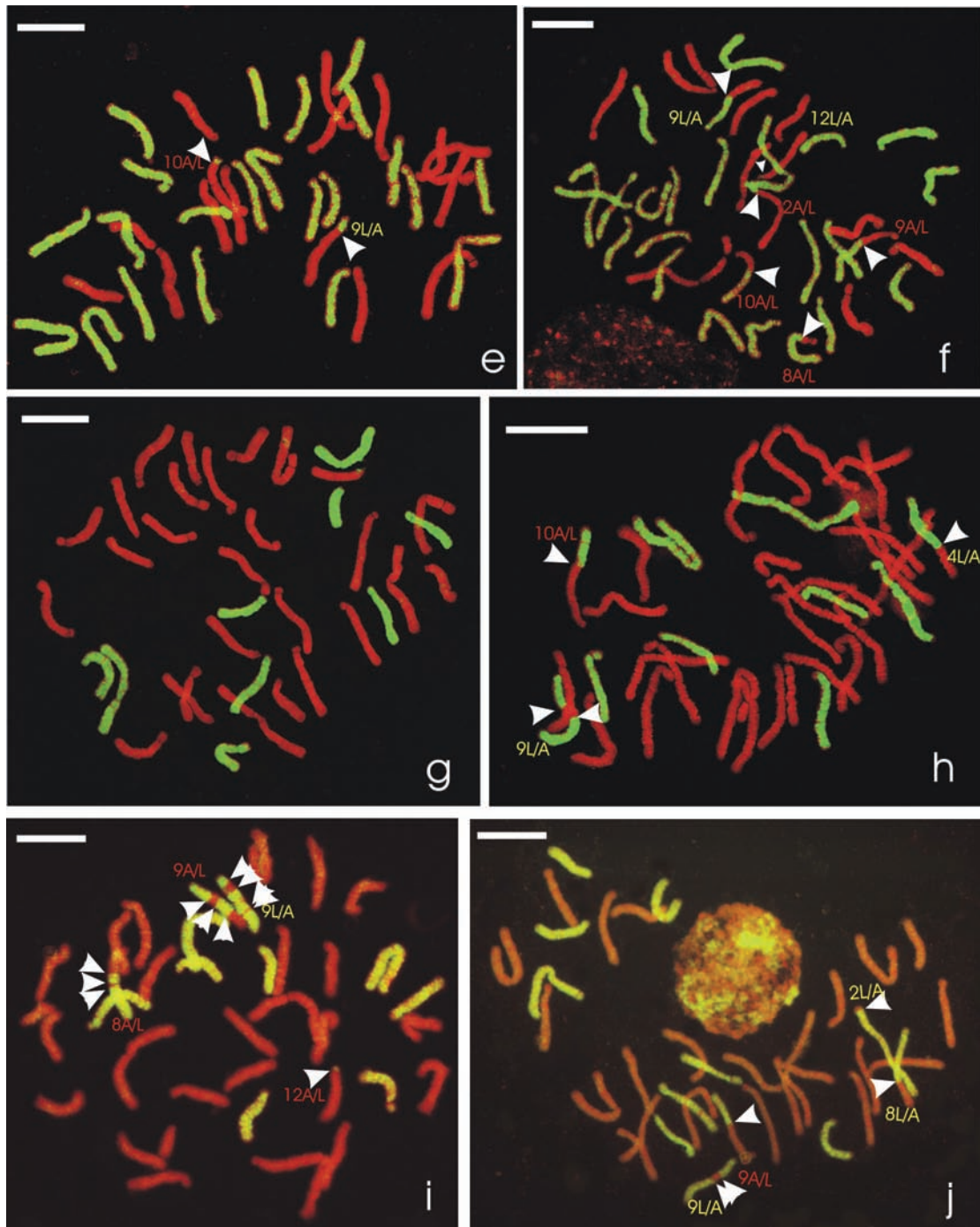
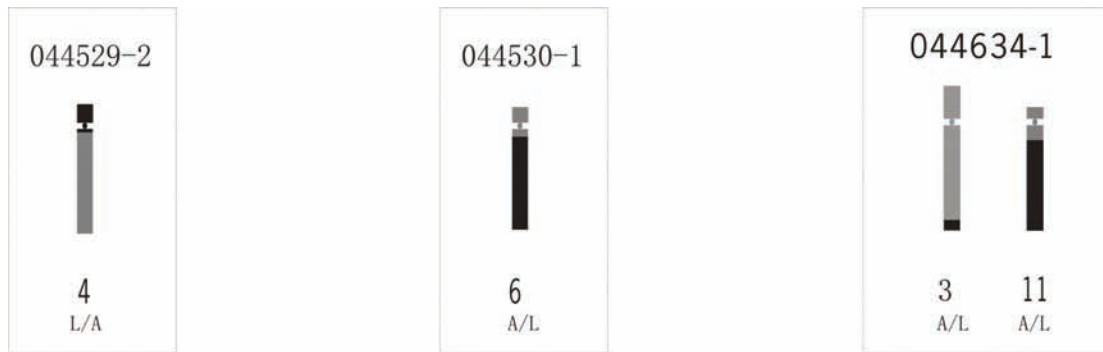
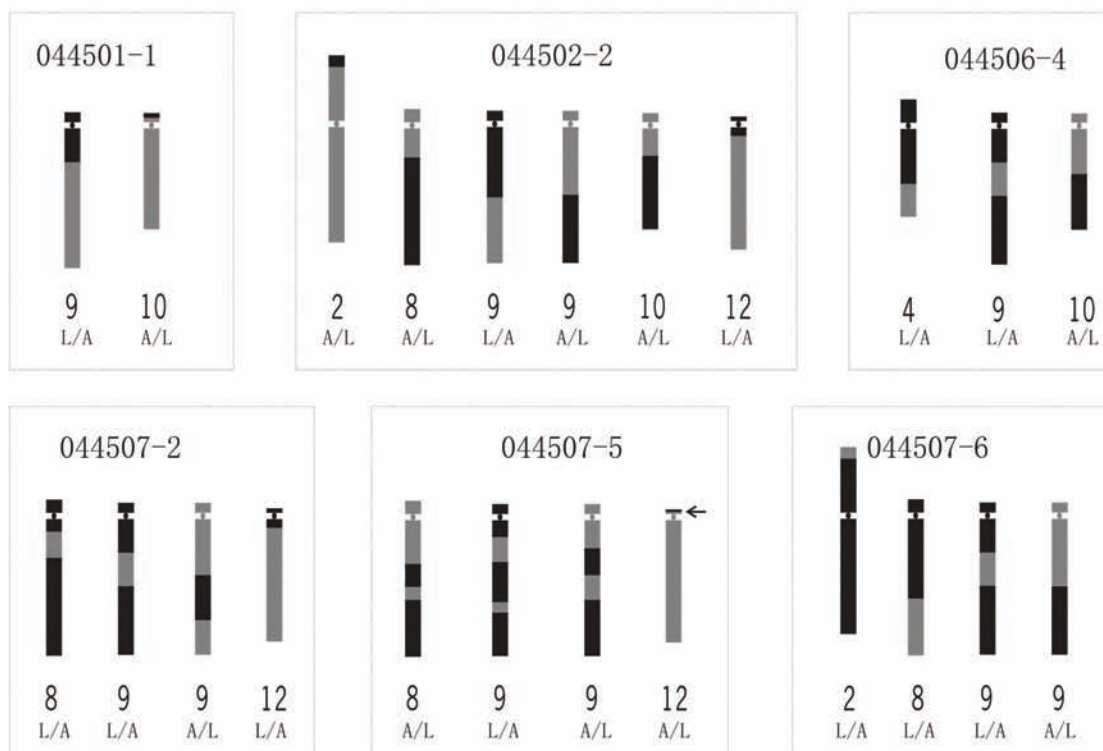


Figure 5-2. GISH results of BC3 progenies. Yellow green chromosomes or segments belong to Longiflorum genome (L) and red chromosomes or segments belong to Asiatic genome (A), because Longiflorum genomic DNA was labeled with digoxigenin and detected with FITC system, and Asiatic DNA was used as block DNA. e. 044501-1 ($2x=3x+1=37$), f. 044502-2 ($2x=3x=36$), g. 044504-3 ($2x=3x=36$), h. 044506-4 ($2x=3x=36$). i. 044507-5 ($2x=3x=36$), and j. 044507-6 ($2x=3x=36$). Intergenomic recombinant chromosomes were indicated with a number and L/A or A/L. The number indicates which chromosome was recombinant, L/A or A/L represents Longiflorum or Asiatic recombinant chromosome respectively. Arrow heads indicate breakpoints. Bar = 10 μ m.



