A molecular cytogenetic analysis of chromosome behavior in *Lilium* hybrids

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Chapter 1

General Introduction

Lily

Lilies belong to genus *Lilium* of Liliaceae family, and consist of about 80 species distributing in the northern hemisphere (Eurasia and North America continent). South-East Asia (China, Korean peninsula and Japan) and North America are two important distribution centers of lily, with 61 and 21 species respectively (Van Tuyl et al. 2011), and the number of native European and Caucasian (Eurasian) species is approximately 10 (Woodcock and Stearn 1950). Based on morphology, physiology, crossing ability and conserved DNA sequences, the species are taxonomically classified into seven sections, these sections are Martagon, Pseudolirium, Lilium, Archelirion, Sinomartagon, Leucolirion and Oxypetalum (Comber 1949; De Jong 1974; Nishikawa et al. 2001; Nishikawa et al. 1999).

Although many lily species have been used as ornamental plants for centuries, systematic breeding of lily cultivars started in the early 20th century, and the number of cultivars exceeds to more than 9000 thousand nowadays (International Lily register, http://www.lilyregister.com/; Leslie 1982; Woodcock and Stearn 1950). Today lilies are important plants that are cultivated for cut flowers and as pot plant, grown in gardens and planted as vegetable or medical use in Eastern Asia. Because of the crossing barriers between different sections, different hybrid groups, which possess distinctive phenotype characters, have been bred since the early twentieth century (McRae 1998). These cultivar groups possess divergent genomes, which cannot crossed with each other by conventional hybridization method. Among which, Longiflorum, Asiatic and Oriental hybrids are of great commercial importance, and hence, are the most widely cultivated:

Longiflorum hybrids (genome L): Cultivars in this group originated from section Leucolirion, and possess trumpet-shaped, pure white flowers, a distinctive fragrance, year-round forcing ability and mostly nodding flowers.

Asiatic hybrids (genome A): Cultivars in this group are derived from interspecific hybridization among about 12 species within Sinomartagon section, and possess a big variation of flower colour (orange, yellow, white, pink, red, purple and salmon), mostly upfacing flowers and early to late flowering (Woodcock and Stearn 1950). Some species, together with part of the cultivars in this group, show resistance to *Fusarium oxysporum* f.sp *lilii* and viruses (McRae 1998).

Oriental hybrids (genome O): Cultivars from this group are bred from interspecific hybridization between six species in section Archelirion. Flowers in this group have large size and strong fragrance (McRae 1998). Most of the cultivars in this group show a fair degree of resistance to *Botrytis elliptica* (Barba-Gonzalez et al. 2005a)

Some basic concepts on genetics

When an interspecific cross is made, the alien genome is introduced into a new genetic background, and the hybrids may undergo genomic shock (Chen and Ni 2006; McClintock 1984; Natali et al. 1998). The instability in new-synthesized interspecific hybrids caused by genomic shock underlies rapid genome changes in the following generations, such genome changes caused by complex intergenomic interaction consists of polyploidization, chromosome rearrangements (structural chromosome aberrations), gene conversion, aneuploidy and so on (Soltis and Soltis 2000), which are considered to be important in plant polyploids. As a result, extensive intergenomic exchanges were conclusively proven to have occurred in many allopolyploids, both revealed by DNA *in situ* hybridization and molecular markers (Brubaker et al. 1999; Osborn et al. 2003; Pontes et al. 2004).

Recently, the so called chromosome rearrangements in allopolyploids were extensively analyzed in a few natural and re-synthesized allopolyploids. Among others, *Brassica napus* supplies a good example in point. *B. napus* is believed to originated from interspecific hybridization between *B. oleracea* (CC, 2n=18) and *B. rapa* (AA, 2n=20) followed by polyploidization (U 1935). When analyzing these natural and synthetic tetraploid *B. napus* populations with molecular markers, various types of "chromosome rearrangements" were detected, such as homoeologous non-reciprocal translocation, homoeologous reciprocal translocation, duplication, deletion and so on (Osborn et al. 2003; Parkin et al. 1995; Sharpe et al. 1995). Later on, it was confirmed that homoeologous recombination during meiosis of the haploid *B. napus* is the main reason of the genetic changes (Gaeta and Pires 2010; Gaeta et al. 2007; Nicolas et al. 2007; Xiong et al. 2011). In addition, genome changes, viz. deletion, duplication, inversion and so on, were also proven to be present by comparing the natural allopolyploids with the re-synthesized allopolyploids or their progenitors, in *Arabidopsis suecica* which is derived from cross between two diploid *Arabidopsis* species (*Arabidopsis thaliana* and *A. arenosa*)(O'Kane Jr et al. 1996; Pontes et al. 2004), in amphidiploid *Nicotiana tabacum* (Kenton et al. 1993), in cultivated *Gossipium* (Brubaker et al. 1999; Reinisch et al. 1994), in *Avena maroccana* (Leitch and Bennett 1997; Soltis and Soltis 1999), in *Avena sativa* (Chen and Armstrong 1994), in allotetraploid *Tragopogon* (Lim et al. 2008b) and many other species.

Genetic changes induced by genomic shock in early generations not only contribute to speciation of hybrids, but also supply diverse materials for plant breeding. Those above mentioned non-Mendelian and rapid genome reconstruction might be a mechanism for generating de novo genomic variation and increasing genetic and morphological complexity, which may partly explain the evolutionary success of allopolyploids over their diploid

counterparts (Finnegan 2002; Liu and Wendel 2002; Pikaard 2001; Rieseberg 2001b; Soltis and Soltis 1999; Song et al. 1995). Since exchange of genetic contents is also critical for transferring traits across distantly related plant species to obtain combinations of desirable characteristics in agriculture and horticulture (Lim et al. 2003), intergenomic chromosome recombination has been extensively induced and utilized in introgression breeding and crop improvement of some main crops. Hexaploid wheat (AABBDD, 2n=6x=42) which contains a translocated chromosome fragment on the long arm of the 1B chromosome from the rye (*Secale cereale*) 1R chromosome are widely used in wheat breeding, this satellite from 1R contains several agronomical important genes including those for seed storage proteins and for disease resistance. In the oilseed *Brassica napus*, lines with the N7-N16 reciprocal recombination harvested a significant higher seed yield compared with that without the reciprocal recombination (Osborn et al. 2003).

Methods used for the detection of chromosome rearrangements

Due to the importance of chromosome structure variation in plants, research on chromosome rearrangements has been a topic of interests for many decades, and the methods used to detect them cover classical cytogenetic methods, molecular marker systems, molecular cytogenetic techniques and sequence-based innovational methods.

A wide range of classical cytogenetic methods have been applied for detecting chromosome rearrangements, both in diploid and polyploid species. Many small chromosome rearrangements that are not detected by mitotic observation can be seen in meiotic analysis according to the meiosis configuration. For example, an inversion heterozygote can be recognized by its association loop at metaphase I and dicentric & acentric fragments at anaphase I. A translocation heterozygote can also be detected by its multivalent formation at metaphase I and the aberrant segregation at anaphase I (reductional or equational segregation), which will cause duplication and deletion in the resultant gametes. Since the mid-20th century, chromosome banding has become one of the main methods to analyze chromosome rearrangements. Because of the different banding karyotypes, some of the introgressed chromosome/segments can be distinguished by their specialized bands (Badaeva et al. 2007), For example, the chromosome 1R from rye demonstrates divergent C bands on the long arm, and as a result, the long arm becomes obviously visible when C banding technique is applied in the translocation lines. Furthermore, some structural variation can also be identified by combined banding techniques. A range of chromosome rearrangements, viz. inversion, deletion, fission and fusion, have been detected in many different species/species hybrids, such as *Equus africanus somaliensis* (Houck et al. 2000) and wheat (Friebe et al. 1996).

With the development of modern techniques, molecular markers are widely used for the detection of genome rearrangements. Compared with the traditional methods, molecular markers have solved the problem of poor resolution in detecting chromosome rearrangements, and have been proved to be a precise and effective way of detecting inter- and intra- specific chromosome rearrangements. Some types of structural variation of a chromosome, such as duplication and deletion, which are difficult to recognize with traditional cytogenetic methods, can be detected and reflected by the presence/absence of bands. One of the advantages is that the non-homologous translocation within the same genome can also be reflected. Furthermore, extensive inter- and intra- genomic rearrangements have been detected in many model plants, and the rates are much higher compared with conventional methods. In wheat, intergenomic translocation between non-homologous genomes can be easily detected using molecular markers (Mickelson-Young et al. 1995). Meanwhile, translocation between wheat and other species has also been characterized using different marker systems (Bonierbale et al. 1988; Boyko et al. 1999; Zhang et al. 1998). Furthermore, the characterization of chromosome rearrangements with molecular markers has also been used in some other plant species. For example, comparative genetics with RFLP mapping has revealed the existence of chromosome rearrangements between different plant species, viz., the comparison among wheat, maize, rice and other grass species(Gale and Devos 1998), between eggplant and tomato (Doganlar et al. 2002). As a result, comparative genetic mapping, in which different marker systems are used, has been proved to be an efficient way for detecting chromosome rearrangements.

However, there are some drawbacks when detecting chromosome rearrangements with molecular markers, which will mislead the real occurrence of chromosome rearrangements. Firstly, markers can just identify the changes in the progeny, which leave the origin of such changes behind, and that is why molecular markers confused recombination from natural meiosis process and real chromosome rearrangements. Secondly, changes in the intensity of bands cannot be well reflected by using DNA profiling method via counting the presence and absence of bands, when the parental bands share the same molecular weight or gene losses/conversion in duplications. Furthermore, balanced chromosome rearrangements such as reciprocal translocation and inversion, cannot be detected by molecular markers. As reported by many researchers, reciprocal recombinations in unreduced gametes produced by some interspecific hybrids could not be detected (Nicolas et al. 2007; Xie et al. 2010). In addition, marker systems require long-term collaborative research and is applicable for a limited number of plants (Badaeva et al. 2007).

DNA *in situ* hybridization, including genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH), was the predominant way and has received a

renewed interest in detecting chromosome rearrangements in recent years (Lim et al. 2008b; Pires and Hertweck 2008; Xie et al. 2010; Xiong et al. 2011). GISH, combined with FISH, allows the discrimination of alien chromosomes/segments and the identification of individual chromosomes in interspecific hybrids and backcrossing progenies (Barba-Gonzalez et al. 2005b; Khan et al. 2009a; Lim et al. 2001b; Schwarzacher et al. 1992; Schwarzacher et al. 1989; Stevenson et al. 1998; Zhou et al. 2008b). Since its successful application in detecting and analyzing intergenomic recombination between homoeologous genomes, the technique has been already used for detecting crossover events through analysis of anaphase I cells (Stevenson et al. 1998; Takahashi et al. 1997; Xie et al. 2010; Zhou et al. 2008a). The particular advantage of this system is that the two chromatids of each homoeologues have the same labeling status, and therefore all crossover exchanges between non-sister chromatids will be visible. As a result, it enables the accurate observation of homoeologous chromosome behaviours during meiosis. As pointed in a previous publication (Xie et al. 2010), the nonreciprocal and reciprocal recombination both originated from a natural meiosis process-chiasmata formation and crossing over between homoeologous chromatids, that is also the reason that the term "translocation" is not accurate in *Brassica napus* (Nicolas et al. 2007; Parkin et al. 1995; Sharpe et al. 1995; Udall et al. 2005); As a result, some genera, which consist of divergent genomes and large chromosomes, viz. *Tulipa, Lilium, Alstroemeria* and so on, are ideal for the GISH analysis. However, several disadvantages are also unavoidable for detecting chromosome rearrangements using DNA *in situ* hybridization. the first one is its poor resolution which made small recombinations invisible. Meanwhile, some kinds of rearrangements like duplication and deletion are, however, very difficult to distinguish; another shortage is that GISH is very experimental demanding and labor-intensive. Beside these, GISH can only detect chromosome variations between homoeologous and nonhomologous chromosomes. With their pros and cons of molecular markers and molecular cytogenetic techniques, there is a tendency that the combining of these two methods will lead to relatively accurate results, which has been used in several reports.

With the development of modern molecular biology, some innovational methods, such as whole genome sequencing and array comparative genomic hybridization (aCGH) which give detailed and informative sequence information, have become available recently. Array-based comparative genomic hybridization allows high-resolution screening of copy number abnormalities in the genome, and becomes an increasingly important tool to detect deletions and duplications in the whole genome (Knijnenburg et al. 2005).

Scope for detection and analysis of chromosomal rearrangements in lilies

Lily has been a model plant for cytogenetic research for more than one century. Lily species are predominantly diploid (2n=2x=24) with the exceptions of *L. tigrinum* and *L. bulbiferum* in which triploids (2n=36) are also present. Since Strasburger's paper on the chromosomes of *Lilium* (Strasburger 1880), many researchers, using lily species, have focused on the study of chromosome morphology and karyotype analysis, meiosis studies, chromosome banding and so on (Anderson et al. 1994; Bach Holm 1976; Fogwill 1957; Noda 1978; Son 1977; Son and Song 1978; Stack et al. 1989; Stewart 1947). Furthermore, some structural aberrations in the diploid species and interspecific hybrids have also been detected by critically observation of mitotic and meiotic chromosome configurations. In an X-ray treated *L. formosanum*, paracentric inversion was detected according to the association configuration and the resultant dicentric and acentric fragments (Brown and Zohary 1955). In a natural population of *L. maximowiczii*, reciprocal translocation was characterized by the multivalent formation and abnormal segregation at anaphase I during meiosis (Noda 1960). In addition, in the intrasectional hybrids of *Lilium martagon* var. *album* × *L. hansonii*, inversion was also observed by abnormalities of meiosis I (Richardson 1936). Since lily is not a leading crop and its long generation time, previous studies only focused on normal cytogenetic research, with little interests in producing cytogenetic stocks like addition and substitution lines.

Current commercial breeding of lily aims at combining desirable traits together through interspecific hybridization and backcrossing. Since the end of 20th century, interspecific hybrids and polyploids have been two main characters of the new lily cultivars (Van Tuyl and Lim 2003), these cultivars are the combination of two or more homoeologous genomes from genetically divergent parental species. Such allopolyploids are ideal for analyzing intergenomic rearrangements using GISH for two main reasons: firstly, the lily genome belongs to the biggest in the plant kingdom (250 fold larger than that of Arabidopsis) and the chromosomes are very big which make the cytological observation easily (Leutwiler et al. 1984; Zonneveld et al. 2005); Secondly, the genomes of different hybrid groups are so highly divergent that make the differentiation of each genome obviously and the structural rearrangements, if any, be detected accurately.

As mentioned above, the occurrence of chromosome rearrangements in the newly formed allopolyploids has been revealed in many polyploid species. Like other plant taxa, how these genomes interact and harmonize with one another in lily interspecific hybrids as well as the backcrossing progenies is a topic of interests for many researchers. Though a critically analysis of the neopolyploids of lily, information about the origin of polyploids, homoeologous genome interaction and the speciation of allopolyploids can be acquired. As a result, GISH has already been successfully applied in lily hybrids for studying intergenomic

recombination, mechanisms of the unreduced gametes production, crossing-over events during meiosis and the construction of cytogenetic recombination maps (Barba-Gonzalez et al. 2005a; Barba-Gonzalez et al. 2005b; Karlov et al. 1999; Khan et al. 2009a; Lim et al. 2000; Lim et al. 2001a; Lim et al. 2003; Xie et al. 2010; Zhou et al. 2008a). Interestingly, it has been found that the so called intergenomic translocation in lily neopolyploids is not a real translocation, but recombination derived from phenomena in natural meiosis: chiasmata formation and crossing over (Xie et al. 2010).

Unreduced gametes

Polyploids with two or more chromosome sets, which consist of autopolyploids and allopolyploids according to the homologous relationship between genomes in the complement, are widespread in flowering plants. It is estimated that up to 70% species in angiosperm are polyploids and the origin is believed to arise commonly through the meiotic-derived unreduced gametes (Bretagnolle and Thompson 1995; Ramanna and Jacobsen 2003; Ramsey and Schemske 1998).

Unreduced (2n) gametes, gametes with a somatic chromosome number, are produced by most of the angiosperms. Since the 80s of the 20th century, the importance of 2n gametes in crop breeding has been fully realized and the mechanisms responsible for 2n gametes production has been well studied in cultivated materials which possess high degree of heterozygosity and genetic variation (Ramanna 1992; Ramanna and Jacobsen 2003; Veilleux 1985). Generally speaking, meiotic abnormalities such as the omission of the first or second meiotic division, abnormal spindle morphology in the second division, or disturbed cytokinesis can lead to the production of viable, unreduced gametes (Bretagnolle and Thompson 1995; Brownfield and Köhler 2011; Ramanna and Jacobsen 2003). Depending on the particular meiotic stages at which nuclear restituted, different restitution mechanisms have been proposed using traditional cytogenetic approaches and molecular cytogenetic techniques. In interspecific hybrids of lily, three different mechanisms viz. first division restitution (FDR), second division restitution (SDR), indeterminate meiotic restitution (IMR) (Lim et al. 2001a) are relevant to the production of viable unreduced gametes and are schematic illustrated in Fig. 1.1.
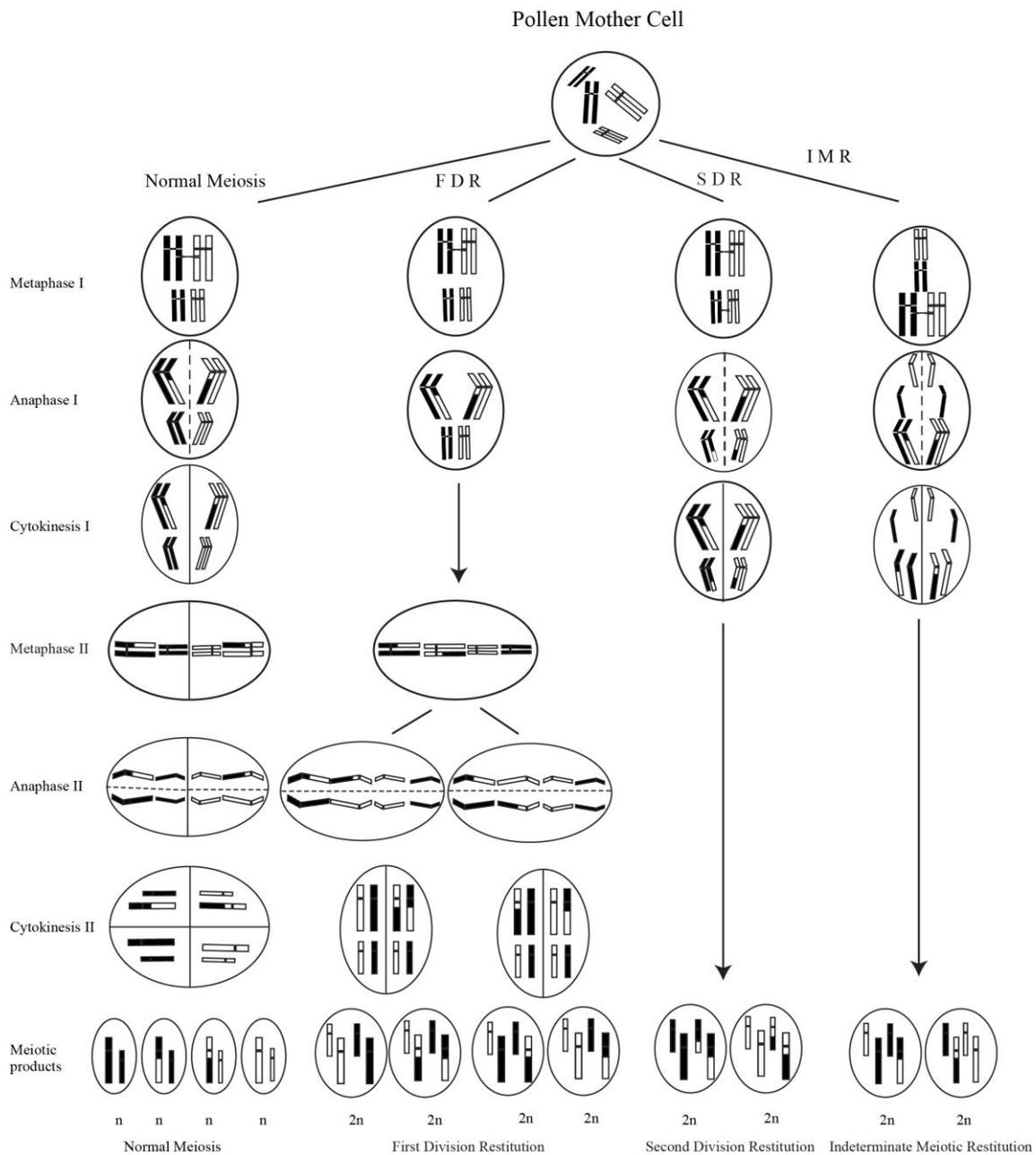
Fig. 1.1. A comparison of normal meiosis and three types of restitution mechanisms during meiosis

Meiotic analysis using cytogenetic and molecular cytogenetic methods has revealed that different types of unreduced gametes can be caused by various meiotic abnormalities. A normal meiosis involves two cell divisions. In the first division, homologous chromosome are segregated which is referred to as a reductional division; and the second division involves the separation of sister chromatids and hence is considered as an equational division. When there

is only an equational segregation in which homologous chromatids segregated during meiosis, FDR gametes will be produced; and when there is only a reductional segregation, SDR gametes will arise. During SDR, homologous and homoeologous chromosomes pair completely, and the chromatids in resultant products do not move to different poles but stay as one gamete without the simultaneous cytokinesis, and hence no formation of cell wall. However, in some of the interspecific hybrids of lily, bivalents disjoin reductionally and univalents divide equally before telophase I. Since there is only one-time division followed by cytokinesis, the resultant products are also 2n gametes, and the mechanism is called indeterminate meiotic restitution (IMR) (Lim et al. 2001a). For FDR-originated offspring, genetic loci that are proximal to the crossover point will be heterozygous, while for SDR, the segments distal to the crossover point will be heterozygous (Fig. 1.1).