(1)The recombinant chromosomes of the BC2 progenies. 044529-2 and 044530-1 originated from the same male parent '041513' ($2n=3x=36$) crossed with different diploid Asiatic cultivars, their recombinant chromosomes were 4L/A and 6A/L respectively. The recombinant chromosomes of 044634-1 were 3A/L and 11A/L.



(2)The recombinant chromosomes of the BC3 progenies.044507-2, 044507-5 (the arrow points the very small recombinant), and 044507-6 originated from the same cross, but their recombinant chromosomes were different, especially in the positions of breakpoints.

Figure 5-3. Diagram of the recombinant chromosomes of BC2 and BC3 progenies.

Discussion

Interploid crosses of autopolyploids have been successful in many plant species (review by Brandham 1982). The present investigation and previous researches on

lilies (Lim et al. 2003, Barba-Gonzalez 2005) showed that interploid crosses involving allopolyploids were also possible using hand pollination and embryo rescue. Based on these results, the significance of interploid crosses on lily breeding is discussed.

The success and failure of interploid crosses in lilies

The results of the present and previous interploid crosses and some intraploid crosses in lilies are summarized in Table 5-5. Clearly, diploid, triploid, tetraploid, and pentaploid lilies could be used as male parents when they had some extent of male fertility; most diploid and triploid lilies could be used as female parents regardless of their male fertility. On the contrary, all allotetraploid and allopolyploid lilies could hardly be used as female parents even though they had good male fertility as estimated from pollen germination test and theoretical analysis. The '1.5 rule' (i.e. ploidy levels of endosperm/embryo=1.5) had been used to explain the reason for failure and success of intra- or interploid crosses in many species (Brandham 1982). Considering that all species explained with the '1.5 rule' have a monosporic eight-nucleate embryo sac, by contrast, *Lilium* species have a tetrasporic eight-nucleate embryo sac. Similarly, we tried to explain lily crosses with the '2.5' rule (i.e. ploidy levels of endosperm/embryo=2.5) because endosperm of diploid *Lilium* species is pentaploid and its embryo is diploid. Obviously, the '2.5' rule can not explain the failure of 4x-4x crosses in *Lilium* (Table 5-5). Actually, the ratio of ploidy levels of endosperm/embryo is 1.6-3.1 in 2x-3x and 1.5-1.7 in 3x-4x crosses in lilies (Lim et al 2003).

Besides the viability of gametes, the ploidy level of the secondary nucleus, i.e. the DNA amount, possibly plays an important role in the lily interploid crosses rather than the ratio of the ploidy levels of endosperm and embryo as in other plant species, because species of *Lilium* have a tetrasporic eight-nucleate embryo sac and have 35 – 36 pg of DNA per haploid nucleus (1C) (Bennett & Smith, 1976; 1991), which is one of the largest genomes among plant species. In the process of tetrasporic eight-nucleate embryo sac formation in *Lilium*, the secondary

nucleus constitute the four megaspores generated by the meiosis during megasporogenesis. This means that the ploidy level of the secondary nucleus is always twice that of its megaspore mother cell regardless of normal or abnormal meiosis. So, diploid, triploid, tetraploid, and pentaploid lilies produce tetraploid, hexaploid, octaploid and decaploid secondary nuclei in their embryo sacs respectively (Table 5-5 & Figure 5-4). From Table 5-5, it can be seen that when secondary nucleus is octaploid or decaploid, or the ploidy level of their endosperm is higher than octaploid, lily inter- and intraploid crosses were hardly successful. Probably, because of too much of DNA in such cases, once the ploidy level of endosperm is higher than octaploid, the endosperm cells did not divide or develop well to meet the need of embryo development.

Based on the results of crosses and above reasoning, it is suggested that the tetraploid secondary nucleus might be ideal for lily endosperm development because it is the same as in normal lily species; the hexaploid secondary nucleus should be acceptable because many $3x-2x$ crosses were successful; but the octaploid or higher secondary nuclei are not ideal because it was difficult to obtain progenies when tetraploids or pentaploids were used as female parents in intra- or interploidy crosses.

The difference between crosses and their reciprocals

The difference between $2x-4x$ and $4x-2x$ or $2x-5x$ and $5x-2x$ is obvious, because one was successful and the other not. A salient difference was also observed between $2x-3x$ and $3x-2x$. Usually, the fruits of $3x-2x$ crosses developed well and 14.56 swollen ovules per fruit were obtained, whereas those of $2x-3x$ were small and only 2.25 swollen ovules per fruit were obtained. On the contrary, the percentage of germinated swollen ovules of $2x-3x$ crosses were much higher than that of $3x-2x$ (Table 5-2). Similar phenomenon occurred between LA x AA ($2x-2x$) and AA x LA ($2x-2x$) (Chapter 4). The difference might be caused by the viability of gametes and the congruity of embryo and endosperm development. They are discussed below (Figure 5-4).

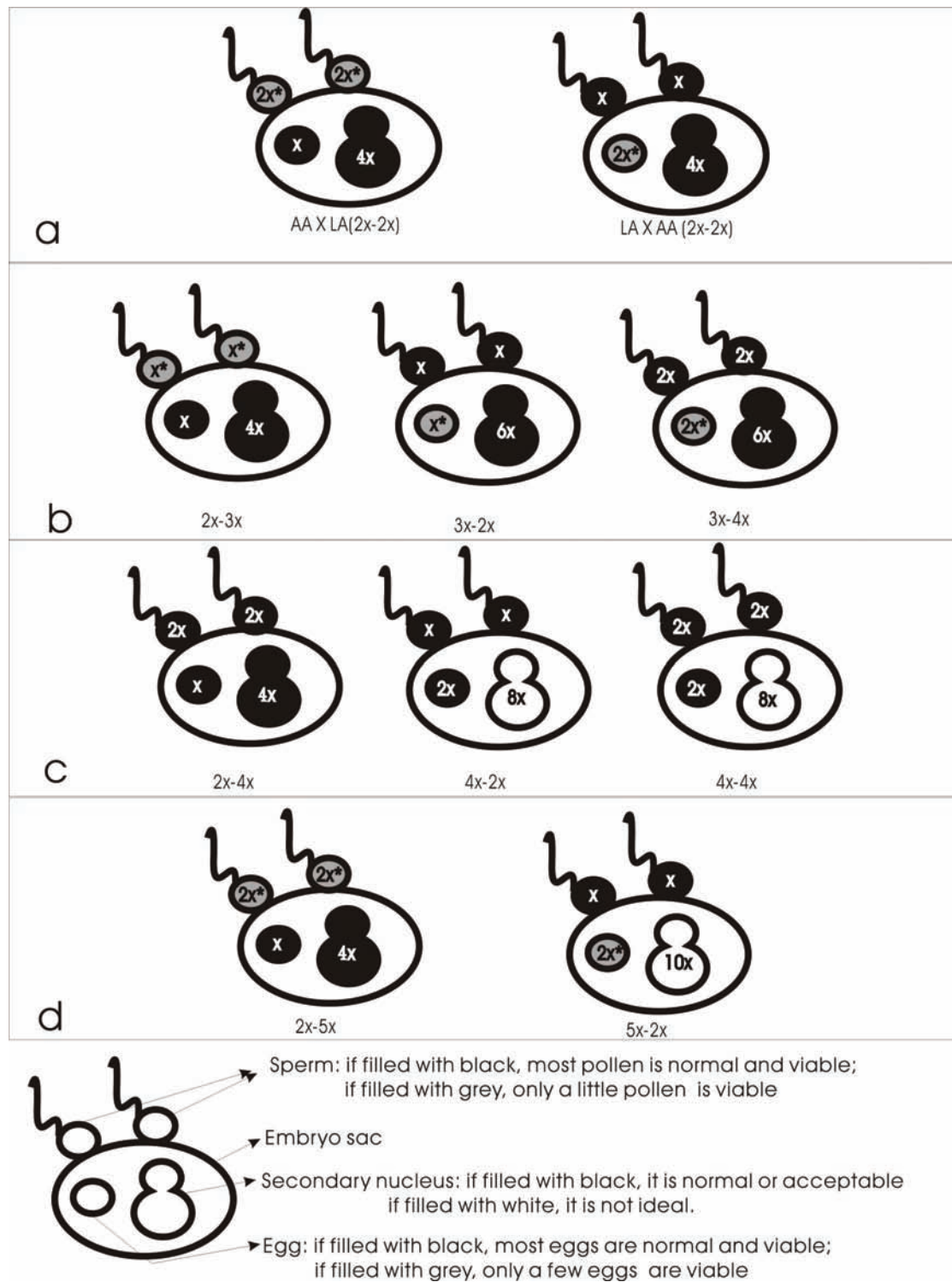


Figure 5-4. A diagram of ploidy levels of sperm, egg cell, and secondary nucleus at double fertilization of different lily crosses and their significance on the development of embryo and endosperm. a. AA x LA and LA x AA; b. 2x-3x, 3x-2x and 3x-4x; c. 2x-4x, 4x-2x and 4x-4x; and d. 2x-5x and 5x-2x. Detailed explanations see text.

(1) AA x LA and LA x AA

In AA x LA (Figure 5-4a: left), the haploid eggs and tetraploid secondary nuclei produced by the Asiatic cultivar (AA) are normal, but most of them could not be fertilized because the viable pollen grains (usually $2n$ pollen) produced by the LA hybrid are very limited due to its abnormal meiosis. This might be the reason that the fruits of AA x LA were usually small. Once some ovules were fertilized by $2n$ pollen, the fertilized eggs and secondary nuclei could develop into embryos ($3x$) and endosperms ($6x$) respectively. This could be the reason that the swollen ovules of AA x LA could easily germinate (Table 4-2). In LA x AA (Figure 5-4a: right), most embryo sacs produced by LA hybrids contained an aneuploid egg cell and a tetraploid secondary nucleus. Probably, most of them could be fertilized by normal n gametes produced by Asiatic cultivars (AA). However, most fertilized aneuploid eggs could not develop into embryos or died early while many fertilized secondary nuclei developed into endosperms ($5x$) to some extent. Thus, many fruits of LA x AA apparently developed well and there were many swollen ovules in each fruit, but only a few of them (usually $3x$) could germinate (Table 4-3).

(2) $2x-3x$, $3x-2x$ and $3x-4x$

Because the hexaploid secondary nucleus was acceptable for endosperm development based on the results of interploid crosses, the difference between $2x-3x$ and its reciprocal could be explained as in AA x LA and LA x AA. Most embryo sacs could not be fertilized in $2x-3x$ crosses because triploid lilies produce a very limited amount of viable pollen due to abnormal meiosis (Figure 5-4b: left). Once a few ovules were fertilized, the fertilized eggs and the fertilized secondary nuclei could develop into embryos ($2x^*$) and endosperms ($5x^*$) respectively. Thus, the swollen ovules obtained from one fruit of $2x-3x$ cross were very limited, but most of them could germinate (Table 5-2). In $3x-2x$ crosses (Figure 5-4b: middle), most embryo sacs produced by triploid lilies contain an aneuploid egg cell and a hexaploid secondary nucleus. Most embryo sacs could be fertilized by fertile n pollen. But most aneuploid embryos could not develop or died early while many fertilized secondary nuclei developed into endosperms ($7x$) to some extent. Therefore, the fruits were large and more swollen ovules per fruit

were obtained in this case, but the percentage of germinated swollen ovules was very low (Table 5-2). 3x-4x is shown in Figure 5-4b (right). It might be similar to 3x-2x. After fertilization, most ovules could be fertilized and many fertilized secondary nuclei could develop into endosperms (8x), but only a few fertilized eggs could develop into embryos (4x* or 5x*).

(3) 2x-4x, 4x-2x and 4x-4x

2x-4x, 4x-2x and 4x-4x crosses are shown in Figure 5-4c. In 2x-4x, many ovules could be fertilized by viable 2x pollen produced by tetraploid lilies. The fertilized eggs and fertilized secondary nuclei could developed into embryos (3x) and endosperms (6x) respectively. So, 2x-4x was very successful (Table 5-2). However, 4x-2x and 4x-4x crosses were hardly successful. This is possibly because the ploidy level of the secondary nucleus (8x) produced by tetraploid lilies is too high for endosperm development.

(4) 2x-5x and 5x-2x.

The success of 2x-5x crosses could be explained as in AA x LA or 2x-3x (Figure 5-4d left). Some ovules could be fertilized because allopolyploid lilies could produce some viable 2x pollen grains. The fertilized eggs and fertilized secondary nuclei developed into embryos (3x) and endosperms (6x) respectively. The reason for the failure of 5x-2x crosses might be similar to that of 4x-2x and 4x-4x, because allopolyploid lilies produced decaploid secondary nuclei (10x) in their embryo sacs (Figure 5-4d right).

Pseudoeuploids and aneuploids of the progenies of lily interploidy crosses

In a backcross such as “diploid Asiatic cultivar (AA) x allotetraploid (LALA)”, the allotriploid BC1 progenies will be normally euploids with 24A+12L chromosomes. Similar results also occur when FDR gametes from LA hybrids are functional in backcross with a Asiatic parent. Apart from such euploids, there are numerous instances in allotriploids of lilies where the chromosome number will be 36, exactly euploid, but the proportion of the parental chromosomes of L and A genomes do not strictly conform to the expected number of 24A+12L. For example, based on their genome compositions, six of the seven BC3 progenies

(Table 5-4) have euploid chromosome numbers, but the proportion of A- and L-chromosomes in different genotypes is variable. This has obviously resulted from substitutions of homoeologous chromosomes during $2x$ gamete formation in the allopolyploid parent. These allotriploids, in spite of having euploid chromosome numbers, possess aneuploid constitution of parental chromosomes and are called pseudoeuploids. Such genotypes have also been found in the BC1 progenies of AA x LA or AA x OA crosses previously (Lim et al. 2001, Barba-Gonzalez et al. 2005a, 2005b).

Some progenies of $2x-3x$ and reciprocal crosses were diploid, some of $3x-4x$ were tetraploid (Barba-Gonzalez 2005) or pentaploid (Lim et al. 2003), and all of $2x-5x$ were triploid. Based on GISH analysis, some of these euploids were pseudoeuploid.

Most progenies generated of $3x-2x$, $2x-3x$ and $3x-4x$ crosses were aneuploid (Lim et al. 2003, Barba-Gonzalez 2005). In the progenies of $3x-2x$, the AOA and ALA contributed around $x+6$ or near haploid eggs, while in those of $3x-4x$, the triploid AOAs usually contributed near diploid eggs (Barba-Gonzalez 2005), but the triploid ALAs contributed near triploid eggs (Lim et al 2003). Theoretically, allotriploid lily, regardless of LAA or ALA or AOA, frequently form 12 bivalent and 12 univalent; similarly, the allopolyploids (ALALA) form about 12 trivalents and 12 bivalents at metaphase I of meiosis. Ramsey & Schemske (2002) pointed out that univalent and trivalent configurations always lead to the formation of aneuploid or pseudoeuploid gametes. This might be the reason that interploidy crosses generate aneuploid or pseudoeuploid progenies. Both the pseudoeuploids and the aneuploids might contribute to genetic variation and are potentially useful for selection.

Intergenomic recombination of the progenies of interploidy crosses

Intergenomic recombinations were observed in the progenies of $2x-3x$, $3x-2x$, and $2x-5x$ crosses. Based on present results, few recombinant chromosomes of the new progenies were directly transferred from their parent. The recombinant chromosomes of 044634-1 (Figures 5-1 & 5-3) were different from those of its

BC1 parent '041552' (Figure 3-2c). And almost all recombinant chromosomes of 044507-2, 044507-5 and 044507-6 (Figures 5-2 & 5-3) were different from those of their male parent '997118-5' (Lim et al. 2003). This implies that new crossovers occurred between homoeologous chromosomes during meiosis of the allotriploid BC1 and allopolyploid BC2. Similar results were reported in other distant lily hybrids (Lim et al. 2003, Barba-Gonzalez 2005). When the recombinant chromosomes of 044506-4 were compared with those of its male parent '997118-12' (Lim et al. 2003), it was noticed that the recombinant chromosome 9L/A of 044506-4 was similar to that of 997118-12. The interstitial recombinant chromosome (9L/A) of 997118-12 were apparently transferred from its male parent '921238-1' (Lim et al. 2003).

6 General discussion

The three common lily cultivar groups, viz., Longiflorum (L), Asiatic (A) and Oriental (O), originate from the sections Leucolirion, Sinomartagon and Archelirion of the genus *Lilium* respectively. It is difficult to make crosses between these different groups. With cut style pollination and embryo rescue, some F1 LA hybrids were obtained, but most of them are highly sterile. The sterility hinders introgression breeding in these lily hybrids. However, the cultivars derived from LA hybrids have become one of the most important lily cultivar groups in the flower market (Leslie 1982-2005, www.vbn.nl). Considering the limited materials reported in previous researches, it is necessary to investigate more genotypes in order to gain more general information on lily LA hybrids. In this thesis, 11 F1 LA hybrids and 19 BC1 cultivars supplied by six Dutch lily breeding companies were analyzed with genomic in situ hybridization (GISH). Intra- and interploid crosses among diploid Asiatic and Longiflorum cultivars, diploid F1 LA hybrids, triploid BC1 cultivars, allotetraploid and allopolyploid lilies were made. Some of the new BC1, BC2 and BC3 progenies were analyzed with flow cytometry and GISH. Based on the results in four experimental chapters (Chapters 2-5), the following topics are discussed:

1. Types of gametes produced by F1 LA hybrids
2. The ploidy levels of backcross progenies
3. The success and failure of lily crosses
4. The difference between crosses and their reciprocals
5. Significance of n gametes and $2n$ gametes for lily breeding
6. The possibility of bilateral sexual polyploidization in lilies

Types of gametes produced by F1 LA hybrids

It is well known that chromosome pairing, crossing over and chromosome distribution are the key events during meiosis (Sybenga 1975). The outcomes of these events are used to predict types of gametes produced by F1 LA hybrids.