The process of polyploidization using unreduced gametes is termed as sexual polyploidization, which has progressed in some crops and contributed to plant breeding and crop improvement dramatically. Superiority of vigor, growth, yield, which are of agronomical importance, has been found in some of the sexual polyploidized progenies in a range of crops, such as banana, sugarcane, potato, alfalfa, lily, which are all triploid or complex polyploids (reviewed by Ramanna and Jacobsen 2003).

Table 1.1. Some mutants that produce high frequencies of unreduced gametes during male meiosis in *Arabidopsis thaliana*

Mutant	Mutation	Type of unreduced gametes	References
Dyad	An equational segregation during meiosis	FDR	(Agashe et al. 2002; Mercier et al. 2001)
Cdka1;2/tam	No meiosis II occurring	SDR	(Cromer et al. 2010; Wang et al. 2010)
Osd1	Failing to enter the second meiosis division	SDR	(d'Erfurth et al. 2009)
Atps1	Disruption of spindle orientation in Meiosis II	FDR	(d'Erfurth et al. 2008)
Tes/stud	Failure of meiosis cytokinesis	Tetraspores	(Yang et al. 2003)

Recently, *Arabidopsis* has become a well-studied species for unreduced gametes formation. A few genes have been proved to be involved in the production of unreduced gametes (Table 1.1). In *Arabidopsis*, a FDR-relevant gene SWI1/DYAD has been characterized, in which this dyad allele can result in an equational segregation without further division during female

meiosis (Mercier et al. 2001; Agashe et al. 2002; Ravi et al. 2008). Interestingly, mutants of two proteins, CYCA1;2 (a member of the cyclin A family) (d'Erfurth et al. 2010; Wang et al. 2010;) and Ommision of Second Division 1 (OSD1) which both impede the entre of meiosis II and control the male and female meiosis (d'Erfurth et al. 2009), lead to the production of SDR gametes. A mutant of *Arabidopsis parallel spindle1* (Atps1) can also disrupt the spindle orientation, which will lead to a mix of dyads and triads (two haploid cells together with a diploid cell) as well as some tetrads during meiosis (d'Erfurth et al. 2008).

The recent discoveries of the genetic mechanisms that unreduced gametes produced in *Arabidopsis* and other species open an exciting avenue to put the knowledge into practice for plant breeding. Indeed, researchers are trying to develop new strategies to induce unreduced gametes by knockdown of specific proteins which have been mentioned before. With the available techniques of targeted gene manipulation, the generation of crops producing designed gametes is becoming realistic. Meanwhile, it will also enhance our understanding of the evolution and speciation of flowering plants.

Meiotic abnormalities and bridges in interspecific hybrids

Interspecific hybridization, which has been used for studying the relationship between different species and making new variation for further breeding, is quite a normal tool in plant breeding. One of the most important features of these distant hybrids is the reduced fertility. The reason of the sterility has been well studied in a few species hybrids and the reasons has been explained as due to the association failure and the abnormal segregation caused by chromosome structure differences at the first cell division during meiosis, which lead to aneuploidy and unviable gametes (Asano 1982; Barba-Gonzalez et al. 2005b; Gopinathan and Babu 1986; Jenkins and Scanlon 1987; Kopecký et al. 2008; Lee et al. 2011; Lim et al. 2001a; Pickering et al. 2004; Zhang et al. 1999; Zhou et al. 2008a).

The main feature of interspecific hybrids during meiosis is the association failure in the first division. In interspecific hybrids, chromosomes from different species are normally partly homologous (homoeologous). During meiosis, these homoeologous chromosomes cannot recognize each other and hence, bivalents cannot be formed (Blanco et al. 1983; Jenkins and Scanlon 1987). As a consequence, univalents will randomly move to one of the cell poles and cause the imbalance of chromosome numbers between the subsequently formed two cells (Lim et al. 2001a; Poggio et al. 1999). Furthermore, it is also noticeable that in some of the distant hybrids, the meiosis is highly irregular due to the difference of basic chromosome number in the crossing parents. Even non-homologous chromosomes successfully paired together, interspecific hybrids could also suffer abnormal segregation at anaphase of the first division. During the process of speciation, genomes of related species are

quite divergent with various chromosome rearrangements. These structure variation can cause abnormal segregation and/or chromosome bridges during meiosis. Gametes from those meiotic divisions possess duplication/deletion and are generally sterile.

Anaphase I bridging has been well documented in a few interspecific hybrids. Together with univalents and multivalents, the presence of anaphase bridges is a relatively normal phenomenon in hybrids, like *Vigna umbellate* × *V. minima* (Gopinathan and Babu 1986), *Pinus* hybrids (Saylor and Smith 1966), *Chorthippus* hybrids (Lewis and John 1963), *Allium* hybrids (McCollum 1974), *Nicotiana tabacum* × *N. glauca* (Trojak-Goluch and Berbec 2003), *Phaseolus vulgaris* × *P. coccineus* (Cheng et al. 1981), *Elymus farctus* × *E. repens* (Heneen 1963), *Guizotia* hybrids (Dagne 1994) and so on. The production of bridges during meiosis had once exclusively explained as the presence of chromosome rearrangements like inversion (McClintock 1931). Later on, another cause-U-type exchanges, became an alternative explanation for the production of bridges (Couzin and Fox 1973; Haga 1953; Jones and Brumpton 1971; Jones 1969; Karp and Jones 1983; Lewis and John 1963; Newman 1967; Rees and Thompson 1955). According to the meiotic configuration, these two causes can be distinguished. Moreover, molecular biology has revealed that two different mechanisms are mainly involved in the repair of double strand breaks (DSBs) in mitosis-homologous recombination (HR) and nonhomologous end joining (NHEJ). Crossovers have been explained as a process of DSBs and the repair with HR (Puchta 2005; Schwacha and Kleckner 1995; Szostak et al. 1983), whereas the relationship between U-type exchanges and NHEJ is not clear yet.

Scope and aim of the thesis

In this research, an attempt will be made to investigate the following four topics:

1. to analyze the genome composition of mitotic and meiotic polyploidized neopolyploids of lily hybrids, and detect, if any, intergenomic chromosome rearrangements as a result of the so-called genomic shock.

2. to elucidate the meiosis process, especially the crossing-over events happened at anaphase I of interspecific hybrids of LA lilies and the gamete formation.

3. to detect the abnormalities of meiosis, including the failure of chromosome pairing, abnormal association and segregation, and any other chromosome rearrangements during meiosis of the interspecific hybrids of LA lilies.

4. to trace the origins and behavior of the aberrant small chromosomes occurring in the backcrossing progenies,.

With those above mentioned purposes, interspecific hybrids were made and distantly related hybrids between Longiflorum and Asiatic cultivars became available. Then the

process of meiosis such as chiasmata formation and crossing over were critically analyzed using GISH and FISH. Some genotypes, which showed a low fertility (others highly sterile), were backcrossed with their Asiatic parent, and the triploid progeny derived from sexual polyploidization were evaluated for their intergenomic recombination. The thesis is structured as follow:

Chapter 2 provides a comparison of intergenomic recombination in different populations (meiotic and mitotic polyploidized progenies), and traces the origin of these recombination by scoring the frequency of reciprocal and nonreciprocal products and analyzing the process of meiosis in the interspecific hybrids of LA lilies.

Chapter 3 presents the GISH-analysis of association and crossing over events in interspecific LA-hybrids, and the statistics of different types of crossing over.

In chapter 4, structural variation was characterized according to the bridge production and chromosome breakage during meiosis, and the bridges was explained as the occurrence of U-type exchanges.

Chapter 5 reports the observation of two types of aberrant small chromosomes (de novo and existing), and characterized them using GISH and FISH with different probes.

In chapter 6 the general discussion the occurrence of chromosome rearrangements as well as polyploidization and their significance in genetic mapping of *Lilium* are discussed and the potential utilization of different chromosome rearrangements were prospected.

Chapter 2

An assessment of chromosomal rearrangements in neopolyploids of *Lilium* hybrids

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Abstract

Two types of newly induced polyploids (neopolyploids) of *Lilium* hybrids were monitored for the occurrence of chromosomal rearrangements through Genomic *in situ* Hybridization (GISH) technique. One of the populations was obtained through crossing an allotriploid Longiflorum × Oriental hybrid (LLO) and an allotetraploid Longiflorum × Trumpet hybrid (LLTT) both of which were derived from somatic chromosome doubling. The other type of allopolyploid population was derived from meiotic chromosome doubling in which numerically unreduced (*2n*) gametes from two different interspecific hybrids, *viz.*, Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA), were used to get backcross (BC) progeny with the Asiatic parents. GISH clearly discriminated the three constituent genomes (L, T and O) in the complements of the progeny obtained from mitotic chromosome doubling. A total of 26 genotypes were analyzed from this population and there was no evidence for any chromosomal rearrangements. However, in the case of meiotically doubled allopolyploid progeny considerable frequencies of chromosomal rearrangements were observed through GISH. The so-called chromosomal rearrangements in meiotic polyploids are the result of homoeologous recombination rather than "translocations". Evidence for the occurrence of meiotic recombination in the LA hybrids has been confirmed with GISH on meiotic chromosomes. Thus, there was evidence that neopolyploids of *Lilium* hybrids did not possess any noticeable chromosome rearrangements.

Keywords: *Lilium*; polyploids; genomic *in situ* hybridization (GISH); homoeologous recombination

Introduction

The occurrence of profound changes in newly synthesized polyploids (neopolyploids) has been recognized for a long time in many plant species (see review, Ramsey and Schemske 2002). Such changes occur in both auto- and allopolyploids and exhibit meiotic complexity including multivalent pairing, multisomic inheritance and the production of unbalanced gametes. More recent investigations have indicated that extensive "chromosomal rearrangements" commonly occur in neopolyploids of some plant species, the chromosomal rearrangements in these cases include translocations, duplications and deletions. Some examples of neopolyploids that have been analysed in detail are: *Brassica* species hybrids (Nicolas et al. 2007; Osborn et al. 2003; Song et al. 1995; Udall et al. 2005) and hybrids between wheat and its related species (David et al. 2004; Feldman et al. 1997; Zhang et al. 2008). The implications of such chromosomal rearrangements for the evolution of polyploids have been reviewed (Leitch and Bennett 1997, 2004; Wendel 2000). Moreover, if extensive chromosomal rearrangements do occur, they might have implications for the speciation of neopolyploids.

Apart from other observations on neopolyploids, molecular cytogenetic analyses using genomic *in situ* hybridization (GISH) technique on some of the allopolyploid crops and their relatives have revealed the occurrence of several intergenomic translocations in their complements. For example, in tobacco (*Nicotiana tabaccum* L.) nine intergenomic translocations have been detected (Kenton et al. 1993); in *Avena maroccana* Gand. five and in cultivated oat (*A. sativa* L.) as many as 18 intergenomic translocations have been identified (Chen and Armstrong 1994; Jellen et al. 1994). It is concluded that such translocations may occur following polyploid formation (Leitch and Bennett 1997). In the case of tobacco and wheat there is convincing evidence that these translocations involve nonhomologous chromosomes of different genomes (Parokonny and Kenton 1995; Zhang et al. 2008).

Unlike translocations that involve nonhomologous chromosomes, the occurrence of so-called "homeologous translocations" have been reported in the case of neopolyploids of *Brassica napus* L. (2n = 4x = 38) (Nicolas et al. 2007; Osborn et al. 2003; Udall et al. 2005). The neopolyploids used in these analyses were produced by crossing dihaploids of *B. napus* (2n = 2x = 19) as female parents with tetraploid male parents. The progenies in these cases originated through the functioning of *2n* eggs from the dihaploids and 2x pollen from the euploid parent. As expected, the progenies were tetraploid. By genotyping these neopolyploids with molecular markers, extensive chromosomal rearrangements that included "homeologous nonreciprocal translocations (HNRT), duplications and deletions were observed (Nicolas et al. 2007). The origin of chromosomal rearrangements was explained as due to recombination between the two distinct but related genomes of *B. napus* (AACC), i.e., A = *B. rapa* (x = 10) and C = *B. oleracea* (x = 9) during the formation of *2n* eggs in the dihaploids. Thus, based on the examples of wheat and tobacco on the one hand and *B. napus*

on the other, two types of translocations can be distinguished: nonhomologous and homoeologous translocations.

During the past several years, a large number of polyploids have been induced by using hybrids of species and cultivars of *Lilium* and the resulting neopolyploids were analysed through GISH (Barba-Gonzalez et al. 2004; Barba-Gonzalez et al. 2005b; Barba-Gonzalez et al. 2006b; Karlov et al. 1999; Khan et al. 2009a; Lim et al. 2000; Zhou et al. 2008b). For the synthesis of polyploids, both somatic chromosome doubling of the F1 hybrids through chemicals such as colchicine or oryzalin as well as sexual polyploidization through numerically unreduced (*2n*) gametes were used. These neopolyploid progeny are ideally suitable for cytological analysis using GISH technique for two important reasons. 1. The chromosomes of *Lilium* species are very large and suitable for cytological analysis. 2. The genomes of the parents used for producing hybrids and their neopolyploids are so well differentiated that structural rearrangements, if any, can be identified accurately through GISH in meiotic as well as somatic cells. The main aim of the present study is to investigate, through GISH analysis, whether chromosomal rearrangements occur in the neopolyploids of *Lilium*. Furthermore, the reasons why intergenomic recombination in hybrids might be mistaken for chromosomal rearrangements are discussed.

Materials and methods

Plant materials

Plant material consisted of polyploids derived from the hybrids of four groups of diploid (2n = 2x = 24) cultivars, *viz*., Longiflorum (L), Asiatic (A), Oriental (O) and Trumpet (T). Because the cultivars are derived from crossing some closely related *Lilium* species (McRae 1998), the specific names of individual species are avoided and the letters in each case indicate the genomes. The first three of these groups (L, A and O) have resulted from crossing of closely related species within each of the three taxonomic sections, *viz*., Leucolirion, Sinomartagon and Archelirion respectively. The last one, the Trumpet group, also belongs to the section Leucolirion, the same as Longiflorum, but forms a separate crossability group within the section and possesses a clearly differentiated genome (Lim et al. 2008a). For the analysis of polyploids derived from somatic chromosome doubling, the progeny of a cross between an allotriploid 'Triumphator' (LLO) with an allotetraploid (LLTT) the latter supplied by one of the Dutch lily companies (Worldbreeding BV) were used. The triploid parent of this cross was produced by backcrossing the allotetraploid, LLOO, hybrid with diploid Longiflorum (LL). Meiotically doubled polyploids were produced by backcrossing Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) F1 hybrids with Asiatic parents in which the F1 hybrids had contributed *2n* gametes and the resulting progenies were triploids (Barba-Gonzalez et al. 2006a; Khan et al. 2009a). Part of the backcross progeny of meiotic

polyploids was supplied by the following Dutch lily breeding companies: De Jong Lelies BV, Royal Van Zanten BV, Testcentrum BV, Vletter and Den Haan BV and Worldbreeding BV.

Mitotic and meiotic chromosome preparations

For mitotic chromosome preparation, young roots were treated with 0.7mM cyclohexamide for 4-6 hours at 4°C then transferred to Carnoy's Solution (Ethanol 3: Acetic acid 1) and stored at 4°C until use. Root tips were incubated in enzyme mixture (1% cellulose RS, 1% Pectolyase Y23, in 2mM citrate buffer, pH 4.5) for 90 minutes at 37°C. Mitotic metaphase chromosomes were spread according to Ross et al. (1996). For meiotic chromosome preparation, young anthers with stages from prophase I to telophase II were collected and fixed in fresh Carnoy's solution for 24 h at 4°C. Part of fixed anthers was squashed in a drop of 2 % acetocarmine to determine appropriate meiotic stage. Anthers with proper meiotic stages were incubated in enzyme mixture containing 1% pectolyase Y23, 1% cellulase RS and 1% cytohelicase in 10mM citrate buffer (pH 4.5) at 37 °C for about 25 − 35 minutes. Subsequently, the procedure used for meiotic chromosome preparations was the same as used for mitotic chromosomes.

GISH procedure

In case of LLO × LLTT population, total genomic DNA was extracted from young leaves of Oriental cultivar 'Sorbonne' and Trumpet cultivar 'Royal Gold' with CTAB method. The DNA was sonicated to 1-10kb fragments and used as probe. The DNA of Longiflorum cultivar 'White Fox' was autoclaved to 200-600bp fragments and used as block. For LA × AA and AA × OA hybrids and interspecific F1 genotypes, sonicated DNA from Longiflorum cultivar 'White Fox' and Oriental cultivar 'Sorbonne' was used as probe respectively, while autoclaved DNA from Asiatic cultivar 'Connecticut King' was used as block. Probe DNA was labelled with either Digoxigenin-11-dUTP or Biotin-16-dUTP by standard Nick translation according to the manufacturer's instruction (Roche, Germany). The GISH procedure was carried out as described previously (Khan et al. 2009a; Lim 2000). Briefly, the hybridization mixture contained 50% formamide, 10% dextransulphate, 2×SSC, 0.25% SDS, 0.6-1.0 ng/μl for each probe and 15-50 ng/μl block DNA. After hybridization and stringency washing, the probes labelled with Digoxigenin-11-UTP and Biotin-16-UTP were detected by anti-digoxigenin and Cy3-streptavidin systems respectively. Then the slides were counterstained with 1 μg/ml DAPI and mounted with Vectashield. Preparation were analysed using a Zeiss Axiophot epifluorescence microscope and photographed with a Canon digital camera.

Chromosome identification and karyotyping

Images of mitotic metaphase chromosomes were measured using the computer program MicroMeasure (Reeves and Tear 2000). In each of the four genomes (L, A, O, T), the

chromosomes were put into sequence according to the decreasing short arm length (Khan et al. 2009a; Lim et al. 2001b; Stewart 1947), and in order to identify the chromosome number in each genome, chromosome length, arm ratio, the centromere index (short arm length/ long arm length +short arm length), and relative chromosome length index (individual chromosome length/total length of a set of chromosomes) were used as identification tools (Barthes and Ricroch 2001).

Statistical analysis

A Chi-square (χ^2) test was used to determine whether observed reciprocal and nonreciprocal product frequencies in the polyploids from meiotic chromosome doubling are significantly different with expectations.

Results

Because the progeny derived from LLO × LLTT crosses were expected to possess chromosomes from three different genomes (L, O and T), GISH with two probes was used to detect three types of chromosomes simultaneously in the complements (Fig. 2.1a). For the interspecific F1 hybrids and meiotically doubled backcross progeny of LA and OA hybrids, only two genomes were involved and they were analysed through an one-probe GISH procedure. The results of the two types of populations are described separately.

Table 2.1. Genome composition of the progeny derived from crossing allotriploid (LLO) × allotetraploid (LLTT) parents derived from somatic doubling determined through GISH

Cross	Number of plants	Number of chromosomes	Genome composition			Number of recombinant chromosomes
			L-genome	O-genome	T-genome	
LLO × LLTT	6	40	24	4	12	0
LLO × LLTT	8	41	24	5	12	0
LLO × LLTT	5	42	24	6	12	0
LLO × LLTT	4	43	24	7	12	0
LLO × LLTT	3	44	24	8	12	0

Chromosome composition of progenies derived from somatic doubling

The progeny of LLO × LLTT cross were expected to be aneuploid, because LLO was an allotriploid and had contributed aneuploid gametes whereas euploid 2x gametes were expected to be functional from the LLTT parent. In all, 26 progeny were analysed through GISH to assess their chromosome constitution (Table 2.1). As expected, all the genotypes of this population were aneuploid with chromosome numbers ranging from 40 to 44. A notable feature was that the chromosomes of the three constituent genomes, *viz.*, L, O and T were clearly distinguishable in individual cells (Fig. 2.1a). Invariably, there were 24 chromosomes

of L genome and 12 of T genome. The number of chromosomes of O genome, however, varied from a minimum of four to a maximum of eight which was the cause of aneuploidy (Table 2.1). A significant feature was that in none of the analyzed 26 progeny there was any evidence for the presence of chromosomal interchanges, either due to intergenomic recombination or translocations. This was expected from the fact that a cursory examination of the parents (*viz.,* LLO and LLTT) had indicated the absence of any chromosomal rearrangements.

Table 2.2. Statistics of genotype number, recombinant chromosomes, reciprocal and non-reciprocal product in the meiotic polyploidized progeny of LA × AA and AA × OA crosses

Group	Number of plants	Recombinant chromosomes	Number of reciprocal products found/Expected	Number of nonreciprocal products found/Expected
LA × AA	64	362	87/90.5	182/181
AA × OA	36	131	28/32.75	77/65.5

Chromosome composition of sexual polyploid progenies of LA and OA hybrids
In the case of BC1 progeny of LA and OA hybrids, 100 (64 + 36 respectively) genotypes were analysed through GISH (Table 2.2). All of these progeny had originated through the functioning of *2n* gametes from the F1 LA and OA hybrids. A common feature of these sexual polyploid progeny was the occurrence of extensive homoeologous chromosomal exchanges due to intergenomic recombination (Fig. 2.1b; Table 2.2) In the backcross progeny, although the number of recombination sites was restricted to one or two in most cases, there were instances in which seven to eight breakpoints per chromosome were present (Fig. 2.1d). The number of recombinant chromosomes varied from a single to as many as 20 per genotype. In all cases the recombinant chromosomes were identified and different types were indicated as follows: In the case of BC1 progeny of LA hybrids, a chromosome with the centromere of L and the recombinant segment of A was indicated as L/A and *vice versa* for its counterpart (i.e., A/L, see Fig. 2.1c). In the case of the progeny of OA hybrids, a chromosome with a centromere of O and a recombinant segment of A was indicated as O/A and *vice versa* for its counterpart (i.e., A/O). The recombinant chromosomes were expected to segregate in the progeny on the observation that almost all *2n* gametes in interspecific hybrids had originated through first division restitution (FDR) in which the sister chromatids of a recombinant chromosome randomly moved , as a rule, to opposite poles during restitution nucleus formation (Fig. 2.2). Thus, when the sister chromatids of a pair of homoeologous chromosomes with a crossover segregated during FDR, two alternative types of segregations were expected: one in which two non-crossover and two crossover chromatids moved to opposite poles (Fig.2.2-I); and another in which only one of the crossover chromatid plus a

non-crossover chromatid moved to the opposite pole (Fig. 2.2-II). The segregants with two non-crossover chromatids could not identified in the progenies, but the three other types,
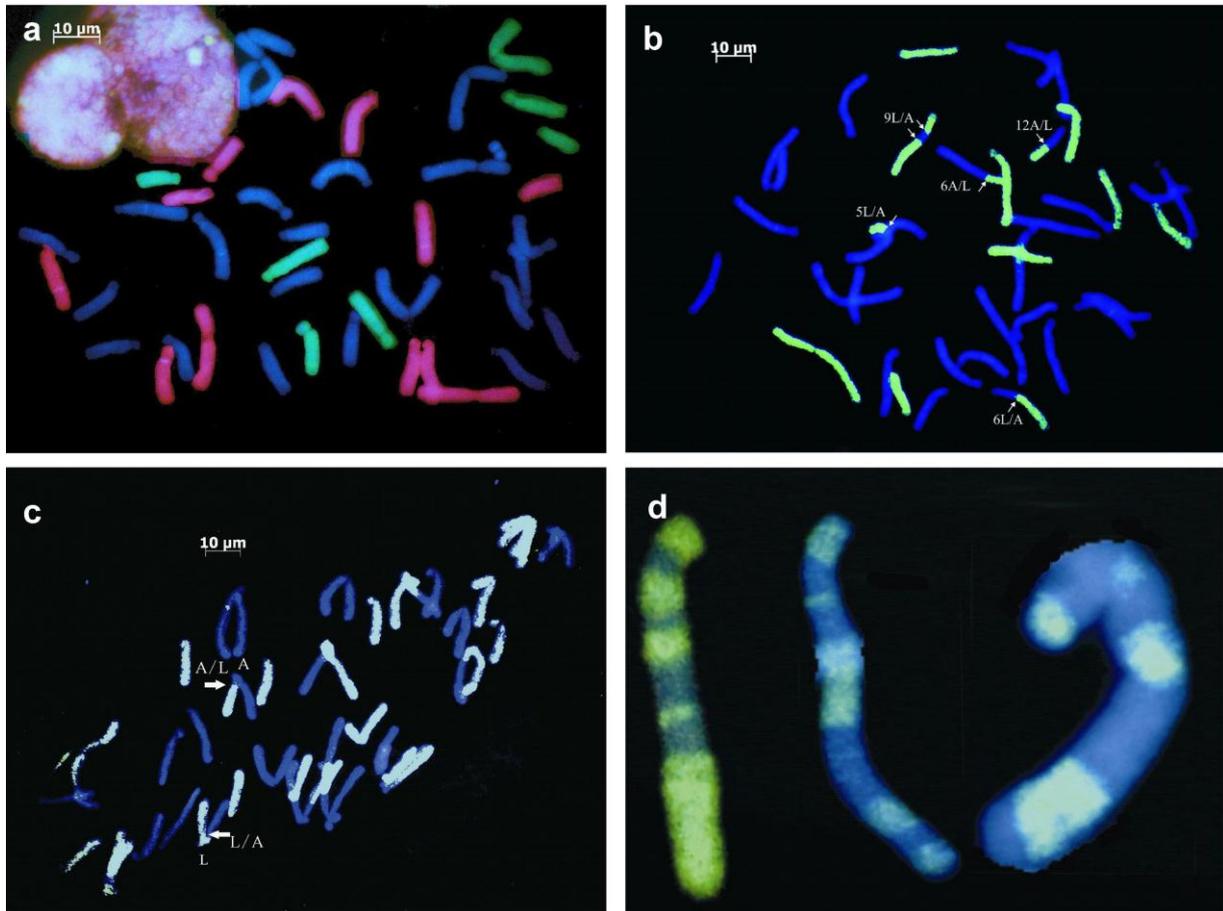

Fig. 2.1. Mitotic and meiotic chromosomes painted by GISH. (**a**) Mitotic metaphase of LLO LT (076928-21), an aneuploid (2n = 4x–5 = 43) derived from crossing allotriploid LLO and allotetraploid LLTT (both were derivatives of somatic chromosome doubling) showing no chromosomal interchanges. GISH clearly identified the chromosomes of the three genomes, T= red (biotin labelled and detected with Cy–3); O= green (digoxigenin labelled and detected with anti-digoxigenin FITC system) and L= blue (DAPI counterstaining). (**b**) Mitotic metaphase of a LA hybrid (074085-12), a triploid (2n = 3x = 36) showing five recombinant chromosomes (arrows) of which one pair represents reciprocal and three are non-reciprocal products L=green (digoxigenin labelled and detected with anti-digoxigenin FITC system) and A=blue (DAPI counterstaining). (**c**) Meiotic chromosomes at Anaphase I of an interspecific hybrid of Longiflorum × Asiatic (LA) lily (006001-16) in which GISH identified intergenomic crossing over between 6 pairs of homoeologous chromosomes (arrows) L=green (digoxigenin labelled and detected with anti-digoxigenin FITC system) and A=blue (DAPI counterstaining). (**d**) Chromosome 9 of LA hybrids from different genotypes showing multiple crossover sites (comparable to 'zebra' chromosomes).

such as L/A-A/L; L/A-L; A/L-A could be identified with GISH. So was the case with the segregations of progeny of OA hybrids: O/A-A/O; O/A-A; A/O-A. After identifying individual recombinant chromosomes, it was possible to detect in each case whether the two reciprocal products of crossover or only one of the two crossover products was present in a

genotype. Assuming that the segregation of crossover and non-crossover chromatids during FDR gamete formation was random, it was expected that the three classes (*viz*., L/A-A/L; L/A-A and A/L-A or O/A-A/O; O/A-A and A/O-A) were expected to be of equal proportion. The segregation in the case of progenies of LA hybrids confirmed the expectation ($\chi^2 = 0.484$, $0.70 < P < 0.80$, Table 2.2). In the case of the progeny of OA hybrids, there was a slight excess of non-reciprocal products ($\chi^2 = 7.12$, $0.01 < P < 0.05$), i.e., O/A-A (Table 2.2). This might well be due to the sample size which was small as compared to the progeny of LA hybrids.
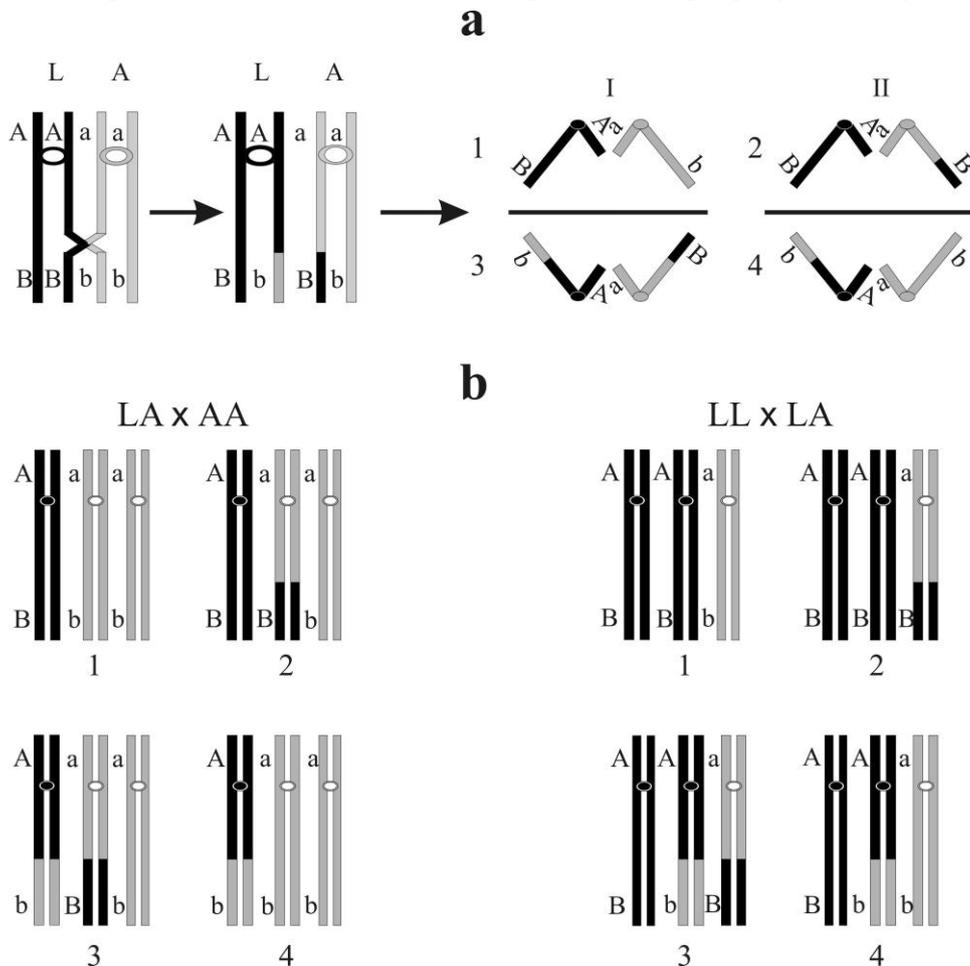

Fig. 2.2 (**a**) Diagrammatic illustration of the segregation of crossover and non-crossover chromatids during FDR gamete formation and (**b**) expected chromosome composition in the backcross progenies (e.g., LA × AA and LL × LA) assuming a single crossover between the two non-sister chromatids of a pair of homoeologous chromosomes. Note: (1) Only three types can be detected in the progenies. (2) Alleles that are distal to the crossover point can conform to the segregation in an autopolyploid, e.g., triplex (BBB), duplex (BBb), simplex (Bbb) and nulliplex (bbb).

In order to verify the origin of recombinant chromosomes in the backcross progenies, meiosis was analysed in the parent LA hybrid through GISH. Especially at anaphase I stages it was possible to identify the half-bivalents that clearly showed the products of intergenomic recombination (Fig. 2.1c). This clearly established the fact that the chromosomal exchanges

observed in the somatic metaphase stages were not *translocations* but the products of recombinations.

Discussion

The absence of any type of chromosomal exchanges in the progeny derived from allotriploid, LLO and allotetraploid, LLTT cross was quite conspicuous in the progeny. In the case of LLO strict autosyndetic pairing between the two L genomes during meiosis excludes the possibility of any intergenomic recombination between L and O genomes. In such cases, the chromosomes of the O genome were left out as univalents during meiosis giving rise to aneuploid BC1 progeny (Table 2.1). In the case of allotetraploid LLTT in which both L and T genomes have their counterparts and the homologues pair normally prevent any possibility of intergenomic recombination. Other than intergenomic recombination, if there were to be any chromosomal translocation, such exchanges should have become visible in GISH preparations. In none of the 26 genotypes that were analysed there was any indications for chromosomal rearrangements. A previous cytological study on the progeny of somatically doubled interspecific hybrids of *L. longiflorum* Thunb. × *L. rubellum* Baker. have indicated that because of autosyndetic pairing during meiosis no intergenomic recombination occurs in such allopolyploid progeny of *Lilium* hybrids (Lim et al. 2000). However, it should be pointed out, however, that intergenomic recombination does occur in allopolyploids derived from somatic doubling as in the case of *Lolium perenne*/*Festuca pratensis* (King et al. 2002). Other than meiotic recombination, the occurrence of chromosomal translocations between the nonhomologous chromosomes of alien genomes has also been reported as in the case of hybrids of *Elymus trachycaulus*/*Triticum aestivum* (Zhang et al. 2008) which gave rise to unusual structures called "Zebra" chromosomes (see later).

In contrast to somatically doubled neopolyploids of lily hybrids, the progeny derived from meiotic doubling possess numerous chromosomal exchanges. In these cases the exchanges result from intergenomic recombination (Table 2.2, Fig. 2.1c) during the origin of *2n* gametes. Such recombinant chromosomes are comparable to those that were reported in the case of neopolyploids produced from the dihaploids of *Brassica napus* in which FDR-like *2n* gametes were functional (Nicolas et al. 2007; Udall et al. 2005). In *Brassica* these authors have detected chromosomal rearrangements such as "homeologous nonreciprocal translocations", duplications and deletions. The detection of these chromosomal rearrangements is, however, based on the use of molecular markers but not through cytological identification of recombinant chromosomes. There are certain drawbacks of drawing conclusions based on molecular marker analysis alone, which will be considered later. But the frequent use of the term 'translocation' to indicate what actually is an intergenomic recombination is confusing. This confusion arises because, in traditional cytogenetic literature, the term translocation is used to imply a chromosomal structural aberration. When a translocation heterozygote segregates during meiosis, it leads to the formation of the so-called

duplication-deletion gametes which are normally lethal resulting in sterility. But, as is evident from meiosis observation and the survival of the nonreciprocal products of recombinant chromosomes in the present study, it may not be appropriate to consider the chromosomal exchanges observed here as translocations. It may be pointed out that whereas translocations are aberrations, recombinant chromosomes occur as a result of a natural phenomenon of intergenomic crossing over. The ratios of reciprocal and nonreciprocal products observed in the segregating progenies (Table 2.2) are of nearly equal proportion in both types of BC1 progenies. This means, the nonreciprocal products of recombinant chromosomes are not similar to duplication-deletion chromosomes that result from the segregation of reciprocal translocations. Furthermore, normal haploid gametes are produced by some genotypes of LA hybrids, with many recombinant chromosomes and are fully viable (Khan et al. 2009b) indicating that there are no deletions in such chromosomes.

There have been extensive discussions regarding the distinction between auto- and allopolyploids in plants (Ramsey and Schemske 1998). When allopolyploids originate strictly through somatic chromosome doubling, they are expected to behave like 'permanent hybrids' due to autosyndetic pairing of homologous pairs of chromosomes. On the other hand, when allopolyploids originate through meiotic doubling, there can be numerous intergenomic recombinant chromosomes in their complements as is evident in the present investigation. In this case, there is a prospect for multivalent formation because of the presence of recombinant segments in the complements. This means, chromosome assortment and segregation of genetic loci that present distal to the recombination point can segregate in allopolyploids. In this sense, even allopolyploids may no longer behave like permanent hybrids but behave like autopolyploids. If this is the case, the allopolyploids synthesized in the case of *Lilium* hybrids through 2*n* gametes can display the attributes of autopolyploids. Because the cytological evidence supports normal Mendelian segregations of reciprocal and nonreciprocal recombinant products in the progenies, it may be not out of place if we use the same terminology as is used in the case of autopolyploids. Thus, for example, the expressions such as triplex (BBB), duplex (BBb), simplex (Bbb) and nulliplex (bbb) can be appropriately used in the case of segregations in allopolyploid in *Lilium* (Fig. 2.2b).