Homoeologous chromosome behavior at metaphase I and anaphase I

Chromosomes that are structurally similar and pair normally at meiosis are termed homologous. Partially homologous chromosomes are called homoeologous. Any diploid interspecific hybrid has two sets of homoeologous chromosomes. The interspecific hybrids in *Lilium* are also highly sterile (Van Tuyl et al. 2002b). Based on previous researches on F1 LA and OA hybrids, it is known that some homoeologous chromosomes partially pair during their meiosis (Lim et al. 2001b, Barba-Gonzalez et al. 2004). However, the configurations at metaphase I of the F1 LA hybrids, which were selected for breeding new cultivars by Dutch lily breeding companies, show quantitative variation ranging from no bivalent to 12 bivalents in different pollen mother cells. This shows that most LA hybrids have an abnormal meiosis and only a few hybrids or pollen mother cells have normal meiosis. Based on the homoeologous chromosome distribution at anaphase I, it is evident that bivalents disjoin and univalents divide simultaneously at anaphase I in most of the pollen mother cells. The products of meiosis are interrelated with chromosome pairing at metaphase I and chromosome distribution at anaphase I. Usually, when all homoeologous chromosomes pair, normal meiosis occurs and produces four haploid microspores; when few homoeologous chromosomes pair, first division restitution (FDR) probably occurs and produces two $2n$ FDR microspores; if bivalents disjoin and univalents divide simultaneously at anaphase I, such meiosis usually produces four aneuploid microspores or indeterminate meiotic restitution (IMR) can occur and produce two $2n$ IMR microspores. Therefore, most LA hybrids have possibilities to produce a high amount of aneuploid gametes and a small amount of $2n$ gametes or n gametes (Chapter 2). The aneuploid gametes are, without any doubt, the reason for the sterility of F1 LA hybrids. As to functional $2n$ gametes, based on the homoeologous distribution at anaphase I, the F1 LA hybrids have more potential to produce IMR $2n$ gametes than FDR $2n$ gametes (Chapter 2). However, most of the BC1 progenies results from FDR $2n$ gametes and less from IMR $2n$ gametes (Lim et al. 2001b, Barba-Gonzalez et al. 2005a, 2005b, Chapters 3 & 4). It might be argued that FDR $2n$ gametes have better viability than IMR $2n$ gametes because chromosomal composition of FDR $2n$ gametes is more balanced than that of IMR $2n$ gametes.

Crossover and intergenomic recombination

Study of crossover events usually resorts to chiasmata counting and molecular mapping (Nilsson et al. 1993, Sybenga 1996). In this thesis, the crossover events, e.g., single, three strand double, four strand double, four strand triple crossover, etc, were elucidated for the first time by analyzing anaphase I of F1 LA hybrids in *Lilium* with genomic in situ hybridization (GISH) (Chapter 2). This technique would be an ideal method to investigate types of crossover in interspecific lily hybrids. This is because lily has large chromosomes and GISH could discriminate the parental chromosomes of lily interspecific hybrids (Karlov et al. 1999, Lim et al. 2001b, Barba-Gonzalez et al. 2004, 2005a, 2005b). Based

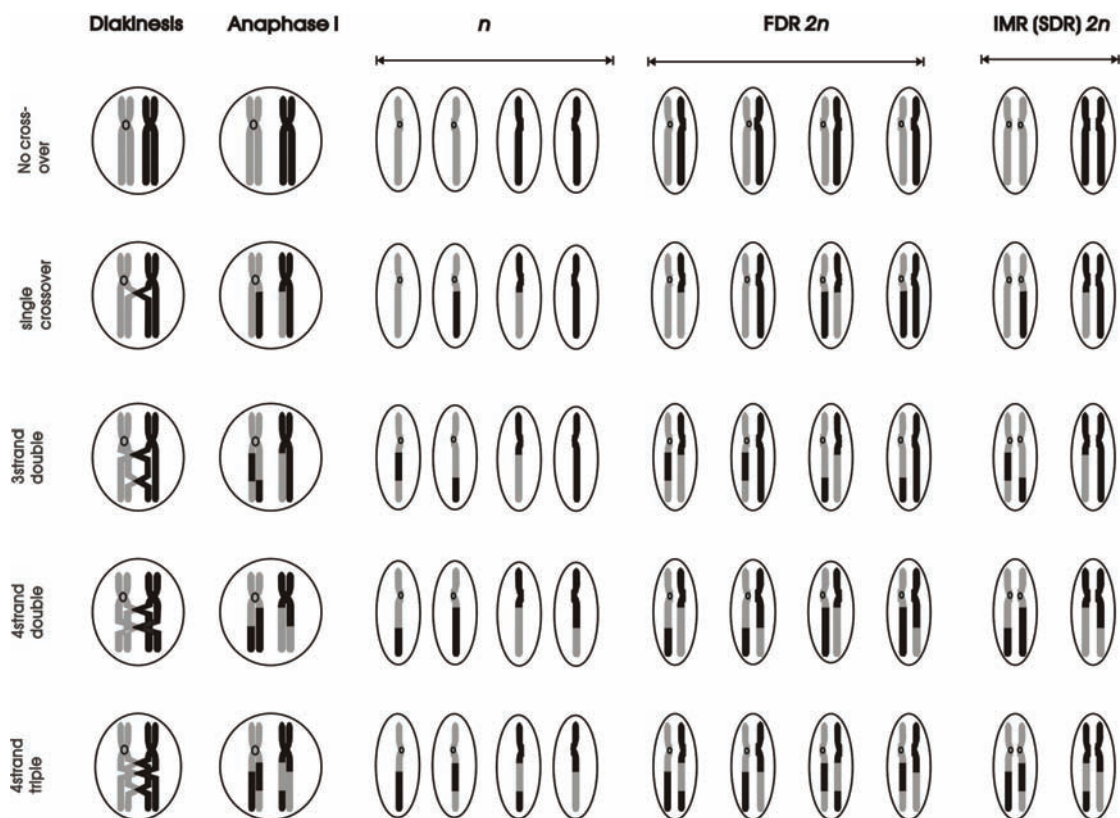


Figure 6-1. Crossover events and their possible consequence in n and $2n$ gametes

on the crossover events which occurred in F1 LA hybrids, the relationship between crossover events and their possible consequence in n and $2n$ gametes of F1 hybrids are illustrated in Figure 6-1.

The ploidy levels of backcross progenies

The ploidy levels of BC1 progenies

Most of the BC1 progenies previously reported, regardless of ALA or AOA, are eutriploid and few of them are eutetraploid. Both the triploids and tetraploids result from $2n$ pollen of F1 LA or OA hybrids (Lim et al. 2001b, Barba-Gonzalez et al. 2005a, 2005b). In this thesis, most of the BC1 cultivars selected by the Dutch lily companies and most of the new BC1 progenies are also eutriploid (Chapters 3 & 4). Most of the triploid BC1 progenies and cultivars result from FDR $2n$ gametes and only a few from IMR. Their intergenomic recombinant chromosomes are usually caused by single crossover. Only few cases possibly result from double crossover or others because of interstitial recombination (Barba-Gonzalez et al. 2005b, Chapter 4). Interestingly, 10 of the 45 new BC1 progenies are diploid (Chapter 4). Based on their genome compositions, it is evident that the recombinant chromosomes were generated from single crossover to multiple crossovers and the maximum number of breakpoints in some chromosomes was found up to 6. This finding showed that introgression breeding with some LA hybrids in lilies can be accomplished at the diploid level, similarly to that demonstrated in hybrids of *Allium cepa* x *A. fistulosum* (Emsweller and Jones 1945; Khrustaleva et al. 2005); *Alstroemeria aurea* x *A. inodora* (Kamstra et al. 1999a), *Lycopersicon esculentum* x *Solanum lycopersicoides* (Chetelat et al. 1997) and *Festuca* x *Lolium* (Zwierzyłowski et al. 1998, Thomas et al. 2003).