As compared to the use of molecular markers for the analysis of chromosomal rearrangements in the case of neopolyploids of *Brassica napus*, the use of GISH in the present study has certain advantages. Molecular marker analysis cannot detect the reciprocal products of recombination but GISH can unequivocally detect such events. Moreover, assessment of the so-called duplications and deletions in *Brassica napus* is based on an indirect quantitative method (Nicolas et al. 2007). Unlike the small chromosomes of *Brassica*, the large and well differentiated chromosomes of *Lilium* are certainly advantageous for GISH analysis and a better insight can be obtained into the chromosomal rearrangements, if any. In the present investigation there seem to be little cytological evidence for the occurrence of extensive chromosomal rearrangements in the neopolyploids of *Lilium.* Finally, there are instances in

which several exchanges of chromosomal segments between the homoeologous chromosomes of L and A genomes (Fig. 2.1d) occurred that resemble "zebra" chromosomes reported in the case of *Elymus trachycaulus/Triticum aestivum* hybrids (Zhang et al. 2008). The latter of these resulted from illegitimate recombination between nonhomologous chromosomes of *Elymus* and *Triticum*. But in the case of LA hybrids the chromosomes with multiple crossovers have originated through crossing-over between homoeologous chromosomes but not due to any aberrations.

Supplement tables

Table S2.1. Ploidy level, chromosome numbers and genome composition of the progeny derived from crossing allotriploid (LLO) × allotetraploid (LLTT) parents derived from somatic doubling

Genotype	Ploidy level**	Number of chromosomes	Genome composition***			Number of recombinant chromosomes
			L-genome	O-genome	T-genome	
076928-1	3.5	43	24	7	12	0
076928-2	3.4	40	24	4	12	0
076928-3	3.4	44	24	8	12	0
076928-4	3.2	41	24	5	12	0
076928-5	3.5	43	24	7	12	0
076928-6	3.5	44	24	8	12	0
076928-7	3.1	40	24	4	12	0
076928-8	3.4	43	24	7	12	0
076928-11	3.4	42	24	6	12	0
076928-12	3.3	41	24	5	12	0
076928-13	3.4	42	24	6	12	0
076928-14	3.2	41	24	5	12	0
076928-15	3.2	40	24	4	12	0
076928-16	3.3	40	24	4	12	0
076928-17	3.3	41	24	5	12	0
076928-18	3.4	41	24	5	12	0
076928-19	3.4	41	24	5	12	0
076928-20	3.3	40	24	4	12	0
076928-21	3.4	43	24	7	12	0
076928-22	3.3	41	24	5	12	0
076928-23	3.3	41	24	5	12	0
076928-24	3.3	42	24	6	12	0
076928-25	3.5	44	24	8	12	0
076928-26	3.4	42	24	6	12	0
076928-28	3.5	40	24	4	12	0
076928-29	3.4	42	24	6	12	0

** determined by flow cytometry

*** determined through GISH

Table S2.2. The number of recombinant chromosomes and segregation of reciprocal and nonreciprocal pairs of homoeologous in 64 genotypes of BC1 progenies of LA × AA crosses

Genotype	No of recombinant chromosomes	No of pairs segregating	Reciprocal products (L/A-A/L)	Non-reciprocal product	
				L/A-A	A/L-A
044525-1	3	3	0	2	1
044539-1	2	1	1	0	0
044571-1	3	2	1	0	1
062035-1	6	6	0	3	3
062035-2	6	5	1	1	3
062071-1	13	9	4	2	3
062071-2	14	10	3	5	3
062074-1	14	8	6	1	1
062074-3	12	8	4	2	2
062074-4	14	8	6	1	1
065051-2	6	6	0	2	4
066828-2	1	1	0	0	1
066960-4	7	6	1	2	3
066960-6	7	5	2	2	1
066960-8	4	4	0	4	0
066960-13	7	5	2	1	2
066960-14	3	3	0	1	2
066960-20	8	5	3	1	1
066963-5	12	9	3	4	2
066963-8	4	4	0	1	3
066994-3	20	11	9	2	0
066994-4	12	8	4	2	2
066994-11	13	9	4	3	2
066994-12	13	11	2	3	6
066995-1	8	7	1	3	3
044595-1	5	4	1	1	2
044601-1	3	3	0	1	2
044601-2	6	6	1	1	3
044601-3	1	1	0	0	1
044601-4	2	2	0	1	1
044601-5	1	1	0	1	0
044601-6	3	2	0	1	0
044601-7	3	3	0	1	2
044601-8	3	2	1	1	0
044638-1	2	2	0	0	2
044638-2	2	2	0	0	2
044638-3	4	4	0	1	3

041552	4	3	1	1	1
041553	3	3	0	1	2
041554	1	1	0	1	0
041555	1	1	0	1	0
041571	4	3	1	1	1
041572	3	2	1	1	0
041575	2	1	1	0	0
041578	1	1	0	1	0
041580	5	3	2	1	0
041581	3	3	0	0	3
041583	2	2	0	1	1
061029	1	1	0	1	0
074051-1	8	6	2	1	4
074051-4	10	8	2	3	2
074051-5	6	5	0	3	3
074051-6	10	7	3	3	1
074051-9	8	5	3	0	2
074051-11	8	8	0	3	5
074051-12	7	5	1	2	1
074085-3	2	1	1	0	0
074085-6	6	3	3	0	0
074085-7	4	3	1	2	0
074085-12	5	4	1	2	0
074085-13	2	1	1	0	0
074085-20	3	2	1	0	0
074085-22	6	4	2	1	1
Total	362	272	87	87	95
No. of expected			90.5	90.5	90.5

Table S2.3. The number of recombinant chromosomes and segregation of reciprocal and nonreciprocal homoeologous in 36 genotypes of the BC1 progenies of AA × OA crosses

Genotype	No of recombinant chromosomes	No of pairs segregating	Reciprocal products (O/A-A/O)	Non-reciprocal product	
				O/A-A	A/O-A
022538-1	7	4	3	1	0
022538-3	6	4	2	2	0
022538-7	6	4	2	1	1
022538-8	4	4	0	2	2
022538-9	4	4	0	2	2
022538-15	4	5	0	1	4
022538-16	8	6	2	3	1
022605-2	2	2	0	1	1
022605-5	2	2	0	1	1
022605-8	3	2	1	1	0
022605-9	7	6	0	4	3
022605-11	2	1	1	0	0
022605-12	2	2	0	1	1
022605-16	4	2	2	0	0
022605-20	6	4	2	2	0
022605-21	8	7	1	3	3
022605-22	2	1	1	0	0
022605-23	5	4	1	2	1
022605-24	4	4	0	4	1
022605-25	5	5	0	3	2
022605-27	2	1	1	0	0
022605-28	1	1	0	1	0
022605-30	4	3	1	1	1
022605-31	2	2	0	2	0
022605-35	7	5	2	3	0
022605-36	2	2	0	2	0
022605-37	1	1	0	1	0
022605-38	2	1	1	0	0
022605-39	4	2	2	0	0
022605-40	4	2	2	0	0
022605-44	2	1	1	0	0
022605-45	3	3	0	0	3
022605-46	6	6	0	3	3
Total	131	103	28	47	30
No. of expected			32.75	32.75	32.75

Chapter 3

Elucidation of intergenomic recombination and chromosome translocation: Meiotic evidence from interspecific hybrids of *Lilium* through GISH analysis

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Abstract

With the aim of tracing the origin of intergenomic exchanges in lily backcross progenies and distinguish differences, if any, between intergenomic recombination and translocation, 13 genotypes of an interspecific hybrids, which were previously used as parents to generate sexual polyploids, were selected for a detailed meiosis analysis. In all genotypes variable numbers of bivalents (0-12) resulting from homoeologous pairing and univalents were observed. But in two genotypes (006001-6 and 006001-13), a multivalent which was either a quadri- or a trivalent, as well as a bivalent involving two Asiatic chromosomes, was observed. An interesting feature of the multivalent in the case of 006001-6 was that two of the Asiatic chromosomes were always found to be associated either in the quadrivalent or the trivalent configurations. This indicated that there was a duplication common to two non-homologous chromosomes within the Asiatic parent. Such a duplication might have resulted from the segregation of a chromosomal translocation between two non-homologous chromosomes in the Asiatic parent 'Connecticut King' which was transmitted to the progeny (006001-6). With the exception of two genotypes, in 11 genotypes that formed variable frequencies of bivalents, the homoeologous chromosome pairing and chiasma formation were similar to that between homologous chromosomes. Especially from the analysis of anaphase I stages it was evident that the expected types of chiasma formation involving non-sister chromatids gave rise to two strand single, two strand double, three strand double , four strand double and multiple exchanges. Whereas these events resulted from locus specific homoeologous exchanges, the translocations resulted from an aberrant form of non-homologous chromosomal exchange of segments. Elucidation of such differences is only possible through the analysis of meiosis using GISH.

Keywords: Recombination; crossing-over; translocation; Lily; meiosis; interspecific hybrids; genomic *in situ* hybridization (GISH)

Introduction

Intergenomic recombination and chromosome translocation are totally different phenomena, although exchanges of chromosome segments occur in both cases. Whereas recombination is the result of crossing-over between homo- or homoeologous chromosomes during meiosis (a natural event), chromosome translocations occur due to chromosome aberrations or mutations (Rieger et al. 1976). Among many other differences, recombinations are locus specific events, whereas translocations are random events – implying that any chromosome segment may be transferred to another location in the genome through breakage and reunion. Despite these differences, in recent years the terms recombination and translocation have been used as synonyms in the cytogenetic literature because little is known about the origin of such genetic changes (Heslop-Harrison 2000; Nicolas et al. 2007; Osborn et al. 2003; Szadkowski et al. 2011; Udall et al. 2005). Especially in the case of newly induced polyploids (neo-polyploids) of species such as *Brassica napus,* intergenomic recombinations have been considered as translocations that lead to extensive chromosomal structural alterations (Gaeta et al. 2007).

Lily (*Lilium*, 2n=2x=24) species have been used for investigating chromosome structural alteration by traditional cytogeneticists during the past century. Together with a few other plant species, especially maize (*Zea mays*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*) and other crops (Burnham 1962; Lewis and John 1963), chromosome rearrangements such as translocations, inversions, duplications and deletions, have been extensively investigated. These studies were not only confined to spontaneous events that occurred in nature but also included aberrations induced by radiations as well as chemical agents. In addition to gaining insights into various aspects of chromosome functions and behaviour, chromosome aberrations were also helpful to establish the relationship between chiasma formation and crossing-over (chiasmatype hypothesis). For this purpose, the plant species with large chromosomes such as those of lilies are especially helpful because they are favourable for critical cytological studies. Some of the examples are: inversion heterozygotes in *Lilium martagon* var. *album* and *L. hansonii* in which spontaneous paracentric inversions were used to test chiasmatype hypothesis (Richardson 1936); x-ray induced terminal deletion and paracentric inversion in *L. formosanum* were used to establish the relationship between chiasma and crossing-over (Brown and Zohary 1955); using a reciprocal translocation in *L. maximowiczii*, chiasmatype hypothesis was confirmed (Noda 1960); by analysing a pair of heteromorphic chromosomes resulting from reciprocal translocations in *Disporum sessile*, chiasmatype hypothesis was also confirmed (Kayano 1960). A favourable feature of reciprocal translocations in the case of *L. maximowiczii* and *Disporum sessile* was the possibility to quantify the frequencies of chiasmata in the interstitial segments (chromosome segments that lie between the centromere and the translocated segment) by estimating equational and reductional separation at anaphase I stages (see later). In all the above cases analyses of meiotic stages in pollen mother cells have been successfully used.

Lily allopolyploids are favorable for the use of genomic *in situ* hybridization (GISH) techniques because of their large chromosomes and well-differentiated genomes. GISH has been used for the study of genome composition (Barba-Gonzalez et al. 2005b; Lim et al. 2003), intergenomic recombination (Barba-Gonzalez et al. 2006b; Zhou et al. 2008b), mechanisms of unreduced gametes production (Lim et al. 2001a) in sexual polyploidized progenies and/or interspecific lily hybrids. In a recent GISH analysis, the phenomenon of intergenomic recombination was evaluated by using somatic metaphase chromosomes of newly synthesized polyploids of interspecific hybrids of *Lilium* (Xie et al. 2010). It was argued that the exchanges of chromosome segments between homoeologous chromosomes of two genomes were recombinations but not translocations. This conclusion is further substantiated through a detailed GISH analysis of meiosis during microsporogenesis in the interspecific hybrids between Longiflorum × Asiatic groups (LA) of lilies in the present study. The observations on the types of chromosome and chromatid segregations are discussed in relation to intergenomic recombination and chromosome translocation. Finally, the significance of chromosome translocation with relevance to gametes formation and genetic mapping is also discussed.

Materials and methods

Plant materials
Interspecific hybrids were obtained through crossing between a Longiflorum (L) cultivar 'White Fox' and an Asiatic (A) cultivar 'Connecticut King' with the use of cut-style pollination and embryo rescue (Van Tuyl et al. 1991).These hybrids were in vitro propagated and then transferred into the greenhouse for maintenance. Most of the hybrids are highly sterile, whereas13 genotypes, which showed a low fertility with the production of functional unreduced (2n) gametes, were selected for the analysis of meiosis.

Meiotic chromosome preparation
Young anthers with proper stages of metaphase I and anaphase I were collected and fixed in fresh-prepared Carnoy's solution (Ethanol : Acetic Acid/ 3:1, v/v) for 24h at 4°C. Part of the fixed anthers were squashed in a drop of 2% acetocarmine to determine appropriate meiotic stage, and the remaining part of anthers were transferred into 70% ethanol and stored at -20°C. For the meiotic chromosome preparation, anthers with proper meiotic stages were incubated in an enzyme mixture containing 1 % pectolyase Y23, 1 % cellulase RS and 1% cytohelicase in 10mM citrate buffer (pH 4.5) at 37 °C for about 25-35 minutes. Digested anther slice was put on a clean slide, then chromosomes were spread according to Ross et al. (1996).

In situ hybridization
Total genomic DNA was extracted from young leaves of Longiflorum cultivar 'White Fox' and Asiatic cultivar 'Connecticut King' according to Fulton et al. (1995). DNA of 'White

Fox' was sonicated to 1-10kb fragments and used as probe. 'Connecticut King' DNA was autoclaved to 100-500bp fragments and used as block. The probe DNA was labelled with Digoxigenin-11-dUTP by standard nick translation according to the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany).

Table 3.1. Chromosome association and segregation abnormalities during meiosis in 13 LA hybrids

Genotype	# of cells analyzed	Chromosome pairing		Remarks
		Range of bivalents	Average bivalents	
006001-6	256	9-12	11.2	Sporadic multivalents
006001-13	132	6-11	8.7	Sporadic non-homologous pairing
006001-9	228	3-11	7.6	
006001-16	143	5-10	6.2	
006001-17	139	4-11	7.2	
006001-36	141	2-9	5.4	
006001-42	133	3-9	4.2	
006001-72	136	4-7	5.6	
006001-80	129	3-9	3.9	
006001-88	126	7-12	10.7	
006001-97	135	4-10	6.2	
041501	133	10-12	10.8	
041502	124	4-10	7.3	

The procedure of *in situ* hybridization was carried out according to Khan et al. (2009) and Xie et al. (2010) with minor modification. The 60μl hybridization mixture contained 50% formamide, 10% dextran sulphate, 2 × saline sodium citrate (SSC), 0.25% sodium dodecyl sulphate (SDS), 1.0-1.5ng/μL probe DNA and 25-50 ng/μL block DNA. The mixture was incubated at 73°C for 10 minutes and ice cooled for 10 minutes, then hybridization mixture was added on each slide. After denaturation the slides for 5 minutes at 80°C, slides were left in a pre-warmed box for overnight hybridization at 37°C. After hybridization, the slides were washed in 2×SSC for 15 minutes, then stringency washing followed with 0.1×SSC at 42°C for 30 minutes. The probes, labelled with digoxigenin-11-dUTP, were detected with the anti-digoxigenin detection system. Then the slides were counterstained with DAPI and mounted

with Vectashield. Finally, photographs were taken with a Canon digital camera attached to a Zeiss Axiophot fluorescence microscope.
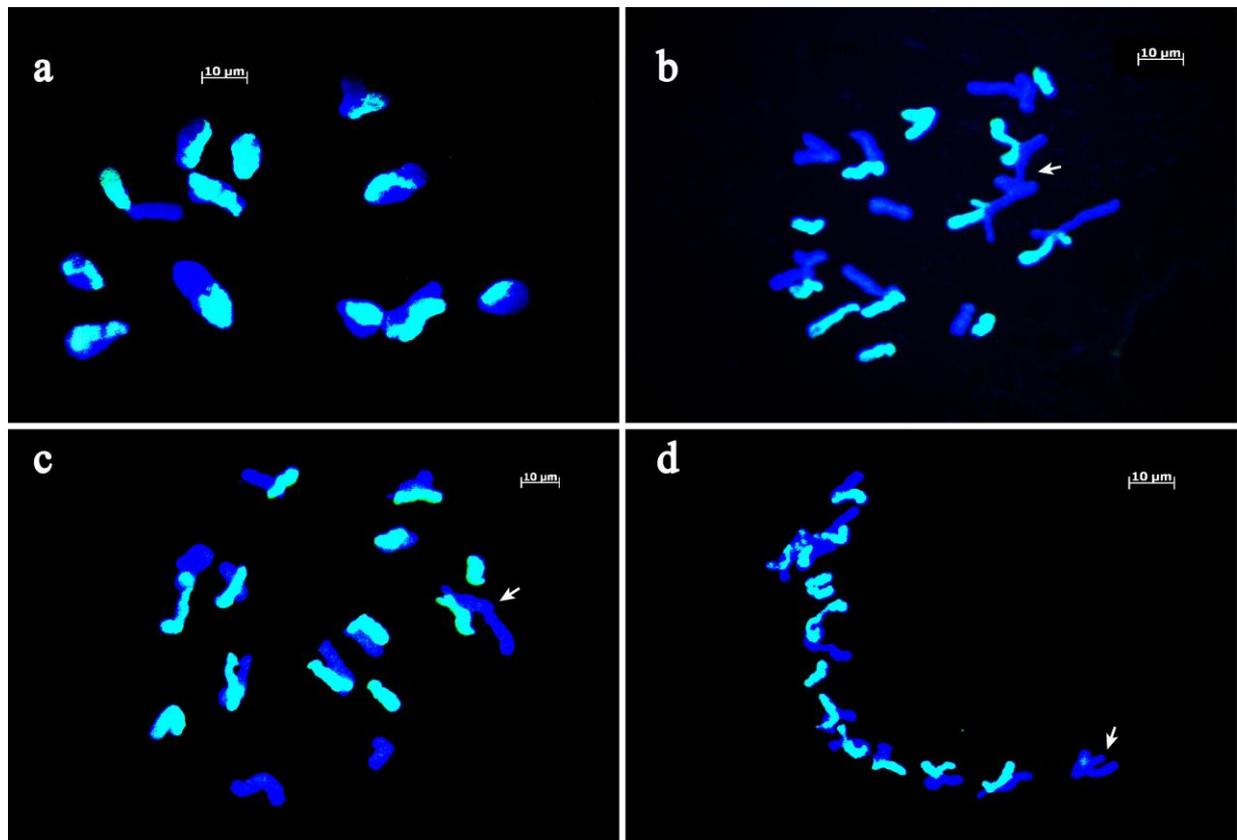

Fig. 3.1. Chromosome pairing at metaphase I during meiosis of interspecific hybrids of lily. (**a**) A pollen mother cell with 12 bivalents involving homoeologous pairing (006001-6); (**b**) Formation of a quadrivalent containing two Asiatic and two Longiflorum chromosomes (white arrow) in one of the pollen mother cells of genotype 006001-6; (**c**) Formation of a trivalent including two chromosomes from Asiatic and one chromosome from Longiflorum (white arrow) in pollen mother cells of genotype 006001-6; (**d**) Formation of a bivalent resulted from non-homologous chromosomes from Asiatic genome (white arrow) in genotype 006001-13. Green fluorescence stands for chromosomes from Asiatic genome and blue fluorescence stands for chromosomes from Longiflorum genome.

Results

Chromosome pairing at metaphase I in LA hybrids

Since the two parents, *L. longiflorum* and Asiatic lilies, belong to two different botanical sections, homoeologous chromosomes were clearly distinguished in the hybrids by GISH (Fig. 3.1a, b, c and d). In order to estimate the extent of chromosome pairing, metaphase I stages were analysed in 13 different genotypes (Table 3.1). In each case approximately 120 to 250 pollen mother cells were analysed. There was a great variation regarding the chromosome associations in different genotypes. The average number of bivalents per cell ranged from 3.9

(006001-80) to 11.2 (006001-6) in these different hybrids. Also, the number of bivalents among different pollen mother cells within a genotype varied considerably: in genotype 006001-6 with high average, for example, the number of bivalents varied between 9 to 12 per cell (for 12 bivalents, see Fig. 3.1a), whereas in another genotype with a low average of bivalents (e.g., 006001-36) they varied between 2 to 9 per cell. With rare exceptions, bivalents invariably resulted from the association of homoeologous chromosomes of the parents that could be clearly identified based on differential fluorescence labelling.

Besides bivalents and univalents, there were also multivalents as well as bivalents resulting from non-homologous association in some of the cells of two genotypes (006001-6 and 006001-13, arrows in Fig. 3.1b, c and d). The common feature of the quadrivalents (Fig. 3.1b), the trivalents (Fig. 3.1c) and the non-homologous bivalents (Fig. 3.1d) was the association of two chromosomes from the Asiatic genome (blue fluorescence). Such non-homologous association between two chromosomes within a haploid set from a parent was normally not expected to occur. In the multivalents of genotype 006001-6, two chromosomes from Asiatic genome as well as at least one chromosome of Longiflorum genome was involved (green fluorescence in Fig. 3.1b and c). The formation of multivalents and nonhomologous bivalents in the F1 hybrid progenies might be due to the presence of a duplication that is common to two non-homologous chromosomes within the Asiatic parent 'Connecticut King'. Alternatively, a reciprocal translocation might be present in the Asiatic parent and a duplication-deficiency gamete (a gametes with duplications as well as deletions) transmitted to the progeny that formed multivalent (see Fig. 3.3). A notable feature of multivalents was the absence of ring multivalents. This was explained from the fact that most of the chromosomes in the karyotypes of *Lilium* species consist of sub-metacentric or sub-telocentric chromosomes they do not form typical ring quadrivalents. Of particular interest was the association of two Longiflorum chromosomes at both ends in the chain quadrivalent (Fig. 3.1b, arrow). The probable explanation for this type of quadrivalent formation is shown in Fig. 3.3 in which both homo- (solid line in Fig. 3.3) and homoeologous (dashed lines in Fig. 3.3) chromosome associations are highlighted. Based on different possibilities of chiasma formation between the four chromosomes that are involved in multivalent formation, different meiotic configurations shown in Fig. 3.1 can be explained as follows: a) If chiasmata are formed in the homoeologous regions but not in the homologous region they result in forming 12 bivalents (Fig. 3.1a). b) If chiasmata are formed in both the homoeologous regions as well as the homologous region it leads to the formation of a quadrivalent in which two Asiatic chromosomes are adjacent each other (Fig 3.1b). c) If a chiasma is formed in the homologous region followed by a chiasma in one of the homoeologous regions, then it results in a trivalent with two Asiatic chromosomes (adjacent to each other) and a Longiflorum chromosome (Fig. 3.1c). d) When there is a chiasma formation only in the homologous region but not in the homoeologous regions, this results in the association of two non-homologous Asiatic chromosomes (Fig. 3.1d). Except for the two genotypes that formed a multivalent and

abnormal bivalents, in all other cases variable frequencies of bivalents and univalents were observed. Because of chromosome pairing abnormalities, the expected metaphase I orientation at the equatorial plate of the cell was rare.

Anaphase I separation

Since anaphase I separation of homoeologous chromosomes occurred regularly only in some of the pollen mother cells, normal metaphase I orientation had occurred in those cases. In other cases, the chromosomes (half-bivalents) were present haphazardly in the cells (Fig. 3.2a, b, c and d). Nevertheless, it was possible to identify the pairs of half-bivalents with and without recombinant segments in the sister and non-sister chromatids. The remarkable feature was that it was possible to identify the types of crossovers that had occurred between the non-sister chromatids of the pairs of homoeologous chromosomes during meiosis based on differential fluorescence. Based on the number and positions of recombinant segments on the non-sister chromatids it was possible to classify the types of intergenomic recombination events that had occurred. These were classified into five classes: a) two strand single (Fig. 3.2a and c), b) two strand double (Fig. 3.2b and c), c) three strand double (Fig. 3.2a and d), d) four strand double (Fig. 3.2a, b and d) and e) multiple cross-overs (Fig. 3.2d). The frequencies of each of these events were estimated in five genotypes (Table 3.2). From an analysis of a total of 637 pairs of half-bivalents it was evident that a large majority (65 %) were two strand single crossing overs, 5.5 % were two strand double, 3.0 % three strand double, 9.3 % four strand double and 17.3 % were multiple crossing over events. Although there was differences in the frequencies of these events, two conclusions could be made from these observations. 1) Regardless of the type of cross-over event, equational separation had occurred for the recombinant segments. 2) All the five crossover types of half-bivalents were similar to the events that are expected (Fig. 3.2) following a normal meiosis in the parent. This evidently indicated that despite genome differentiation between the genomes of the species of *L. longiflorum* and Asiatic lilies they retained homoeology that enabled normal crossing-over between the homoeologous pairs of chromosomes. In the two genotypes (006001-6 and 006001-13) with multivalent and nonhomologous bivalent formation there was no deviation at anaphase I that could be observed with regard to disjunction as compared with other genotypes without multivalent formation.

Table 3.2. Crossover events in 166 anaphase I pollen mother cells from five progenies of an interspecific LA hybrid

Genotype	Nr. of cells	Pairs of recombinant chromosomes	Two strand		Three strands double	Four strands double	Multiple crossover
			Single	Double			
006001-6	52	194	115	5.	11	15	48
006001-9	15	75	41	6	0	9	19
006001-13	4	9	8	0	0	1	0
006001-16	66	266	180	20	5	23	38
006001-88	29	93	70	4	3	11	5
Total	166	637	414	35	19	59	110
Frequency (%)			65	5.5	3.0	9.3	17.3

Discussion

In order to resolve the difference between intergenomic recombination and chromosome translocation, we have investigated two genotypes with translocations (006001-6 and 006001-13) and 11 genotypes without translocations (Table 3.1). Because of disturbed chromosome pairing between L and A genomes during meiosis in the F1 hybrids, the pairing configurations reported in *L. maximowiczii* which had normal chromosome synapsis (Noda 1960) could not be found in any of the genotypes used in the present study. Nevertheless, non-homologous bivalents and multivalent formation (Figs. 1 and 2) were clear enough evidence for the presence of translocations in two genotypes. A unique feature of the translocation in *L. maximowiczii* was that as a result of translocation it had given rise to two pairs of heteromorphic chromosomes whose modes of distribution during anaphase I stage could be identified morphologically due to the inequality of the arms. Taking advantage of the easily identifiable interchanged chromosomes, Noda (1960) quantified the frequencies of crossing-over in the interstitial segments based on equational segregations at anaphase I. Unlike in traditional staining techniques, however, GISH provides opportunities to identify the modes of chromosome segregation during anaphase I due to differential fluorescence. This facilitates
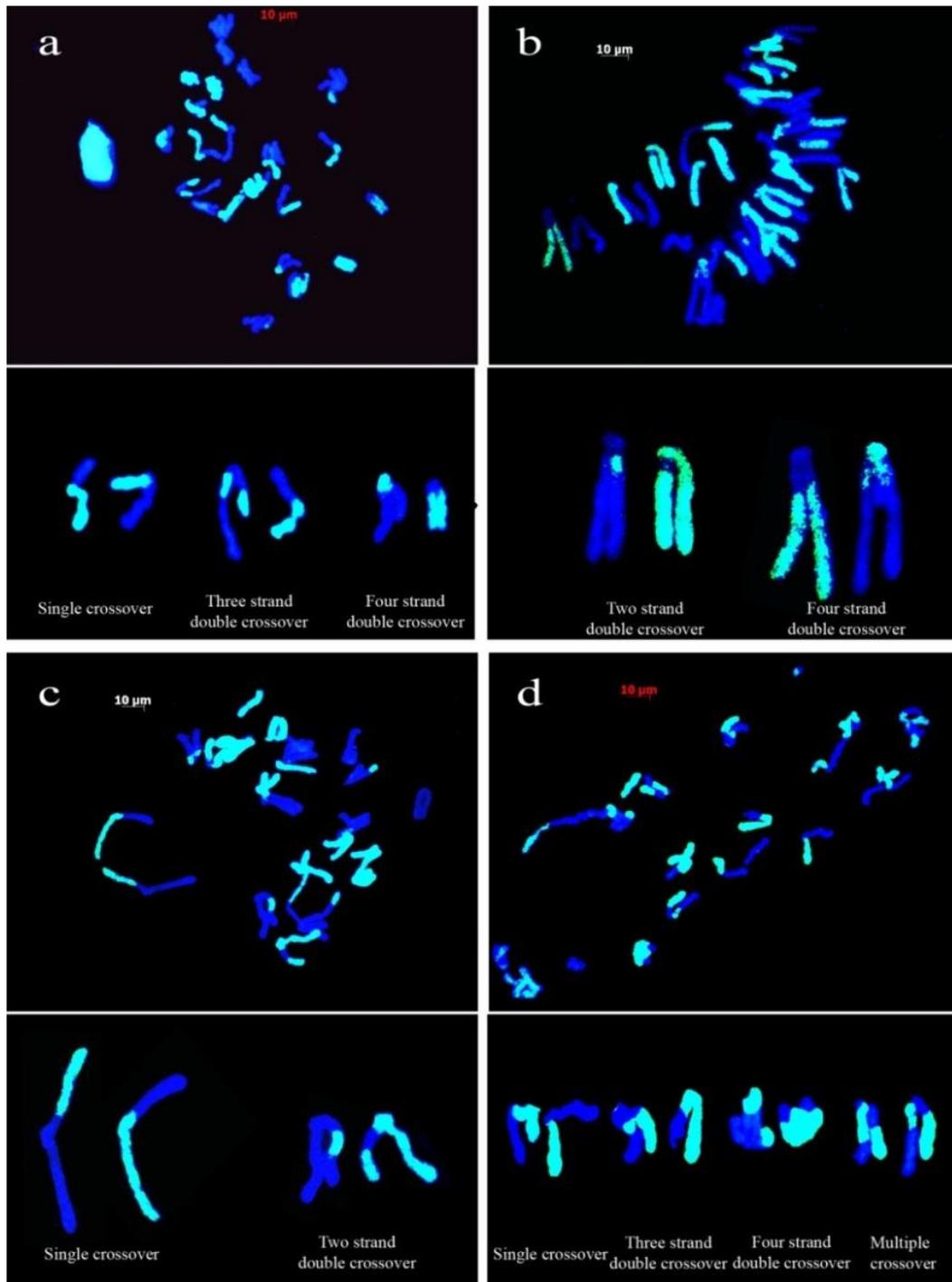
Fig. 3.2. Homoeologous chromosome segregation and crossing over ar anaphase I during meiosis of interspecific hybrids of lily. (a) Anaphase I segregation of homoeologous chromosomes confirmed the occurrence of single crossover, three strand double and four strand double crossovers. (b) Anaphase I segregation of homoeologous chromosomes illustrated the happening of two strand double and four strand double crossovers. (c) Anaphase I segregation of pollen mother cells indicated the happening of single crossover and a two strand double crossover; (d) Anaphase I segregation of pollen mother cells in meiosis revealed the occurrence of single, three strand double, four strand double and multiple crossovers

the detection of not only reductional and equational segregation of chromosomes in some cases but also identify different types of cross-overs between the non-sister chromatids of homoeologous chromosome pairs. Assuming normal chromosome pairing and crossing-over in LA hybrids, one can expect different types cross-overs to occur, viz., two strand single, two strand double, three strand double, four strand double and multiple cross-overs (Fig. 3.3). All the expected types have been found in the present study (Fig. 3.2a, b, c and d). Then the question arises whether these exchanges of segments between the non-sister chromatids of each pair of homoeologous chromosomes should be considered as recombinations or translocations? Undoubtedly the latter possibility must be ruled out because cross-over events are locus specific events between homoeologous pairs of chromosomes. Moreover the frequencies of exchanges per cell are so high that it is inconceivable that such high rates of chromosome mutations (translocations) can occur spontaneously in any organism.

Based on difficulty of crossing, F1 hybrid sterility, reduced chromosome pairing and clearly differential fluorescence of chromosomes, the genomes of *L. longiflorum* and Asiatic species are well differentiated. Despite this, however, there is almost completely normal crossing-over between some of the pairs of homoeologous chromosomes. Considering the crossover types observed in this study as well as those reported earlier (Zhou et al. 2008a), the genomes of different groups of *Lilium* species appear to be homo-sequential. So much so, that it has enabled the construction of cytological maps of three different genomes, viz., Longiflorum, Asiatic and Oriental groups (Khan et al. 2009a). In view of the high degree of homoeology between the genomes, the exchanges of chromosome segments through crossing-over events cannot be considered as translocations.

The occurrence of either duplication or translocation in the cultivar 'Connecticut King' is of considerable importance because attempts have been made to construct molecular maps in recent years (Abe et al. 2002; Khan 2009; Shahin et al. 2011; Van Heusden et al. 2002). A main problem to establish molecular maps is that the number of linkage groups exceed the basic chromosome number. One cause of this problem is the presence of very large genomes in *Lilium* species with a large basic chromosome number (x=12), which means larger number of markers and mapping individuals are needed. On the other hand, chromosome rearrangements provide another candidate reason for the mapping problem of lily. Although the relationship between chromosome structure variation and genetic mapping has not been well studied, limited reports have showed that reciprocal translocation can also cause the variation of linkage groups (Farré et al. 2010; Kamphuis et al. 2007; Larson et al. 2011). Until now, none of the chromosomes of *Lilium* species have been associated with any of the genes or molecular markers so far. In this context, it might be essential to carefully analyse the genome of 'Connecticut King' and identify the aberration that has been encountered.
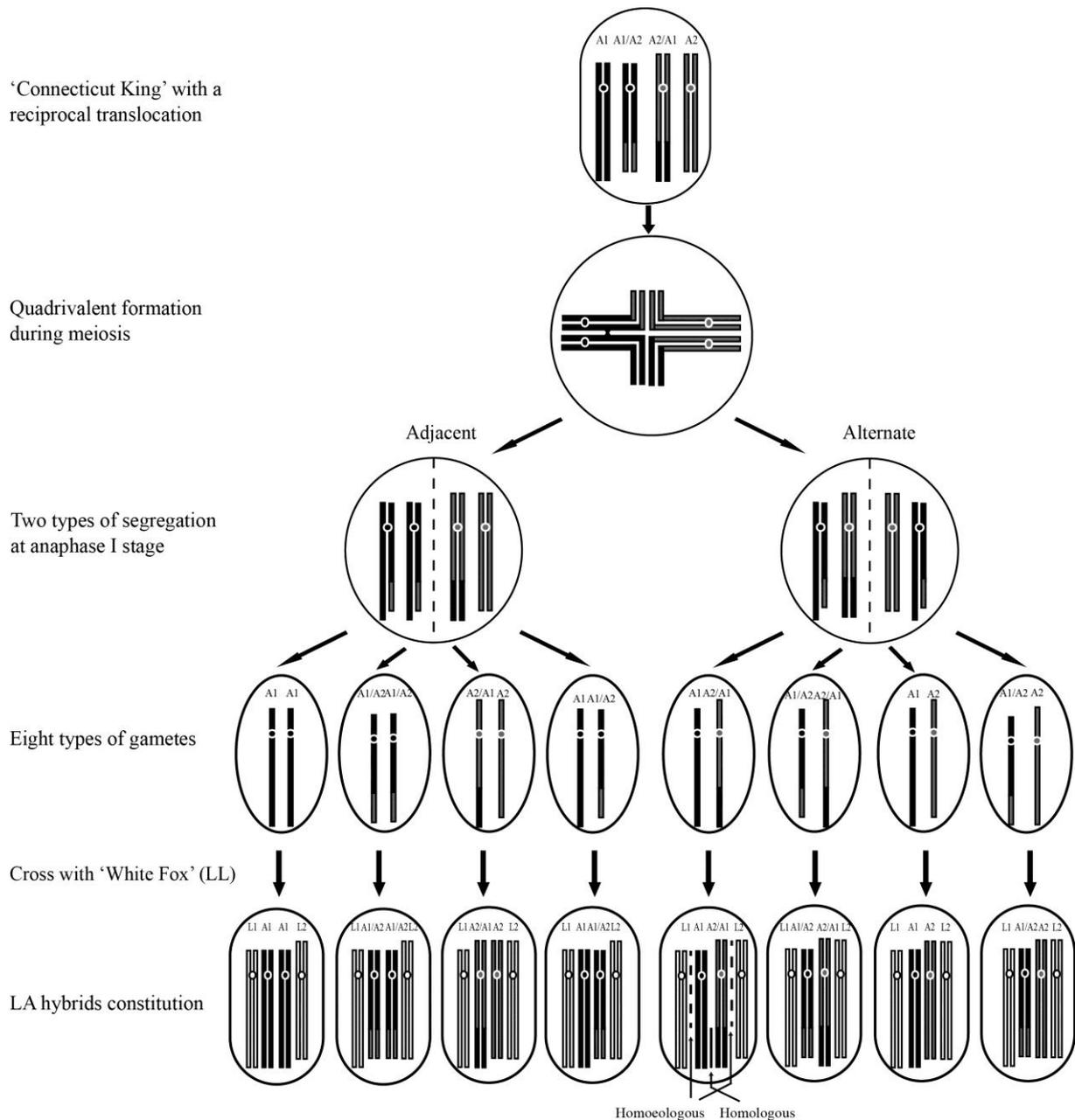
Fig. 3.3. Gametes production and the resultant hybrids in the parental parent 'Connecticut King' with a reciprocal translocation. If a single crossover happened between two non-sister chromatids in the interstitial segment (the segment between the centromere and the translocated segment on the chromosome with a translocation), both adjacent and alternate segregation lead to duplication-deficiency gametes.

The occurrence of multivalents and nonhomologous bivalents in two genotypes is explained due to the presence of a duplication between two chromosomes from the Asiatic genome. It is noticeable that this duplication is large enough to form chiasma(ta) in the homologous segment so frequently that it results in forming multivalents (quadri- and trivalent) or abnormal bivalents involving two non-homologous Asiatic chromosomes. This

duplication indicates that there is either a duplication or a reciprocal translocation in the paternal parent 'Connecticut King', and in the latter, a so-called duplication-deficiency gametes has been transmitted to the progeny successfully (Burnham 1962). These two alternative possibilities need cytological confirmation through the analysis of the Asiatic parent. From the available observations and previous reports (Abe et al. 2002; Khan 2009; Shahin et al. 2011; Van Heusden et al. 2002), it appears that the presence of a reciprocal translocation may be more likely. During meiosis of reciprocal translocation, quadrivalents are normally formed at metaphase I. Chiasma formation and crossing over in such cases will be suppressed in the area close to the translocation breakpoints, both alternate and adjacent segregation lead to reduced fertility. When progenies from these gametes are used for genetic mapping, markers will show skewed segregations, which has been found when these LA population were used for mapping (Shahin et al. 2011). Furthermore, two translocated chromosomes usually lead to the formation of 'pseudolinkage' (Albrecht and Chetelat 2009; Beeman et al. 1986; Farré et al. 2010; Kamphuis et al. 2007; Larson et al. 2011). One example is the linkage maps of an interspecific F2 *Solanum ochranthum* × *S. juglandifolium* population. Chromosome 8 and 12 were connected in one large linkage groups, which indicating a likely reciprocal translocation (Albrecht and Chetelat 2009). In the maps of LA lily, linkage group 1 (219 cM) is more than two times longer compared with the average longth of the linkage grous (95 cM)(Khan 2009), which also indicate a likely reciprocal translocation in 'Connecticut King'. Meanwhile, if crossovers happen in the interstitial segment of the translocated chromosome, different types of duplication-deficiency gametes, which are generally sterile, will be produced (Fig. 3.3).