The ploidy levels of BC2 progenies

Only limited BC2 progenies, which originated from crosses between triploid BC1 cultivars and diploid Asiatic cultivars, were analyzed with GISH. They were predominantly diploid and contained very few Longiflorum chromosomes or segments. This implies that some specific Longiflorum traits are possibly transferred into Asiatic cultivars (Chapter 5). It should be pointed out that most of the previous BC2 progenies were aneuploid. The triploid ALA or AOA lilies

contribute around $x+6$ chromosomes to most BC2 progenies (Lim 2000, Barba-Gonzalez 2005).

The ploidy levels of BC3 progenies

The allopolyploid lilies, which originated from crossing between triploid BC1 (ALA) and allotetraploid (LALA) (Lim et al. 2003), were successfully crossed with Asiatic cultivars and Longiflorum cultivars. Most of the BC3 progenies were pseudoeuploid and possessed euploid chromosome number ($2n=3x=36$) but the parental genomes were aneuploid as a result of chromosome substitutions, whereas apart from this, one aneuploid ($2n=3x+1=37$) was found. Both the pseudoeuploids and the aneuploids might contribute to genetic variation and are potentially useful for selection.

The success and failure of lily crosses

Usually, the success or failure of interploid crosses is attributed to the ratio of endosperm and embryo ploidy level in many other plant species (Brandham 1982). In this thesis, interploid or intraploid crosses were made after normal pollination and embryo rescue. It was found that the degree of success or failure of lily crosses depends on the ploidy level of secondary nucleus and viability of the gametes. Based on the results of lily crosses, diploid, triploid, tetraploid, and pentaploid lilies could be used as male parents when they had male fertility to some extent; most diploid and triploid lilies could be used as female parents regardless of their male fertility. On the contrary, allotetraploid and allopolyploid lilies could hardly be used as female parents even though they had good male fertility as estimated from pollen germination. Therefore, a crossing polygon of lily intra- and interploid crosses related with LA hybrids is proposed (Figure 6-2). It might be valuable for lily breeding.

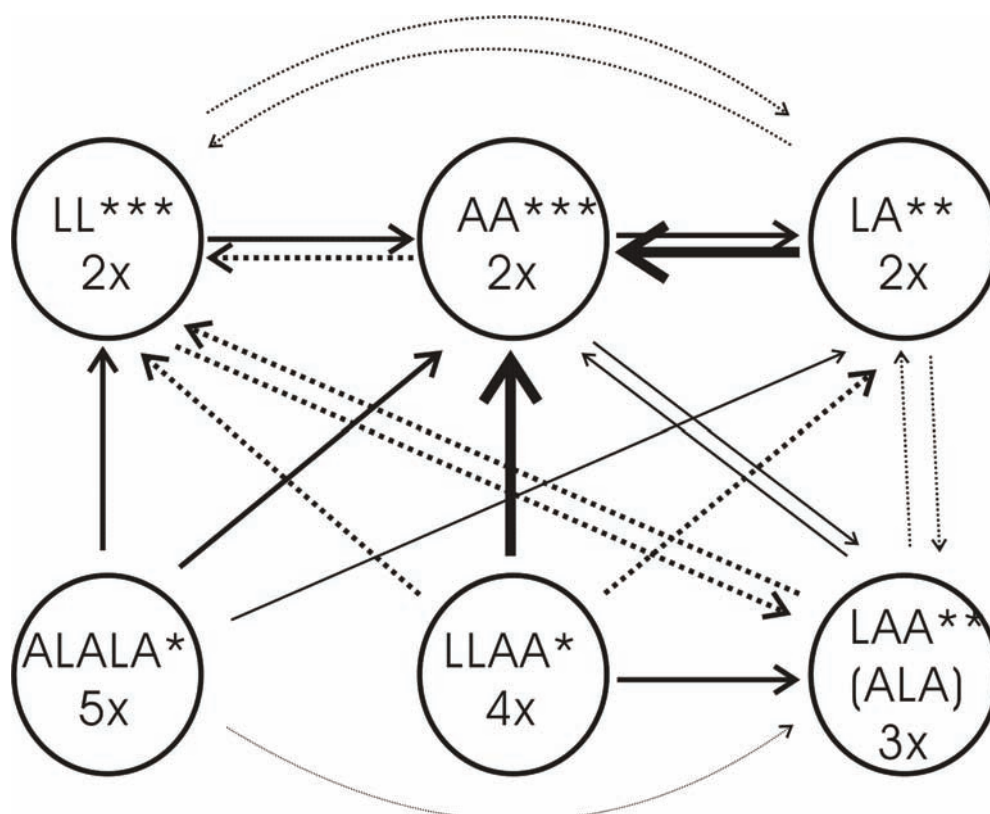


Figure 6-2. A crossing polygon of intra- and interploid crosses related with Longiflorum and Asiatic hybrids in *Lilium*. In this figure, the successful crosses were connected with dash arrows, and the expected successful crosses with dotted arrows. The arrows point towards female parents. The thinner, the less successful. The prerequisite for any cross is that its male parent is fertile.

* means the self crosses were hardly successful;

** means the self crosses might be possible if some genotypes have good male and female fertility;

*** means the selfed crosses were highly successful.

The difference between crosses and their reciprocals

Many reciprocal differences in interploid crosses were reported (review, Ramsey & Schemske 1998). In lily, the difference between 2x-4x and 4x-2x or 2x-5x and 5x-2x is obvious, because one direction was successful and the other not. A salient difference was also observed between 2x-3x and 3x-2x and between AA x LA and LA x AA. The difference is possibly caused by the frequency of functional

gametes, ploidy level of endosperm and the interaction between embryo and endosperm development (Chapter 5).

Significance of n gametes and $2n$ gametes for lily breeding

F1 distant hybrids usually have abnormal meiosis and are highly sterile. In all previous lily researches, only functional $2n$ gametes produced by lily distant interspecific hybrids were found (Van Tuyl et al. 1989, Asano 1982a, Lim et al. 2001b, Barba-Gonzalez et al 2005a, 2005b). In this thesis, with a more precise screening, not only the genotypes which produce functional $2n$ gametes were selected, but the genotypes which produce n gametes as well (Chapter 4). The advantages of $2n$ gametes over mitotic doubling in lily breeding have been well elucidated and confirmed (Lim et al. 2001b, Barba-Gonzalez et al 2005a, 2005b, Chapters 3 and 4). The variation caused by $2n$ gametes considerably increases the chance of selection from BC1 progenies. This might be the reason why Dutch lily breeders have selected many cultivars directly from the BC1 LA progenies (Chapter 3). The occurrence of n gametes in the F1 LA hybrids and the production of diploid BC1 progenies have been reported for the first time in this thesis. The diploid BC1 progenies have more interstitial recombinant chromosomes than triploid BC1 progenies, and it is expected that they may have a relative good fertility. This finding might open a new way for lily LA introgression breeding.

Possibility of bilateral sexual polyploidization possible in lilies

Bilateral sexual polyploidization results from the occurrence of both male and female $2n$ gametes simultaneously as has been shown in, for example, *Triticum-Aegilops* hybrids (Fukuda & Sakamoto 1992), *Triticum-Hynaldia* hybrids (Stefani 1986), *Avena sativa* haploids (Rines & Dahleen 1990), *Triticum turgidum* haploids (Jauhar et al. 2000), *Alstroemeria* (Ramanna et al. 2003), etc. Because the F1 hybrids are highly sterile, such bilateral sexual polyploidization from crosses between two lily F1 interspecific hybrids has not been accomplished. If two F1 hybrids are highly sterile, it is expected that the crossing between them is not

successful. However, the male and female fertilities of different F1 LA hybrids are highly variable based on their pollen germination and the results of backcrosses (Chapter 4). It is expected that bilateral sexual polyploidization is possible when a F1 hybrid with good female fertility (i.e. 041558) is crossed with another F1 hybrid with good male fertility (i.e., 041502). The expectation has been confirmed with the crosses between some F1 LA hybrids, e.g., “041546x041502”, “041548x041502” and “041558x041502”. The main advantage of bilateral sexual polyploidization is that the tetraploid progenies with intergenomic recombination would be fertile as illustrated in *Alstroemeria* (Ramanna et al. 2003). Such progenies could be used as male parent to cross with diploid cultivars for producing much more triploid plants with considerable genetic variation than via unilateral sexual polyploidization.

From the results presented in this thesis, the following conclusions can be drawn:

1. Different genotypes of LA hybrids have variable configurations at metaphase I during their meioses. Most of them have abnormal meiosis and produce huge amounts of aneuploid gametes and small amounts of $2n$ gametes. Only a few of the pollen mother cells of some genotypes have normal meiosis and produce n gametes.
2. The variation of BC1 progenies caused by intergenomic recombination of $2n$ gametes might be one of the main reasons why Dutch lily breeders could have selected new cultivars directly from the progenies.
3. Some interploid crosses involved in allopolyploids are successful in lilies. Usually, diploid and triploid lily can be used as female in the interploid crosses regardless of their male fertility. On the contrary, tetraploids and pentaploids hardly can be used as female, although they are good male parents in $2x-4x$, $3x-4x$, and $2x-5x$ crosses.
4. The phenomena of the differences between reciprocal crosses are elucidated. They are caused by the viability and the frequency of the gametes, the ploidy level of secondary nuclei, and the interaction between the endosperm and embryo development.