In conclusion, intergenomic recombination in lily allopolyploids are derived from crossing over events during meiosis; while the non-homologous chromosome pairing in multivalents and bivalents potentially lead to the production of gametes with real chromosome rearrangements.

Chapter 4

Cytogenetic analysis of interspecific *Lilium* LA hybrids reveals chromatid bridges caused by U-type exchanges during meiosis

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Abstract

Meiotic abnormalities were investigated in interspecific lily hybrids using genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH). At metaphase I, the bivalents involving homoeologous chromosomes and unpaired univalent were the main configuration for most of the pollen mother cells. Besides these, also multivalents as well as bivalents involving non-homologous chromosome pairing in the same genome, were also observed. Moreover, broken chromosomes were sporadically detected at metaphase I using GISH and FISH with telomere repeats as probe. At anaphase I, chromatid bridges accompanied with fragments were present. GISH and FISH revealed that these bridges involved not only non-sister chromatids but also sister-chromatids. This strongly suggests that the bridges and the fragments found were derived from U-type exchanges. In conclusion, U-type exchanges, including spontaneous chromatid breakage and fusion, leads to anaphase bridging at meiosis in interspecific hybrids of lily. It is argued that during meiosis of interspecifc hybrids of lily, both homologous recombination (HR) and nonhomologous end joining (NHEJ) were both involved to repair double strand breaks (DSBs). U-type exchanges, together with association failure, will cause reduced fertility, and lead to aneuploidy and production of isochromosomes during sexual polyploidization.

Keywords: Anaphase bridging; interspecific hybridization; meiosis; lily; nonhomologous end joining (NHEJ); double strand breaks (DSBs), GISH; FISH

Introduction

Anaphase bridging during mitotic division has been found to be due to the erroneous repair of double strand breaks (DSBs). The formation of bridges during mitosis involves two process: DSBs and the repair. Other than homologous recombination (HR) in the repair of DSBs, nonhomologous end joining (NHEJ) can also restore chromosome integrity (Rothkamm et al. 2003). Since NHEJ can ligate any broken ends of chromosomes, this mechanism of repair can result in chromosome rearrangements, including translocation, inversion, isochromosome formation, chromosome bridges and so on (Acilan et al. 2007; Hartlerode and Scully 2009; Yu and Gabriel 2004). The dicentric chromosomes or ring chromosomes caused by fusion of dysfunctional telomeres and broken chromosome ends in maize, yeast, mammals and human tumour cells, have been revealed to be due to the repair of DSBs by NHEJ (Gisselsson 2008; Rai et al. 2010). Thus, NHEJ is considered as an error-prone mechanism of DSB repair (Gorbunova and Levy 1999).

Other than mitotic bridges, anaphase I bridges at meiosis have been well documented by cytogenetists. Two causes have been explained as the origin of dicentric bridges in meiosis. One of the main causes of anaphase bridging is the existence of (paracentric) inversion heterozygote. When a single crossover happens within the inversion loop, bridges and fragments will arise at anaphase I. This phenomenon has been observed in many species like maize (*Zea mays*) (McClintock 1931), *Drosophila* (Matzkin et al. 2005), sunflower (*Helianthus*) (Rieseberg et al. 1999), wheat (*Triticum aestivum*) (Lukaszewski et al. ; Qi et al. 2006) and many others. U-type exchange is thought to be the other important cause of anaphase bridging. This process involves spontaneous chromatid breakage (DSBs) at prophase I of meiosis and fusion of broken ends before separation, which will also lead to the production of dicentric bridges and acentric fragments at anaphase I (Couzin and Fox 1973; Haga 1953; Jones and Brumpton 1971; Jones 1969; Karp and Jones 1983; Lewis and John 1963; Newman 1967; Rees and Thompson 1955). However, compared with mitosis, repair mechanisms of DSBs during meiosis were rarely studied, and the limited results showed that homologous recombination exclusively took responsibility of the DSBs and lead to crossing over (Keeney 2001; Puchta 2005; Szostak et al. 1983). Combining the two causes of anaphase I bridging with the DSB repair mechanisms, it seems that HR and NHEJ are both involved during meiosis, in which the former takes responsibility of bridges from inversion heterozygote and the latter leads to the bridges from U-type exchange.

There are some criteria to distinguish the bridges and fragments with respect to their different origins. Bridges from paracentric inversion and U-type exchanges result in different meiotic configuration at the first meiotic division, which can be recognised through a critical meiotic observation. The first difference is that bridges and fragments from inversion heterozygote involve non-sister chromatids, while U-type exchanges can happen between both sister and non-sister chromatids. Another feature caused by inversion is the invariable size of the fragments. No matter where the crossover happened in the inversion loop, the resultant acentric fragments should be of constant size. On the contrary, asymmetrical bivalents, fragment size variation and side arm bridges are all evidence for the occurrence of U-type exchanges. Moreover, bridges and fragments in some species and species hybrids, which had been considered to originate from inversion, have been proven to be derived from U-type exchanges. In the species of *Tradescantia* and *Paeonia brownii*, "the occurrence of inversion was presumptive and circumstantial" and the presence of bridges and fragments have finally been explained as due to U-type exchanges (Lewis and John 1963). In conclusion, inversion heterozygote, as well as U-type exchanges, lead to anaphase bridging with different configuration at meiosis.

Meiotic bridges not only occur spontaneous, but can also be induced by genomic shock, including radiation treatment and interspecific hybridization. Radiation treatment, which is probably the most efficient method, leads to chromosome breakage and various types of anaphase bridging in a number of species like *Lilium longiflorum* (Mitra 1958), *Zea mays* (Viccini and De Carvalho 2002), *Triticum* (Wu and Yu 2001) and many others. Interspecific hybridization is another cause of the dicentric bridge production in a wide range of species hybrids like *Vigna umbellate* × *V. minima* (Gopinathan and Babu 1986), *Pinus* hybrids (Saylor and Smith 1966) and so on. Interestingly, the bridges and fragments in F1 hybrids found between some species in the genus *Chorthippus*, which were once thought to have originated from paracentric inversion, have been proven to arise from spontaneous chromosome breakage and reunion (Lewis and John 1966). In all of the above mentioned species and species hybrids, meiotic bridges were studied using traditional cytogenetic methods.

Genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) have the potential to give more convincing results about the origin of anaphase bridging. These two methods, which enable the localization of labelled probes after DNA hybridization, can not only distinguish non-sister chromatids in hybrids and allopolyploids (GISH), but also check

the intactness of chromosomes (FISH), which is essential for chromosome breakage detection. As a result, GISH and FISH can identify bridges and fragments and trace their origins.

In the present chapter, meiosis irregularities at metaphase I and anaphase I stages were investigated in interspecific hybrids of lily using GISH and FISH, and the origin of anaphase bridging was analysed according to the anaphase I configuration. Finally, the significance of the meiotic bridges and fragments was discussed.

Materials and methods

Plant material
Interspecific hybrids were obtained through crossing between a Longiflorum (L) cultivar 'White Fox' and an Asiatic (A) cultivar 'Connecticut King' with the assistance of cut-style pollination and embryo rescue (Van Tuyl et al. 1991). These hybrids were in vitro propagated and then transferred into the greenhouse for maintenance. Thirteen genotypes, which had been successfully used to produce sexual polyploidized progenies, were selected for the analysis of meiosis.

Meiotic chromosome preparation
Young anthers in putative meiotic stages from metaphase I to telophase I were collected and fixed in freshly prepared Carnoy's solution (Ethanol : Acetic Acid/ 3:1, v/v) for 24h at 4°C. A part of the fixed anthers was squashed in a drop of 2% acetocarmine to determine the appropriate meiotic stage, whereas the rest of the anthers were transferred into 70% ethanol and stored at -20°C. Anthers with proper stages were incubated in enzyme mixture containing 1 % pectolyase Y23, 1 % cellulase RS and 1% cytohelicase in 10mM citrate buffer (pH 4.5) at 37 °C for about 25-35 minutes for meiotic chromosome preparation,. Digested anther slices were put on a clean slide and chromosomes were spread according to Ross et al. (1996).

Probes for GISH and FISH
Total genomic DNA was extracted from young leaves of Longiflorum cultivar 'White Fox' and Asiatic cultivar 'Connecticut King' according to Fulton et al. (1995). DNA of 'White Fox' was sonicated to 1-10kb fragments and used as probe. 'Connecticut King' DNA was autoclaved to 100-500bp fragments and used as block. The probe DNA was labelled with digoxigenin-11-dUTP by standard nick translation according to the manufacturer's instruction (Roche Diannostics GmbH, Mannheim, Germany).

FISH experiments were performed using two different probes, 1) clone pTa71 which contains the EcoRI fragment of 45S ribosomal DNA from wheat (9kb) (Gerlach and Bedbrook 1979); 2) a probe of telomere repeat sequence generated by PCR according to Cox et al. (1993) with minor modifications. In brief, two oligomer primers 1fw (5'-TTTAGGG-3')$_5$ and 1rev (5'-CCCTAAA-3')$_5$ were synthetized by Isogen Life Science, the Netherlands. PCR reactions were set-up in the absence of template DNA. Each 100 μL PCR reaction comprised of 10 μL of $10 \times$ Taq buffer (Promega), 1.5 mM $MgCl_2$, 2 units of Taq polymerase (Promega), 2.5 mM dNTPs and 10 pmol of each primer 1fw and 1rev. Temperature cycling was performed according to Ijdo et al. (1991) with a final extension step of 10 min at 72°C. Probes of different genomic DNA were labelled with either digoxigenin-11-dUTP or biotin-16-dUTP by nick translation according to the manufacturer's instruction (Roche Diannostics GmbH, Mannheim, Germany).

In situ hybridization

The procedure of *in situ* hybridization was carried out according to Khan et al. (2009a) and Xie et al. (2010) with minor modification. For GISH, the hybridization mixture contained 50% formamide, 10% dextransulphate, $2 \times$ saline sodium citrate (SSC), 0.25% sodium dodecyl sulphate (SDS), 1.0-1.5 ng/μL for the probe and 25-50 ng/μL block DNA. While for FISH, the hybridization mixture contained 50% formamide, 10% dextransulphate, $2 \times$ saline sodium citrate (SSC), 0.25% sodium dodecyl sulphate (SDS), 2.0-2.5 ng/μL for the probe and 50-100 ng/μL block DNA. The mixture was incubated at 73°C for 10 minutes and ice cooled for 10 minutes, then 60μl hybridization mixture was added on each slide. After denaturation for 5 minutes at 80°C , slides were left in a pre-warmed box for overnight hybridization at 37°C. After hybridization, the slides were washed in $2 \times$ SSC for 15 minutes then stringency washing was followed with $0.1 \times$ SSC at 42°C for 30 minutes. The probe labelled with digoxigenin-11-dUTP was detected with the anti-digoxigenin detection system and probe labelled with biotin-16-dUTP was detected by cy3-streptavidin system. Then the slides were counterstained with DAPI and mounted with Vectashield. At last, photographs were taken with a Canon digital camera attached to a Zeiss Axiophot epifluorescence microscopy.

Table 4.1. Chromosome associations and segregation abnormalities during meiosis in 13 LA hybrids

Hybrid genotype number	# of cells analyzed	Chromosome pairing		Bridge formation frequency at anaphase I *(%)	Remarks
		Range of bivalents	Average bivalents		
006001-6	256	9-12	11.2	17	Sporadic multivalents
006001-9	228	3-11	7.6	6	
006001-13	132	6-11	8.7	1	Sporadic non-homologous/homoeologous pairing
006001-16	143	5-10	6.2	11	
006001-17	139	4-11	7.2	3	
006001-36	141	2-9	5.4	7	
006001-42	133	3-9	4.2	5	
006001-72	136	4-7	5.6	6	
006001-80	129	3-9	3.9	2	
006001-88	126	7-12	10.7	9	
006001-97	135	4-10	6.2	0	
041501	133	10-12	10.8	4	
041502	124	4-10	7.3	0	

*Bridge information was scored according to the anaphase I and telophase I pollen mother cells

Results

Chromosome breakage at metaphase I

Chromosome association was observed at metaphase I during meiosis using GISH. In these hybrids, 120 to 260 pollen mother cells were observed and analysed. The main character in these 13 interspecific hybrids is the occurrence of bivalents involving homoeologous chromosomes, as well as univalent with failed association. Meanwhile, few quadrivalents, trivalents and bivalents involving non-homologous association between two Asiatic chromosomes were also detected, indicating the existence of chromosome translocation. Furthermore, chromatid breakage was also sporadically observed at metaphase I stage. In one of the pollen mother cells with 12 bivalents, an Asiatic chromosome in one of the bivalents

was apparently shorter, with additional fragments present nearby (Fig. 4.1a). This obviously indicated that the Asiatic chromosome was broken into two pieces.
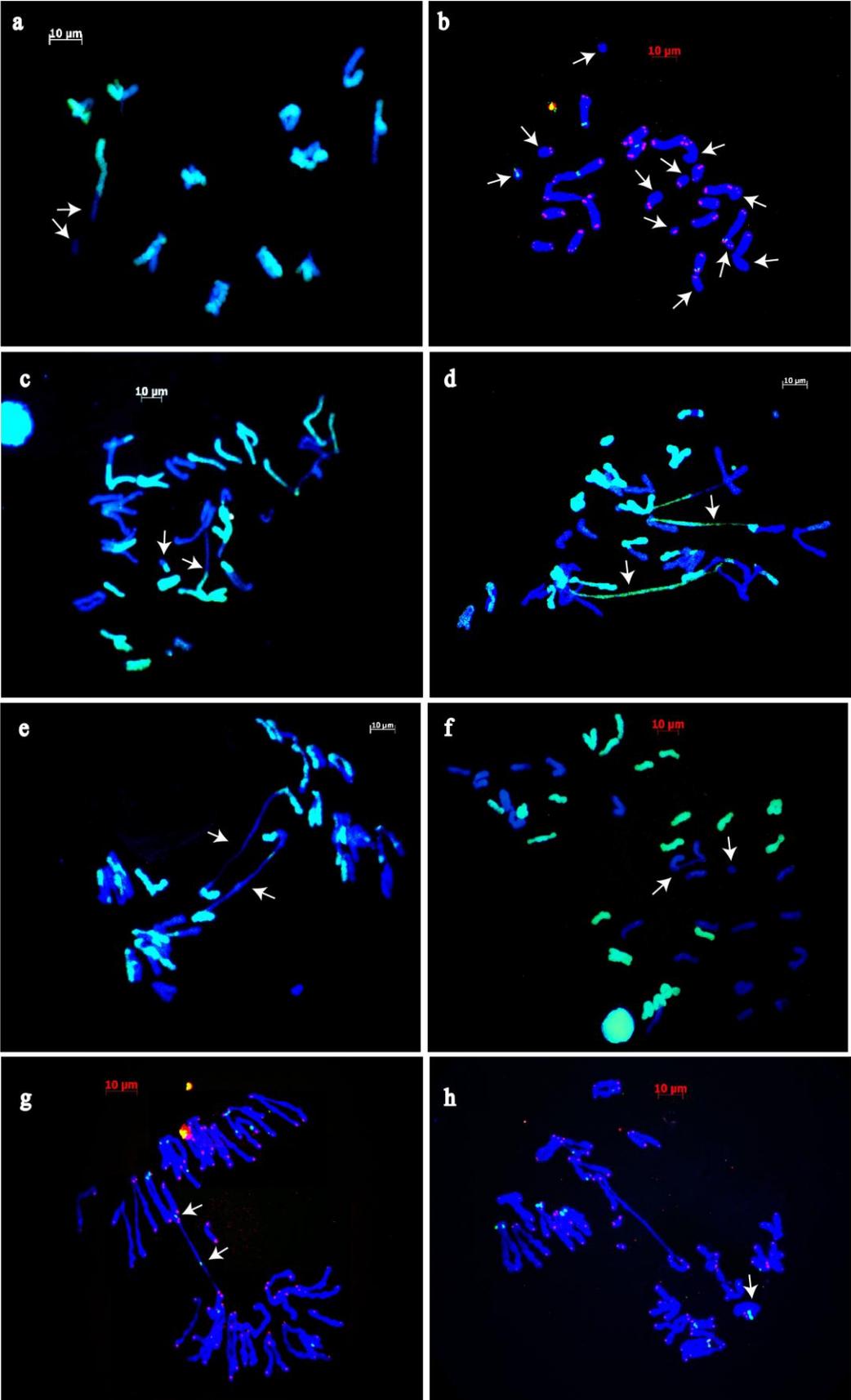
Fig. 4.1. Chromosome breakage at metaphase I and anaphase I bridging during meiosis of interspecific hybrids of lily (006001-6). (**a**) a pollen mother cell with successful association showed 12 bivalents, one of which had an apparently shortened Asiatic chromosome as well as an additional fragment nearby (white arrows); (**b**) FISH analysis with telomere repeat and 45s rDNA as probes revealed the presence of a number of broken chromosomes (white arrows); (**c**) a dicentric bicolour bridge and an acentric bicolour fragment at anaphase I stage in one of the pollen mother cells (white arrows); (**d**) two unicolor bridges (green fluorescence, white arrows), as well as one bicolour bridge at anaphase I in one of the pollen mother cells; (**e**) two unicolour bridges at anaphase I stage in one of the pollen mother cells (blue fluorescence, white arrows); (**f**) anaphase I bridging, accompanied by a fragment, between two nonhomologous chromosomes from the Asiatic genome (white arrows); (**g**) two 45s rDNA loci on the bridge indicate the bridge happened in two sister chromatids (green fluorescence, white arrows); (**h**) a putative ring chromosome with two telomere signals on one end and two telomere missing on the other end (white arrow). Green fluorescence stands for chromosomes from Longiflorum genome, and blue fluorescence represents chromosomes from Asiatic genome

FISH with telomere repeats as probe also confirmed the occurrence of chromosome breakage at metaphase I. Normally an intact chromosome consists of two chromatids and possesses four telomeres at meiosis, any breakage of a chromosome or chromatid can be characterized by the absence of telomeric signals in FISH. In a few pollen mother cells at metaphase I, a number of broken chromosomes were detected that lacked half of the telomere signals, indicating chromosome breakage (Fig. 4.1b).

Dicentric bridges and fragments at anaphase I stage

Bridges and fragments were detected at anaphase I and telophase I stages during meiosis. In the 13 genotype investigated (Fig. 4.2), diverse bridging frequencies were scored by traditional cytogenetic observation (Table 4.1). In one genotype (006001-6), the frequency of bridging formation reached 17%, followed by around 11% in genotype 006001-16. Most genotypes showed relatively low bridging frequencies (less than 10%), while two genotypes (006001-97 and 041502) didn't show any anaphase I bridging.

FISH and GISH revealed two types of bridges at anaphase I. One type of bridges and fragments involved non-sister chromatids (Fig. 4.1c). Such bridges and fragments both showed two different fluorescences (Fig. 4.1c). Since their variable size of the accompanying bicolor fragments of the bicolour bridges, paracentric inversion was excluded. In addition, a common feature of the bicolor fragments is that two fluorescences have the same lengths in all cases (Fig. 4.1c), which was also rejecting the existence paracentric inversion. This type of

bridges is explained as U-type exchange between non-sister chromatids. In this process, two nonsister chromatids from homoeologous chromosomes broke respectively, two broken chromatids, which possessed centromeres, mismatched together and formed a bridge, and two acentric chromatids formed a fragment at anaphase I (Fig. 4.2). The other type of bridges and fragments involved sister-chromatids (Fig. 4.1d). This type of bridging not only happened with Asiatic chromosomes (Fig. 4.1d), but could also be detected in Longiflorum chromosomes (Fig. 4.1e). In this case, the bridges and fragments only showed one fluorescence. FISH experiments with telomere repeats and 45s rDNA as probes revealed that the fragments possessed two normal telomeres in all cases, and the two 45s rDNA loci on the bridge further confirmed that the bridge involved sister-chromatids, (Fig. 4.1g). According to the configuration of the bridge-linked homoeologous chromosomes revealed by GISH and FISH, formation of this type of bridges & fragments involved a U-type exchange between sister chromatids and a single crossing over between non-sister chromatids (Fig. 4.2).