Especially, the occurrence of functional haploid gametes of F1 LA hybrids and some diploid BC1 progenies were obtained for the first time. These diploid BC1 progenies have three advantages over the triploid BC1 progenies for introgression breeding: 1) they usually contain more recombinant chromosomes, especially interstitial recombinant chromosomes because haploid gametes result from normal meiosis; 2) introgression can be accomplished at the diploid level; 3) the diploid BC1 progenies are expected to have better fertility than triploid BC1 progenies. So, this finding might open a new way for LA hybrid introgression breeding in lilies.

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Summary

Lily, one of the economically most important ornamental crops, belongs to the genus *Lilium* of the family Liliaceae. There are about 80 species in *Lilium* which are categorized into seven sections, i.e., *Lilium*, *Martagon*, *Pseudolirium*, *Archelirion*, *Sinomartagon*, *Leucolirion* and *Oxypetala*. Usually, it is not so difficult to cross between the species within each section and the hybrids are fertile. However, it is very difficult to cross the species belonging to different sections. With cut style pollination followed by embryo rescue techniques, such distant interspecific crosses can be possible, but the hybrids are highly sterile. All modern lily cultivars have originated from hybridization among wild lily species. The three main lily cultivar groups, viz., Longiflorum, Asiatic and Oriental, originated from hybridization within one section, i.e., *Leucolirion*, *Sinomartagon* and *Archelirion* respectively. Up to now, about 150 Longiflorum, 4000 Asiatic cultivars, and 2000 Oriental cultivars have been registered. The genomes of Longiflorum (*Leucolirion*), Asiatic (*Sinomartagon*) and Oriental (*Archelirion*) are represented as L (Longiflorum), A (Asiatic) and O (Oriental) genome respectively. They possess quite different valuable traits, and one of the main goals of modern lily breeding are to combine the three distinctive groups in new cultivars.

In this thesis, crosses among diploid Asiatic and Longiflorum cultivars, diploid F1 LA hybrids, triploid BC1 cultivars, allotetraploid and allopolyploid lilies were made. 11 diploid F1 LA hybrids and 19 triploids BC1 cultivars which were supplied by the Dutch lily breeding companies, and 23 new BC1, five BC2 and seven BC3 progenies were analyzed with conventional cytological methods, flow cytometry and genomic in situ hybridization.

The configurations of metaphase I during meiosis of the F1 LA hybrids are quantitatively variable, ranging from no bivalent to 12 bivalents in different pollen mother cells. This implies that LA hybrids have abnormal meiosis and normal meiosis, and indicates that LA hybrids have possibilities to produce aneuploid gametes, $2n$ gametes and n gametes (Chapter 2). Because the bivalents disjoin and the univalents divide simultaneously at anaphase I of the observed pollen mother cells, it is concluded that F1 LA hybrids have more potential to produce

IMR $2n$ gametes than FDR $2n$ gametes (Chapter 2). However, most of the BC1 progenies result from FDR $2n$ gametes and less from IMR $2n$ gametes. Probably, FDR $2n$ gametes have better viability than IMR $2n$ gametes because of chromosome and gene imbalance in the latter (Chapters 3 & 4).

Besides the mode of $2n$ gamete formation, some crossover events, e.g., single, three strand double, four strand double, four strand triple crossover, etc, are clearly elucidated based on the GISH results from anaphase I of F1 LA hybrids (Chapter 2). The intergenomic recombinant chromosomes of triploid BC1 progenies mainly originate from single crossover (Chapters 3 & 4). The intergenomic recombinant chromosomes caused by other crossover events are confirmed in diploid BC1 progenies (Chapter 4).

Based on GISH analysis of 19 BC1 cultivars from the Dutch lily breeding companies, 17 of the BC1 cultivars are eutriploid and two hypertriploid (Chapter 3). Nevertheless, among 45 new BC1 progenies, 10 of them are diploid, while the others are triploid (Chapter 4). This is the first reported finding that LA hybrid can produce functional haploid gametes. This finding might be valuable for lily introgression breeding.

Only limited BC2 progenies, which originated from crosses between triploid BC1 cultivars and diploid Asiatic cultivars, were analyzed with GISH. They were predominantly diploid and they contained very few Longiflorum chromosomes or segments (Chapter 5).

Allopentaploid lilies have relatively good male fertility as determined from their pollen germination. They were successfully crossed with Asiatic cultivars and Longiflorum cultivars. Most of the BC3 progenies were pseudoeuploids that possessed euploid chromosome numbers ($2n=3x=36$) but the parental genomes were aneuploid as a result of chromosome substitutions. Apart from this, one aneuploid ($2n=3x+1=37$) was also present. Both the pseudoeuploids and the aneuploids might contribute to genetic variation and are potentially useful for selection.

Based on the results of lily interploid crosses ($2x-3x$, $2x-4x$, $2x-5x$ and their reciprocals), diploid, triploid, tetraploid, and pentaploid lilies could be used as

male parents when they had some degree of male fertility. Most diploid and triploid lilies could be used as female parents regardless of their male fertility. On the contrary, allotetraploid and allohexaploid lilies could hardly be used as female parents even though they had good fertility as estimated from pollen germination tests. The success and failure of interploid crosses in lilies depends on the viability of the gametes and the ploidy level of the secondary nucleus. From the process of tetrasporic eight-nucleate embryo sac formation in *Lilium*, it is derived that diploid, triploid, tetraploid, and pentaploid lilies produce tetraploid, hexaploid, octaploid and decaploid secondary nuclei in their embryo sacs respectively. Thus, It is suggested that tetraploid secondary nucleus might be ideal for lily endosperm development; hexaploid secondary nucleus are acceptable; but octaploid or higher secondary nuclei are not ideal (Chapter 5).

The difference between $2x-4x$ and $4x-2x$ or $2x-5x$ and $5x-2x$ is obvious, because one was successful and the other not. A clear difference was also observed between $2x-3x$ and $3x-2x$ and between $AA \times LA$ and $LA \times AA$. The difference is possibly caused by the frequency of functional gametes, ploidy level of endosperm and the interaction between embryo development and endosperm development (Chapter 5).

$2n$ gametes and n gametes might play different roles in lily breeding. The former is more useful for polyploid breeding; the latter may be valuable for introgression breeding. We could not over- or underestimate either of them, because the LA hybrids which could produce functional $2n$ gametes or n gametes are very limited. Especially, it is more difficult to find LA hybrids which produce functional n gametes. The advantages of $2n$ gametes over mitotic doubling in lily breeding have been well confirmed (Chapters 3 and 4). One of the further tasks is how to use the LA hybrids which produce viable n gametes in lily breeding.

Samenvatting

Lelie, één van de economisch belangrijkste siergewassen, behoort tot het geslacht *Lilium* en de Liliaceae familie. Het geslacht *Lilium* is onderverdeeld in 7 secties met in totaal ca 80 species, t.w. *Lilium*, *Martagon*, *Sinomartagon*, *Pseudolirium*, *Archelirion* en *Oxypetala*. In het algemeen is het niet heel moeilijk om soorten binnen een sectie met elkaar te kruisen en de hybriden bezitten meestal enige fertiliteit. Het is echter niet eenvoudig om leliesoorten afkomstig uit verschillende secties, met elkaar te kruisen. Door gebruik te maken van de afgesneden stijlbestuiving en embryocultuur methoden toe te passen zijn zulke kruisingen mogelijk, maar de hybriden zijn in hoge mate steriel. Het moderne leliecultivar-sortiment is hoofdzakelijk afkomstig van kruisingen tussen verschillende wilde leliespecies. De cultivars worden ingedeeld in drie hoofdgroepen, t.w. de Longiflorum-, de Aziatische- en de Oriental-hybriden, die respectievelijk afkomstig zijn uit kruisingen binnen de secties *Leucolirion*, *Sinomartagon* en *Archelirion*. Momenteel zijn circa 150 Longiflorum-, 4000 Aziatische- en 2000 Oriental-hybriden geregistreerd. De genomen van de Longiflorums (*Leucolirion*), de Aziaten (*Sinomartagon*) en de Orientals (*Archelirion*) worden respectievelijk aangeduid als L- (Longiflorum), A- (Azaat) en O- (Oriental) genom. Zij bezitten zeer verschillende waardevolle eigenschappen en één van de hoofddoelstellingen van de moderne lelieveredeling is het verenigen van de eigenschappen van deze drie groepen in nieuwe cultivars. In dit proefschrift is kruisingsonderzoek verricht met divers uitgangsmateriaal t.w. diploïde Aziatische- en Longiflorum-cultivars, diploïde F1 LA hybriden, triploïde BC1 LA cultivars en allotetraploïde en allopentaploïde LA-hybriden. 11 Diploïde F1 LA hybriden en 19 triploïde BC1 cultivars, die door de Nederlandse lelieveredelingsbedrijven ter beschikking werden gesteld, en 23 nieuwe BC1 vijf BC2 en zeven BC3 nakomelingen werden onderzocht met behulp van conventionele cytologische methoden, flow cytometrie en genomische in situ hybridisatie. De metafase I configuraties gedurende de meiose van de F1 LA hybriden bleken in verschillende pollenmoedercellen kwantitatief te variëren van 0 tot 12 bivalenten.

Dit betekent dat LA-hybriden de mogelijkheid bezitten om aneuploïde, $2n$ -gameten en n -gameten te produceren (Hoofdstuk 2). Omdat de bivalenten uit elkaar gaan en de univalenten zich gelijktijdig verdelen tijdens de anafase I in de waargenomen pollenmoedercellen is geconcludeerd dat F1 LA hybriden meer gelegenheid hebben om IMR $2n$ -gameten dan om FDR $2n$ -gameten te produceren (Hoofdstuk 2). De meeste BC1 nakomelingen zijn echter afkomstig van FDR $2n$ -gameten en veel minder van IMR $2n$ -gameten. Waarschijnlijk hebben FDR $2n$ -gameten een hogere levensvatbaarheid dan IMR $2n$ -gameten vanwege een onevenwichtige chromosoom- en genbalans in laatstgenoemde gameten (Hoofdstuk 3 & 4).

Naast de wijze van $2n$ -gameet vorming, zijn enkele overkruisingsgebeurtenissen t.w. de enkelvoudige, de driestrengs dubbele, de vierstrengs dubbele, de vierstrengs drievoudige etc., duidelijk verhelderd op basis van de GISH resultaten afkomstig van de anafase I van F1 LA-hybriden. De intergenomische recombinante chromosomen van triploïde BC1 nakomelingen zijn hoofdzakelijk afkomstig van een enkelvoudige overkruising (Hoofdstuk 3 & 4). De intergenomische recombinante chromosomen afkomstig van andere typen van overkruising zijn bevestigd in diploïde BC1 nakomelingen (Hoofdstuk 4).

Op grond van de GISH-analyse van 19 BC1 cultivars afkomstig van de Nederlandse veredelingsbedrijven, bleken 17 BC1 cultivars triploïd ($3x$) en twee hypertriploïd ($3x+1$) (Hoofdstuk 3). Desondanks werden er na analyse van 45 nieuwe BC1 nakomelingen naast triploïden ook 10 diploïden gevonden (Hoofdstuk 4). Dit is de eerste keer dat aangetoond is dat LA-hybriden functionele haploïde gameten kunnen produceren. Deze ontdekking kan van groot belang zijn voor de introgressie van eigenschappen bij de lieweredeling.

Slechts een beperkt aantal BC2 nakomelingen, afkomstig van kruisingen tussen triploïde BC1 cultivars en diploïde Aziatische cultivars, werd geanalyseerd met behulp van GISH. Deze bleken voor het merendeel diploïd en zij vertoonden slechts enkele Longiflorum chromosomen of segmenten ervan (Hoofdstuk 5).

Allopentaploïde lelies bleken op grond van een goede pollenkieming een relatief goede manlijke fertiliteit te bezitten. Zij werden met succes gekruist met Aziatische en Longiflorum-cultivars. De meeste BC3 nakomelingen bleken pseudoeuploid in die zin dat zij een euploïd chromosoomaantal bezaten ($2n=3x=36$), maar de

ouderlijke chromosomen waren aneuploïd vanwege chromosoom substituties. Daarnaast werd er ook één aneuploïde nakomeling ($2n=3x+1=37$) gevonden. Zowel de pseudoeuploïden als de euploïden kunnen bijdragen aan de genetische variatie en zijn in principe gunstig voor selectie.

Op grond van de resultaten van de interploidie kruisingen ($2x-3x$, $2x-4x$, $2x-5x$ en de reciproke combinaties) konden diploïde, triploïde, tetraploïde en pentaploïde lelies gebruikt worden als vaders indien er voldoende manlijke fertiliteit aanwezig was. De meeste diploïde en triploïde lelies konden als moeder gebruikt worden, ongeacht hun manlijke fertiliteit. Daarentegen konden allotetraploïde en allopentaploïde lelies nauwelijks als moeder gebruikt worden ook al bezaten zij een goede pollenfertiliteit. Het slagen of mislukken van interploidie kruisingen bij lelies hangt af van de levensvatbaarheid van de gameten en het ploïdieniveau van de secundaire kern. Vanuit het principe van de tetraspore achtkernige embryozakvorming in *Lilium* kan worden afgeleid dat diploïde, triploïde, tetraploïde en pentaploïde lelies respectievelijk tetraploïde, hexaploïde, octaploïde en decaploïde secundaire kernen in hun embryozakken vormen. Daarom is het voor de hand liggend dat een tetraploïde secundaire kern ideaal is voor de vorming van lelie-endosperm; een hexaploïde secundaire kern zou acceptabel kunnen zijn, maar secundaire kernen met een octaploïde of een nog hoger ploïdieniveau zijn verre van ideaal. (Hoofdstuk 5).

Het verschil tussen $2x-4x$ en $4x-2x$ of $2x-5x$ en $5x-2x$ kruisingen is duidelijk, want de één slaagt en de ander niet. Een duidelijk verschil werd ook waargenomen bij $2x-3x$ en $3x-2x$ en bij AA x LA en LA x AA combinaties. Het verschil wordt mogelijk veroorzaakt door de frequentie van de functionele gameten, het ploïdieniveau van het endosperm en de interactie tussen de ontwikkeling van embryo en endosperm. $2n$ -gameten en n -gameten kunnen een verschillende rol spelen in de lelieveredeling. De eerste is meer geschikt bij veredeling op een hoger ploïdieniveau, terwijl de laatste waardevol kan zijn bij de introgressie van bepaalde eigenschappen. We kunnen geen van beide over- of onderschatten, want de LA-hybriden die $2n$ - of n -gameten kunnen vormen zijn zeer beperkt. Het is vooral heel moeilijk om LA-hybriden te vinden die functionele n -gameten produceren. De voordelen van $2n$ -

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gameten boven mitotische verdubbeling bij de lieweredeling is duidelijk bevestigd (Hoofdstukken 3 en 4). Eén van de volgende uitdagingen is vast te stellen hoe de LA-hybriden, die functionele n-gameten produceren gebruikt kunnen worden bij de lieweredeling.

中文摘要

百合为重要的经济观赏作物，位居荷兰十大切花第四位，隶属于百合科百合属。该属共约有 80 余种，依其生物学性状，归为七组(即: *Lilium*, *Martagon*, *Pseudolirium*, *Archelirion*, *Sinomartagon*, *Leucolirion* 和 *Oxypetala*)，皆分布于北半球的山区。一般情况下，组内杂交较易成功且杂种可育；而组间杂交较难成功且杂种高度不育。现在切花百合主要有麝香百合、亚洲百合、东方百合和麝亚杂种百合四大品种群，其中前三大品种群分别源于 *Leucolirion*, *Sinomartagon* 和 *Archelirion* 的组内杂交。迄今，约有 150 个麝香百合品种，4000 个亚洲百合品种和 2000 个东方百合品种登记注册。由于源于不同组的百合品种拥有差异更大的农艺性状，因此，组间杂交的百合品种更具商业前景，如：麝亚杂种百合品种较少，但其生产面积和产值在四大品种群中仅次于东方百合。现在百合的育种目标主要是将三大不同组的优良农艺性状整合到新的栽培品种中。

本文在二倍体的亚洲百合和麝香百合、二倍体的麝亚百合杂种一代、三倍体的回交一代品种、异源四倍体和异源五倍体的麝亚百合之间进行了杂交，采用传统细胞学、DNA 含量测定和基因组荧光原位杂交的方法，对荷兰百合育种公司提供的 11 个二倍体麝亚百合杂种一代和 19 个三倍体回交一代品种，以及我们自己获得的 23 个回交一代、5 个回交二代和 7 个回交三代进行了分析。