There were two additional indications for the occurrence of U-type exchanges. The first one is that U-type exchanges occured not only between homoeologous chromosomes, but also between two non-homologous chromosomes from Asiatic genome (Fig. 4.1f). In this case, both the bridge and the fragment showed the same fluorescence. The second proof was the presence of a putative ring chromosome with an additional fragment, except another bridge and fragment (Fig. 4.1h). This broken chromosome was recognized as chromosome number 2 from Asiatic genome, which was sub-metacentric and showed a very strong 45s rDNA locus on the short arm near the second constriction. It was deduced that a U-type exchange happened between two sister chromatids, and no crossover (or rarely with even number of crossovers) happened between non-sister chromatids of these two homoeologous chromosomes. As a result, one part of the broken chromosome formed a ring chromosome and two other arm fragments fused together and formed an acentric fragment with two telomere (Fig. 4.2). The ring chromosome will cause anaphase bridging in the second meiotic division. In conclusion, the bridges produced at anaphase I during meiosis of the interspecific lily hybrids were the outcome of chromosome breakage and fusion, with or without crossing over between homoeologous chromatids.

Discussion

In the present study, anaphase bridges with fragments between sister and non-sister chromatids were observed and the origin was found to be due to U-type exchanges. There are a number of reasons for this conclusion: 1) chromosome breakage was found at metaphase I;

2) bridges involved, not only non-sister chromatids, but also sister-chromatid; 3) breakage and fusion between sister chromatids without crossing over (or rarely even number of crossovers) lead to a ring chromosome together with a fragment, indicating that for U-type exchange crossing over is not always needed; 4) chromosome breakage and fusion occured not only between two nonsister chromatids of two homoeologous chromosomes, but also between the two non-sister chromatids of two non-homologous chromosomes; and 5) in view of the variation in fragment size, paracentric inversion was excluded. As a result, bridges and fragments at anaphase I during meiosis of these lily hybrids were derived from spontaneous chromosome breakage and fusion, and similar to many species hybrids in which inversion heterozygote was usually expected, judge these bridges from paracentric inversion is arbitrary.

DNA *in situ* hybridization is a powerful technique in studying bridges, which enables the discrimination of sister U-type exchanges from non-sister U-type exchanges. GISH revealed that anaphase I bridges and fragments are not sufficient proof for non-sister U-type exchanges. In classical cytogenetics, non-sister U-type exchange was characterized by the configuration of anaphase I bridges and fragments, while sister U-type exchanges were recognised by the anaphase I loops with fragments, univalent loops and univalent bridges at meiosis (Haga 1953; Jones and Brumpton 1971; Jones 1969; Karp and Jones 1983; Walters 1956). Our results revealed that not only non-sister U-type exchanges, but also sister chromatid U-type exchanges, with a single crossing over, can also give rise to the production of an anaphase I bridge together with an acentric fragment (Fig. 4.1d and 1e; Fig. 4.2). The only difference of these two is that bridges and fragments derived from non-sister U-type exchanges are the merger of chromatid segments from two genomes that are differentially labelled by the fluorescence labelling . On the other hand, anaphase I loops with fragments, univalent loops and univalent bridges at meiosis are of course proof of sister U-type exchanges, but loops are difficult to be identified at meiosis since chromosomes are so condensed. However, FISH with a telomere repeat as probe can simultaneously detect the number of telomeres on individual chromosomes, which provides convincing proof for ring chromosomes.

Anaphase bridging in interspecific hybrids results in reduced fertility, aneuploidy and probably the production of isochromosomes in the progeny. During male meiosis which will give rise to haploid pollen, a chromatid bridge will break at one or multiple locations, resulting in chromosome structural changes and/or loss of chromosome material. This will cause half of the gametes from the pollen mother cell to be unbalanced and lethal, and explain the remarkable reduction of fertility. Since unreduced gametes can endure aneuploidy, some

of the sexual polyploidized progenies can be aneuploids. That is why a few aneuploids were found with one or two chromosomes missing in backcross progenies of lily after sexual polyploidization (Khan 2009; Khan et al. 2009a; Zhou 2007). However, anaphase bridging is not the only factor that contributes to aneuploids, at metaphase I during meiosis, numerical univalent were also present in most of the pollen mother cells (chapter 3), the random movements of these univalents in the first meiotic division can also lead to the production of aneuploids (Zhou 2007). In conclusion, meiosis with anaphase bridges mostly produced unviable gametes due to chromosome number or structure variation.
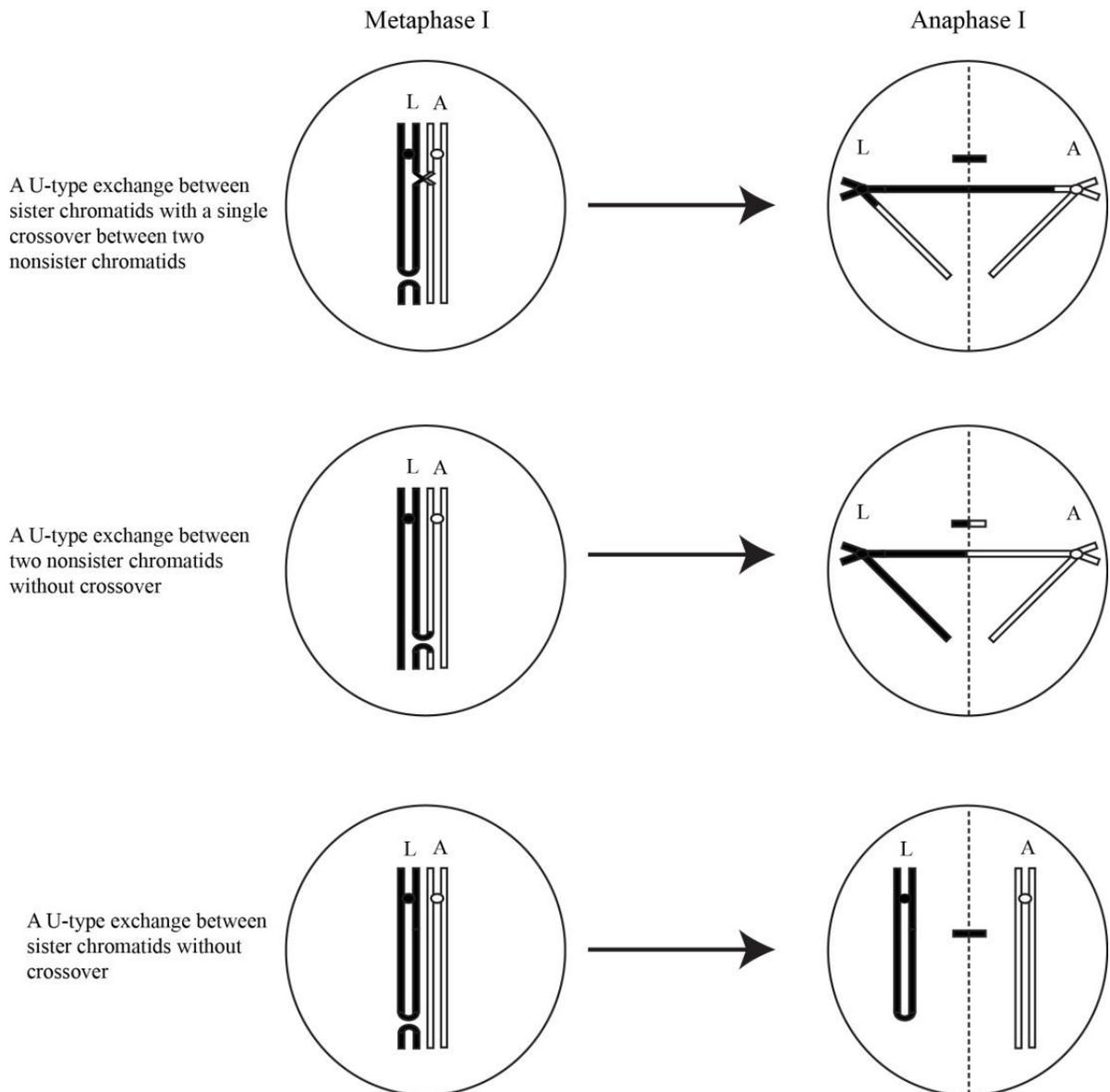
Fig. 4.2. Illustration of the production of chromosome bridges with different configurations at anaphase I stage during meiosis of interspecific lily hybrids

Chromatid breakage is probably a genetic response to genomic shock caused by interspecific hybridization in lily. Like radiation, interspecific hybridization can cause meiotic instability, which is common in many species hybrids. Our results showed that univalents, multivalents, non-homologous bivalents, bridges as well as ring chromosomes were present during meiosis of these lily hybrids. Similarly, univalent, chain and ring multivalents and anaphase bridges were found in the pollen mother cells of a F1 hybrid between *Vigna umbellate* and *V. minima* (Gopinathan and Babu 1986). Non-homologous chromosome pairing has also been found in the hybrids of *Lolium temulentum* × *L. perenne*. In the hybrids of *Helianthus annuus* × *H. tuberosus*, genomic alterations were revealed to be the response to genomic shock following the interspecific cross (Natali et al. 1998). These meiotic abnormalities all involved chromatid breakage. Since normal meiosis can be found in both of the parents of the hybrids, the meiotic irregularity is probably due to interspecific hybridization. Indeed, during allopolyploid formation, interspecific hybridization, rather than polyploidization, is likely the reason of extensive genetic and epigenetic changes (Wang et al. 2006). Furthermore, if chromosome breakage occurs at the centromere position, fusion of two broken chromatids from one chromosome arm will probably lead to the production of isochromosomes (see chapter 5), which has been also presumed as a mechanism leading to B chromosomes.

We propose that U-type exchanges in lily hybrids are DSBs and the repair mediated by NHEJ. It has been revealed that crossovers are indeed DSBs followed by the repair by HR (Keeney 2001; Puchta 2005; Szostak et al. 1983). In mitotic cells, DSB repair with the sister chromatid appears to be preferred, whereas interhomolog recombination is favoured during meiosis (Pradillo and Santos 2011). Sequence repeats comprise a large fraction of lily genome and, although they can be quite divergent from each other, their enormous number and dispersal throughout the genome also makes them potential repair templates. Increase of HR mediated events—such as unequal sister-chromatid exchange and ectopic HR between non-allelic repeated DNA fragments can result in chromosomal rearrangements (Aguilera and Gómez-González 2008). As a result, altered karyotypes in yeast have been explained as due to DSBs repaired either by reciprocal unequal sister chromatid recombination or ectopic recombination between non-homologous chromosome (Loidl and Nairz 1997). However, such explanation doesn't fit the current results for two reasons. Firstly, none of reciprocal unequal recombination and ectopic recombination can produce bridges and fragments like what has been found in lily (Fig. 4.1). Like in yeast, two mechanisms normally lead to variation of chromosome size. Even there was an inversion, the chance that two fluorescence of the

fragment have the same length would be rare. In addition, ectopic recombination is mostly nonreciprocal. Secondly, as shown in the results, the allelic homologies/homoeologies were still available in the pollen mother cells that formed bridges at anaphase I. On the contrary, bridges happened not only between sister chromatids but also nonsister chromatids from homoeologous chromosome pairs (Fig. 4.1). Moreover, isochromosomes from centric fission and fusion have also been found in the backcross progenies of LA lilies. All of these evidence indicates that the repair of DSBs in bridge & fragment formation is nonhomologous. Thus, NHEJ is proposed to be involved in the repair of DSBs during meiosis of interspecific LA lilies.

Chapter 5

Characterization of ancient and potentially new B chromosomes in *Lilium* hybrids through GISH and FISH

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Abstract

Supernumerary (B) chromosomes and small aberrant chromosomes were detected in *Lilium* hybrids and characterized through genomic *in situ* hybridization (GISH) and florescence *in situ* hybridization (FISH). Two small, supernumerary or B chromosomes were detected as extra chromosomes in a tetraploid plant derived from chromosome doubling of a hybrid (2n=2x=24) between a cultivar of the Longiflorum (L) and the Trumpet (T) group. When this tetraploid LLTT hybrid was crossed with a triploid LLO hybrid (O=Oriental), the B chromosome was transmitted to 73.4% of the progenies. Based on GISH and FISH characterization it was shown that the B chromosome found consisted of two identical arms, with 5S rDNA hybridizing to the majority of it, which were flanked by normal telomeres, suggesting that this is an isochromosome. In another population, which is a backcross progeny between a F1 hybrid of Longiflorum × Asiatic (LA) and its Asiatic parent, the former produced functional *2n* gametes which resulted in a triploid LAA progeny (2n=3x=36), in which three exceptional plants possessed 35 normal chromosomes and a small aberrant chromosome instead of the expected normal number of 36. In all three cases the small aberrant chromosomes were isochromosomes which had obviously originated during the first backcross generation. These three chromosomes showed normal telomeres and mitosis. In addition, one of the new generated chromosomes possessed two 45S rDNA sites in the proximal positions. These new arisen isochromosomes were proposed to originate from centric breakage and fusion of two short arms of the missing chromosome in three genotypes respectively, based on the comparison of arm lengths as well as rDNA loci. Their relevance to the origin of Bs is discussed.

Keywords: lily, B chromosomes, isochromosome, centromere misdivision, multicolour GISH, rDNA

Introduction

B chromosomes (Bs) have been reported to occur in more than a thousand flowering plant species (Jones and Houben 2003; Jones and Rees 1982). The survey is by no means complete, but the available literature suggests that some families (e.g. Compositae, Graminae and Liliaceae) possess larger numbers of species with Bs as compared with others (Jones 1995; Levin et al. 2005; Trivers et al. 2004). Based on cytological studies, certain general trends for Bs are detected: 1. Bs are incidental, i.e. present only in some of the individuals in a sampled population; 2. during meiosis, they might pair among themselves but not with standard chromosomes (As); 3. their inheritance is normally non-Mendelian and their number can vary among individuals of a species; 4. except for rDNA genes, no other major gene loci have been found; 5. they occur predominantly in plants with large chromosomes; and 6. they are derived from As, but "their mode of origin remains a mystery" (Jones et al. 2008a; Jones et al. 2008b). Despite the wide occurrence of Bs among plants, critical analysis of their molecular structure, organization and genetics has been investigated in only a few species such as: maize, rye, *Brachycome dichromosomatica*, *Crepis capillaries* (Donald et al. 1995; Jones et al. 2008b; Maluszynska and Schweizer 1989). In most of these cases, already existing Bs have been investigated. However, the origin of an apparent new B chromosome has been clearly detected and characterized in detail in *Plantago lagopus* (Dhar et al. 2002). This origin involved a "mutation (aneuploidy), chromosome fragmentation, specific DNA sequence amplification, addition of telomeric repeats, and centromeric misdivision" (Dhar et al. 2002). This obviously indicates that the origin of Bs involves a series of events which cannot always be traced or clearly defined.

The genus *Lilium* is well known to possess one of the largest genomes and chromosomes among flowering plants (Bennett and Smith 1976; Zonneveld et al. 2005). In this genus the occurrence of accessory chromosomes has been reported in at least 17 species (reference in Brandram 1967). These so-called accessory chromosomes vary in size from very minute to as large as the normal A chromosomes. Their numbers vary from one in *L. davidii* var. *willmottiae* to as many as eight in the hybrid *L. leichtlinii* var. *maximowiczii* × *L. amabile* var. *unicolor*. Although the Bs in *Lilium* species have not been characterized in great detail, the presence of sub-median, telocentric as well as median chromosomes has been detected in different species. Besides establishing the occurrence of B chromosomes in several species, preferential transmission and maintenance in EMCs (embryo-sac-mother cells) and Mendelian transmission in pollens have been investigated in wild populations of *L. callosum* (Kayano 1957; Kimura and Kayano 1961). In all the investigations on *Lilium* species, there appears to be no detailed investigation on the structure and organization of B chromosomes so far. While investigating the karyotypes of *Lilium* hybrids we have detected B chromosomes as well as small aberrant chromosomes (newly originated), the latter of which resemble potential Bs. In order to compare the existing Bs and aberrant small chromosomes in lily hybrids, and trace

their origin, the structure of these small chromosomes is analyzed using GISH and FISH techniques and the relevance of these structures to the probable origin of B chromosomes is discussed.

Materials and methods

Plant materials

Two types of lily populations were investigated for extra chromosomes in this study. In one case, a population consisting of 26 genotypes was derived from crossing an allotriploid (2n=3x=36) and an allotetraploid (2n=4x=48). The allotriploid was the backcross progeny between a Longiflorum cultivar (LL) and a somatic chromosome doubled Longiflorum × Oriental hybrid (LOLO), and was denoted as LLO; while the allotetraploid was obtained through somatic chromosome doubling of a cross between a Longiflorum (LL) and a Trumpet (TT) cultivar, and accordingly was denoted as LLTT. All the progenies of the LLO × LLTT combination were aneuploid in which chromosome numbers varied from 40 to 45 due to variable numbers of chromosomes from the O genome (Table 5.1). The other population consisted of 25 progenies derived from crossing a 2n gamete producing Longiflorum × Asiatic hybrid (2n=2x=24) with its Asiatic parent (2x) and was denoted as LAA. The progenies were predominantly triploid (3x) except for three aneuploids (2n=3x-1=35).

Mitotic chromosome preparation

Young roots were collected from in vitro plants and treated with 0.7mM cyclohexamide for 4-6 hours at 4°C after which they were transferred to freshly prepared Carnoy's solution (ethanol : acetic acid, 3:1 v/v) and stored at 4°C until use. Root tips were washed and incubated in an enzyme mixture (1% cellulose RS and 1% Pectolyase Y23 in 2mM citrate buffer, pH 4.5) for 90 minutes at 37°C. Mitotic metaphase chromosomes were spread according to Ross et al. (1996).

DNA preparation for GISH and FISH experiments

Genomic DNA was extracted from young leaves using the protocol described by Murray and Thompson (1980). For GISH in the progeny of LLO × LLTT, DNA of Oriental cultivar 'Sorbonne' and Trumpet cultivar 'Royal Gold' was sonicated to 1-10kb fragments and used as probes. Genomic DNA extracted from Longiflorum 'White Fox' was autoclaved to 200-600 bp fragments and used as block. In the case of the LAA progeny, genomic DNA from Longiflorum cultivar 'White Fox' was sonicated to 1-10kb fragments and used as probe, and the genomic DNA from Asiatic cultivar 'Connecticut King' was autoclaved to 200-600 bp fragments and used as block.

FISH was performed using three different probes, 1) clone pTa71 which contains the 9kb EcoRI fragment of 45S ribosomal DNA from wheat (Gerlach and Bedbrook 1979); 2) clone pScT7 which contains the 462bp BamHI fragment of 5S ribosomal DNA from rye (Lawrence

and Appels 1986); 3) a probe of telomere repeat sequence generated by PCR according to Cox et al. (1993) with minor modifications. In brief, two oligomer primers 1fw (5'-TTTAGGG-3')$_5$ and 1rev (5'-CCCTAAA-3')$_5$ were synthesized by Isogen Life Science, Netherlands. Concatemers were produced during a PCR reaction in which the primers also serve as template. Each 100 µl reaction comprised 10µl of 10×Taq buffer (Promega) 1.5mM MgCl$_2$, 2 units of Taq polymerase (Promega), 2.5mM dNTPs and 10pmol of each primer. Temperature cycling was performed according to Ijdo et al. (1991) with a final extension step of 10 min at 72°C.