不同麝亚百合杂种一代减数分裂中期构型存在数量差异，不同花粉母细胞中的 24 条近源染色体(12 条属亚洲百合，12 条属麝香百合)从不能形成任何二价体到可形成 12 对二价体不等。这意味着麝亚百合杂种一代既有异常的减数分裂又有正常的减数分裂，同时也表明了麝亚百合杂种一代既有产生非整倍体配子和体细胞配子的可能性，又有产生单倍体配子的可能性。由于观察到的花粉母细胞减数分裂的后期 I 阶段通常为二价体和单价体同时分开并移向两极，因此可推论为麝亚百合杂种一代产生不明确减数恢复(IMR)的体细胞配子的潜力大于产生第一次减数恢复(FDR)的体细胞配子的潜力(第二章)。然而，较多的回交一代源于第一次减数恢复的体细胞配子，而较少的回交一代源于不明确减数恢复的体细胞配子。造成这种现象的原因可能为由于第一次减数恢复的体细胞配子有平衡的染色体和基因组成，其生活力强于不明确减数恢复的体细胞配子(第三章和第四章)。

通过对麝亚百合杂种一代减数分裂后期 I 的基因组原位杂交分析，不仅对体细胞配子的形成模式，而且对联会近源染色体间的单交换、双交换和三交换等进行了清晰的阐述(第二章)。三倍体回交一代中的重组染色体多源于单交换(第三章和第四

章)。由其它交换造成的重组染色体在二倍体的回交一代中得到了证实(第四章)

荷兰百合育种公司提供的 19 个回交一代品种中, 17 个为整三倍体($2n=3x=36$), 两个为超三倍体($2n=3x+1=37$)(第三章)。然而, 在 45 个新回交一代中, 10 个为二倍体, 其余为三倍体(第四章)。麝亚百合杂种一代能产生有活力的单倍体配子为首次报道。这一发现会对百合种质渗入育种有较大意义。

在基因组原位杂交所分析的有限的回交二代中, 它们主要为二倍体, 且麝香百合的染色体大都被淘汰, 仅个别麝香百合的染色体或片段被保留在这些后代中(第五章)。

异源五倍体的麝亚百合的花粉有较高的萌发率, 即其雄性可育, 且与二倍体的亚洲百合品种和麝香百合品种成功杂交。这些回交三代多为拟三倍体, 即它们有整倍体的染色体数目($2x=36$), 但其中的亲本基因组因染色体的取代而非整倍体。除此之外, 也发现了一个非整倍体。无疑, 这些拟三倍体和非整倍体引起的遗传变异对育种选择会有较大的意义。

根据百合倍性间的正反交结果, 如果二倍体、三倍体、四倍体和五倍体百合根据花粉萌发表明雄性可育, 一般皆可作父本。一个有趣的现象是: 无论雄性是否可育, 二倍体和三倍体百合一般可作杂交母本; 相反, 即使四倍体和五倍体百合雄性可育, 它们一般难作杂交母本。百合倍性间杂交的成败取决于配子的活力和次级核(胚乳核)的倍性。从百合四孢子八核胚囊的形成过程可以推断二倍体、三倍体、四倍体和五倍体百合在其胚囊中分别形成四倍体、六倍体、八倍体和十倍体的次级核。因而, 杂交结果意味着四倍体的次级核对百合胚乳的发育是最理想的, 其次为六倍体的次级核, 而八倍体和十倍体的次级核不利于百合胚乳的发育(第五章)。

通常情况下, $2x-4x$ 杂交和 $2x-5x$ 杂交极易成功, 而其反交很难成功, 因此, $2x-4x$ 与 $4x-2x$ 的差异和 $2x-5x$ 与 $5x-2x$ 的差异是显而易见的。同样, $2x-3x$ 和 $3x-2x$ 之间, 以及 $AA \times LA$ 和 $LA \times AA$ 之间的差异也是显著的。这种差异可能是功能配子的频率、胚乳的倍性和胚与胚乳发育的相互作用引起的。

远缘杂种一代产生的体细胞配子和单倍体配子在百合育种中可起不同的作用。前者更利于多倍体育种, 后者更利于种质渗入育种。我们不可过分强调或忽视其中任何一方, 因为能产生有功能的体细胞配子或单倍体配子的麝亚百合杂种一代非常有限, 特别是发现能产生有功能的单倍体配子的麝亚百合杂种一代更加困难。体细胞配子相对于染色体加倍在百合育种中的优势得以确认(第三章和第四章)。下步的重要研究工作之一是如何利用这些可产生有功能单倍体配子的麝亚百合杂种一代。

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Twenty years ago when I studied in Horticulture Department of Nanjing Agricultural University, Dutch flower industry imprinted in my mind though all its stories were from textbook or other media. It had been a dream that I could go to the Netherlands to learn the knowledge and techniques of flower breeding or/and flower production. At this moment when I finish my PhD thesis research on lily breeding, it is my great honour to express my gratefulness to everybody that helped me in different ways.

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Curriculum vitae

Shujun Zhou was born in Zhucheng, Shandong Province, China on December 20, 1965. He studied at Nanjing Agricultural University, China and obtained his BSc in Ornamental Horticulture in 1987. He studied at Beijing University (Peking University), China and obtained his MSc degree in Botany in 1992. As a visiting scientist, he worked for one year in 1998-1999 at the Plant Breeding Laboratory, and for a half year in 2002 at Plant Research International, Wageningen University, The Netherlands. As a teacher (lecturer and associate professor), he was employed at Shandong Agricultural University, China from 1987 -1989 and 1992-2004. Since May 2004, he worked as PhD-student at Wageningen University and Research Center. The results of his research on lily cytogenetics and breeding are described in this thesis. His personal email address is: shujunzhou@msn.com.

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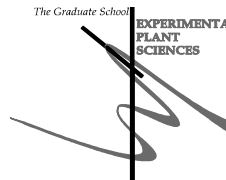
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Education Statement of the Graduate School

Experimental Plant Sciences

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Issued to: Zhou Shujun
Date: 27 March 2007
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase		<i>date</i>
▶ First presentation of your project		
Analysis of genome compositions of BC1 Cultivars with GISH (at cytogenetic lab meeting)		Oct 22, 2004
▶ Writing or rewriting a project proposal		
Introgression breeding through the use of sexual polyploids of LA lily hybrids		May-Aug 2004
▶ Writing a review or book chapter		
Interploid crosses in lily breeding', in Floriculture, Ornamental and Plant Biotechnology Global Science Books		Nov-Dec 2006
▶ MSc courses		
GEN-30306: Genetic analysis, tools and concepts (GATC)		Oct-Nov 2006
▶ Laboratory use of isotopes		
<i>Subtotal Start-up Phase</i>		19.5 credits*
2) Scientific Exposure		<i>date</i>
▶ EPS PhD student days		
PhD day, Wageningen		September 19, 2006
PhD day, Radboud University		Dec.8, 2006
▶ EPS theme symposia		
EPS Theme 3 symposium 'Genome Plasticity', Wageningen		Dec 9, 2005
EPS Theme 3 symposium, Science Park, Amsterdam University		Dec 10, 2005
▶ NWO Lunteren days and other National Platforms		
ALW meeting 'Experimental Plant Sciences'		April 03 - 04, 2006
▶ Seminars (series), workshops and symposia		
European flying seminar Rob Martienssen		Oct 23, 2006
▶ Seminar plus		
▶ International symposia and congresses		
European Life Science Conference for Young Scientists (ELSYS), Enschede (NL)		Feb 25-27, 2007
▶ Presentations		
A brief introduction on GISH and LA lily breeding (Lily company meeting)		Jul 19, 2004
Different gametes of LA hybrids and their significance in lily breeding (Lily company meeting)		Nov 28, 2005
Difference of lily reciprocal crosses (lily company meeting)		Jul 14, 2006
▶ IAB interview		September 18, 2006
▶ Excursions		
Visit lily, tulip companies		May 18, 2004
Visit lily, tulip companies		May 13, 2005
Visit lily, tulip companies		May 17, 2006
<i>Subtotal Scientific Exposure</i>		5.5 credits*
3) In-Depth Studies		<i>date</i>
▶ EPS courses or other PhD courses		
Advanced Biochemistry (Shandong Agricultural University, China)		Sep-Dec 2003
Gateway to Gateway technology (4 days)		Nov 20-24, 2006
System Biology (4 days)		Dec 11-14, 2006
▶ Journal club		
member of the literature discussion group at Plant Breeding Group		2004-2007
▶ Individual research training		
Microscope technique for chromosome research, Genetics, Wageningen (two weeks)		Jul 2005
<i>Subtotal In-Depth Studies</i>		9.9 credits*
4) Personal development		<i>date</i>
▶ Skill training courses		
Advanced English (Shandong Agricultural University, China)		Sep-Dec 2003
▶ Organisation of PhD students day, course or conference		
▶ Membership of Board, Committee or PhD council		
<i>Subtotal Personal Development</i>		1.5 credits*
TOTAL NUMBER OF CREDIT POINTS*		36.4

* A credit represents a normative study load of 28 hours of study