Probes were labelled with either digoxigenin-11-dUTP or biotin-16-dUTP using standard nick translation according to the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany).

In situ hybridization
GISH was carried out according to Barba-Gonzalez et al. (2005b) and Khan et al. (2009a), the 40µl hybridization mixture contained 50% (v/v) deionized formamide, 10% (w/v) sodium dextran sulphate, 2×SSC, 0.25% (w/v) sodium dodecyl sulphate, 0.6-1.0 ng/µL for each probe and 15-50 ng/µL block DNA. FISH was carried out according to Lim et al. (Lim et al. 2001b) with a 40µl hybridization mixture of 50% (v/v) deionized formamide, 10% (w/v) sodium dextran sulphate, 2×SSC, 0.25% (w/v) sodium dodecyl sulphate, 2-2.5 ng/µL for each probe and 100-200 ng/µL sheared herring sperm DNA, the latter was used as block DNA. The hybridization mixture for GISH or FISH was incubated at 73°C for 10 minutes and ice cooled for at least 10 minutes, and then was added on each slide, the slides were covered with cover slips and denatured at 80°C for 5 minutes after which slides were transferred to a pre-warmed hybridization chamber for overnight incubation at 37°C. After hybridization, stringency washing was performed using 0.1×SSC at 42 °C for 30 minutes. The probes labelled with digoxigenin-11-dUTP or biotin-16-dUTP were detected with the anti-digoxigenin-FITC or Cy3 respectively. After detection the slides were counterstained with 1 µg/mL 4',6-diamidino-2-phenyl-indole (DAPI) and mounted with Vectashield (Vector Laboratories, Inc., Builingame, USA). Preparations were photographed with a Canon camera attached to a Zeiss Axiophot epifluorescence microscopy.

Chromosome identification and karyotyping
Images of mitotic metaphase chromosomes were measured using the computer program MicroMeasure (Reeves and Tear 2000). In all four genomes (L, A, O, T), the chromosomes were put into sequence according to decreasing short arm length (Stewart 1947). In order to identify the chromosome in each genome, the chromosome length, arm ratio, centromere index (short arm length/ long arm length + short arm length), relative chromosome length index (individual chromosome length/total length of a set of chromosomes) and 45S rDNA locus were used as identification tools (Barthes and Ricroch 2001; Lim et al. 2001b).
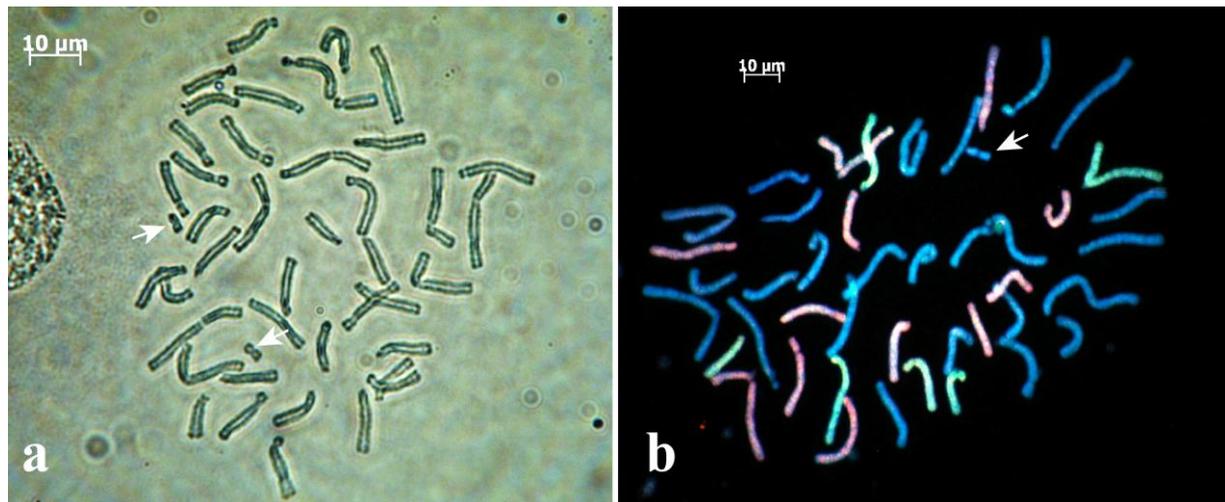
Fig. 5.1. Discovery of B chromosomes in the male parent (LLTT) and its multicolour GISH analysis in the progeny. (**a**) Two Bs in the male (L) parent of the tetraploid LLTT (white arrows). (**b**) B chromosome (white arrow) was blocked by L genome DNA in multicolour GISH in genotype 076928-21 (LLO × LLTT) in which GISH clearly identified the chromosomes of the three genomes. T= red (biotin labelled and detected with Cy3– streptavidin); O= green (digoxigenin labelled and detected with anti-digoxigenin FITC system) and L= blue (DAPI counterstaining)

Results

Extra chromosomes in progenies of LLO × LLTT
As a first step, the karyotypes of the two parents were investigated for their chromosome constitution. Whereas the triploid LLO possessed the expected 36 chromosomes without any extra chromosomes, there were two small extra chromosomes in the LLTT parent in addition to the normal chromosome complement (Fig. 5.1a). As expected, all the progenies from LLO × LLTT combination were aneuploid with chromosome number varying from 39 to 45. Beside the standard chromosomes (As), the small extra chromosomes were also detected in the progeny. Besides their small size, the extra chromosomes were clearly metacentric and present in all somatic metaphase cells of the root meristem. Because chromosomes of three different genomes (i.e., L, T and O) were expected to be present in the progenies of the LLO × LLTT cross, multicolour GISH analysis was used to analyze these progenies. Results showed that chromosomes of the three genomes could be clearly distinguished and there was indication that the small extra chromosome was blocked by Longiflorum DNA (Fig. 5.1b). Out of the analyzed 26 offspring plants, 19 genotypes possessed either one or two extra chromosomes, which will be mentioned as B chromosomes (Bs), whereas seven of the 26 progenies had no Bs (Table 5.1). This indicated that the transmission of Bs through the male parent LLTT was very high (73.4 %).

Table 5.1. Distribution, size and characters of small chromosomes in two different types of lily populations

Genotype	# of A chr.	# of small chr.	Origin	Length (μm)	Remarks	
LLO × LLTT population	Aneuploid 39-45	1-2	Male (LLTT)	6.29	existing	5S rDNA
074051-9	35	1	Female (LA)	8.41	de novo	45S rDNA
084798-2	35	1	Female (LA)	4.91	de novo	No rDNA
084798-6	35	1	Female (LA)	6.77	de novo	No rDNA
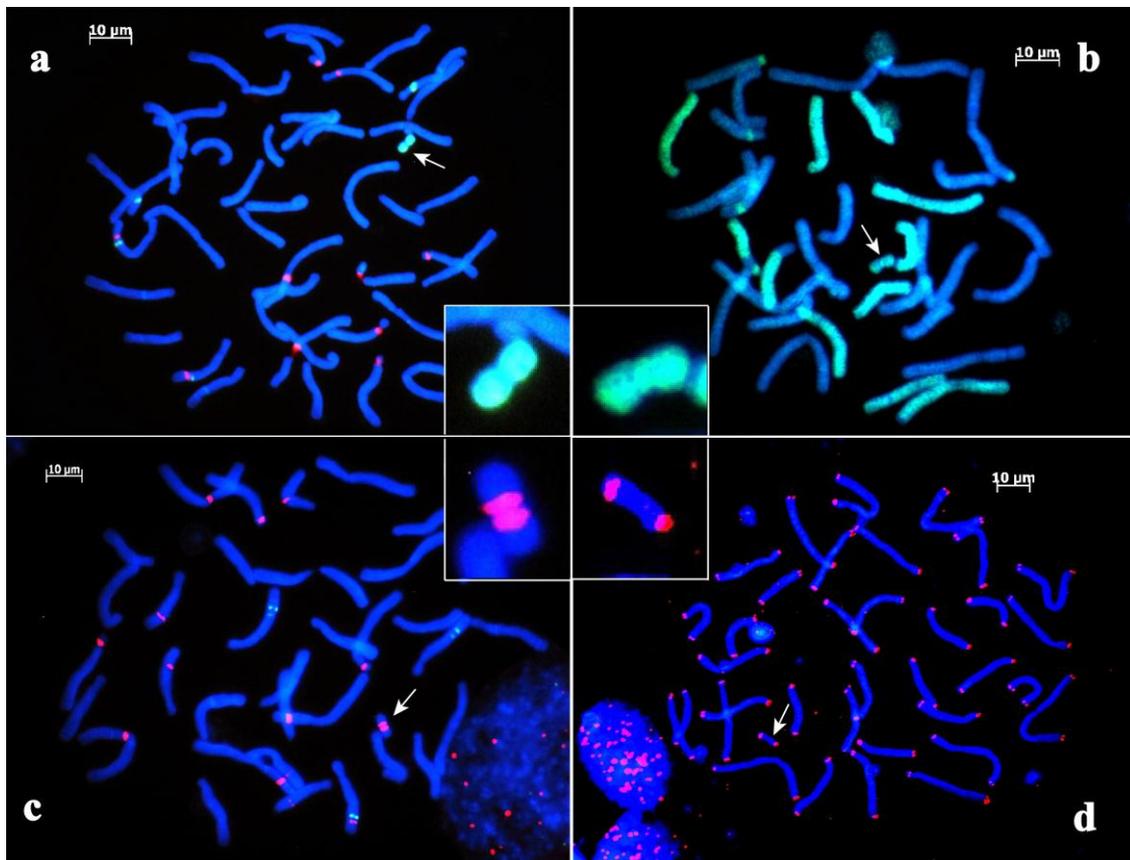
Fig. 5.2. Cytogenetic structure of small chromosomes in different genotypes of lily revealed by GISH and FISH. (**a**) B chromosome (LLO × LLTT, 076928-23) is wholly hybridized by 5S rDNA probe (red represents 45S rDNA loci and green represents 5S rDNA loci). (**b**) B chromosome can be hybridized by genomic DNA probe of Longiflorum (LAA, 074051-9, green represents L genome and blue represents A genome). (**c**) 45S rDNA loci on B chromosome (LAA, 074051-9, red represents 45S rDNA loci and green represents 5S rDNA loci). (**d**) Telomere labelling with telomere repeat sequences as probe in 074051-9; the small aberrant chromosome showed a normal telomere signals (white arrow and inset).

In order to investigate the structure of these Bs in more detail, FISH was applied using 5S rDNA and 45S rDNA as probes (Fig. 5.2a). In addition, the telomeric repeat sequence was used as a probe to detect the status of the chromosome ends. A notable feature was that FISH clearly detected several hybridization sites of both 5S rDNA (green) and 45S rDNA (red) sites in the standard chromosomes (Fig. 5.2a). The B chromosomes clearly contained two equal arms and possessed blocks of 5S rDNA on both arms (inset in Fig. 5.2a) flanked by telomeric sequences (result not shown). In view of the identical morphology of both arms of these B chromosomes it was concluded that they are isochromosomes.

Small aberrant chromosomes in progeny of LA × AA cross

 Out of 25 triploid progenies derived from LA × AA cross, 22 genotypes were euploids with the expected 36 chromosomes. None of these normal triploids possessed any extra chromosomes or fragments, similar to both of the parents from which the progeny was derived (results not shown). In the other three genotypes of this progeny, viz., 074051-9, 084798-2 and 084798-6, however, all the somatic cells possess 35 chromosomes together with a small chromosome in all the somatic cells. Because the small chromosome occurred in all three genotypes together with 35, instead of 36 normal chromosomes, the small structures which are probably related to the missing A chromosomes in each genotype are called 'aberrant'. The size of the aberrant chromosomes varied from 4.9 to 8.4 μm in different genotypes (Table 5.2). The structural organization of these three small chromosomes was investigated through GISH and FISH using 5S rDNA, 45S rDNA and telomeric sequences as probes. The results of GISH and FISH analyses of the aberrant chromosome in genotype 074051-9, are shown in Fig. 5.2b, c and d. GISH results indicated that the small aberrant chromosome in genotype 074051-9 (Fig. 5.2b) and 084798-2 originated from Longiflorum whereas the aberrant chromosome in 084798-6 was derived from Asiatic genome. By using two probes, 45S rDNA and 5S rDNA, different hybridization sites were detected through FISH in the complement (Fig. 5.2c). The striking feature, however, was that the small aberrant chromosome in genotype 074051-9 possessed a hybridization site of 45S rDNA repeat (red fluorescence) on each of its two arms in proximal positions (arrow and inset in Fig. 5.2c). When probed with telomeric sequences, FISH clearly demonstrated the presence of telomeres in the small aberrant chromosome (Fig. 5.2d, arrow and inset). Thus, each arm of this aberrant chromosome has a block of 45S rDNA proximally followed by a non-hybridized region and a telomere. In two other genotypes, 084798-2 and 08798-6, the small aberrant chromosomes were clearly median chromosomes without any rDNA repeats but possessed normal telomeres as revealed by FISH (results not shown). In view of the similar morphology of the arms of small chromosomes in all three genotypes, they were concluded to be isochromosomes as well.

Table 5.2. Comparison of arm length between the aberrant small chromosome and the missing chromosome in three LAA genotypes

Genotype	Arm length of aberrant chr. (µm)	Missing chromosomes	Short arm length of missing chr. (µm)**	Other similarity
074051-9	4.21±0.17	L4	4.33	45s rDNA
084798-2	2.45±0.08	L9	2.36	
084798-6	3.38±0.06	A6	3.73	

Note: L4 and L9 stands for chromosome 4 and 9 from Longiflorum genome respectively. Similarly, A6 represents chromosome 6 from Asiatic genome. ** data from Khan et al. (2009a)
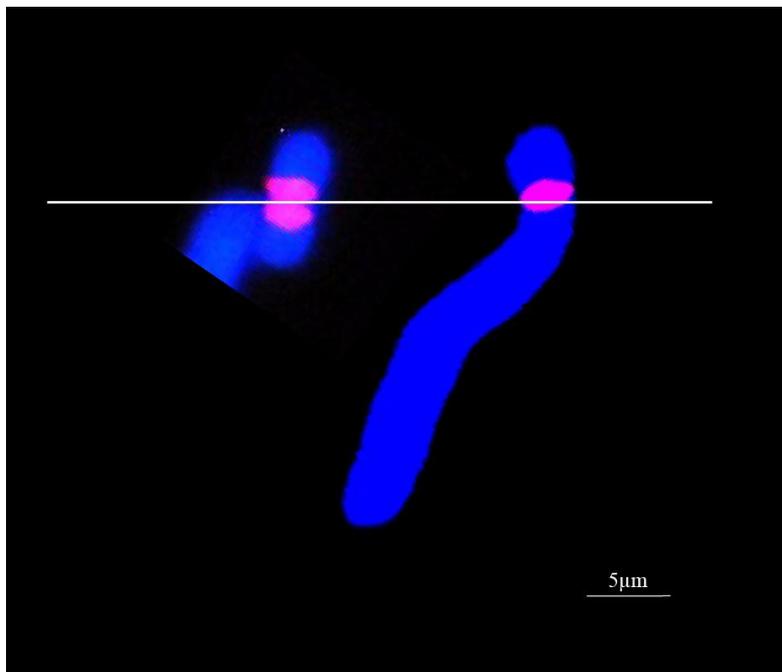
Fig. 5.3. A comparison of the small aberrant chromosome and the missing chromosome (L4) in genotype 074051-9.

*Centric breakage and fusion lead to the production of isochromosomes in progeny of LA ×
AA cross*

As mentioned before, a common feature of the three genotypes with isochromosomes in the
progeny of LA × AA cross is that they were all aneuploids with a loss of a normal
chromosome. Interestingly, GISH results revealed that the new generated isochromosomes
were from the same genome as the missing chromosome in all of the three genotypes. As a
next step, a comparison was made between the isochromosomes and the corresponding
missing chromosomes according to the arm length and FISH analysis. Results showed that the
arm length of the new generated chromosome was the same as the short arm length of the
missing chromosome in these three genotypes respectively (Table 5.2). Furthermore, 45S
rDNA signals were detected in the proximal position on the isochromosome in genotype
074051-9, which was exactly the same as the short arm of the missing chromosome L4 (Fig.
5.3). In view of these facts, it was proposed that these new generated isochromosomes
originated from a centric breakage and fusion of two short arms of the missing chromosomes
during meiosis.

Discussion

The small aberrant chromosomes in three genotypes of LA hybrids have been proposed to
originate from centric fission and fusion of two short arms of the missing chromosomes.
Firstly, there are reliable indications that the small aberrant chromosomes in the progeny of
LA × AA backcross have originated independently in the BC1 generation. None of the parents
possessed any small aberrant chromosome comparable to those observed in the three progeny
BC1 plants; secondly, all three genotypes that possessed aberrant chromosomes had an
aneuploid chromosome number of 35 instead of the expected 36 As; thirdly, the small
aberrant chromosome and the missing chromosome in each of the three genotypes are
respectively from the same genotype; fourthly, as what has been shown in the results part, the
similarity of arm length relationship and 45S rDNA distribution also strongly support the
hypothesis; and finally, chromosome breakage and fusion have been found during meiosis of
interspecific hybrids of Longiflorum × Asiatic (LA) lilies (see chapter 4). All these evidence
indicates that due to misdivision of the centromere, two telocentric chromosomes are formed.
The telocentric long arm is probably eliminated whereas the short arm has given rise to an
isochromosome which has survived. This survival might be due to the fact that, in one step, a
stable chromosome with a functional centromere and telomeres at both ends are formed. It
means that the species of the genus *Lilium* are well positioned to generate aberrant small
chromosomes such as the ones reported in this study. This is because, the karyotypes of
Lilium species possess two pairs of median or sub-median chromosomes while the other 10
pairs are highly asymmetrical with very small or minute short arms relative to the long arms
(Lim et al. 2001b; Stewart 1947). Furthermore, there are some other proofs to support that

chromosome centric fission and fusion lead to the production of isochromosomes. In maize and wheat, meiotic univalents not only randomly move to one pole when segregating at anaphase I, but also have a tendency to misdivide at the centromere (Lukaszewski 2010). Such centromere misdivision gives rise to centric translocation, production of telocentric and isochromosomes (Kaszas et al. 2002; Lukaszewski 2010).

The occurrence of telocentrics and isochromosomes has been reported previously in *Lilium* species (Brandram 1967). They have been called accessory chromosomes. Whether they behave similar to B chromosomes from other species is not known. Because the origin of Bs has been considered as a 'mystery', it might be worthwhile to investigate the origin of these small aberrant chromosomes as the ones observed in this study in more detail. One instance in which the mode of origin of a B chromosome has been investigated is in *Plantago lagopus* which involves the formation of a minichromosome, amplification of 5S rDNA, stabilization of telomeric repeats and formation of an isochromosome (Dhar et al. 2000; Jones et al. 2008b). Compared to this mode of origin, the formation of isochromosomes from the short arms following misdivision of the centromere, as described in this investigation, is a more simple mechanism for the potential origin of Bs.

The presence of rDNA repeats in two cases deserves a comment. In more than 30 plant species the presence of rDNA sequences on Bs has been recorded (Dhar et al. 2002; Donald et al. 1995; Flavell and Rimpau 1975; Friebe et al. 1995; Jones 1995; Maluszynska and Schweizer 1989). A good example resembling the aberrant chromosome in genotype 074051-9 is the B chromosome found in *Allium cernuum*. Using Ag-NOR banding, the B chromosome was found to be median and possessed rDNA sites with nucleoral activity on both arms (Friebe 1989). Furthermore, there is information suggesting that NOR regions are prone to chromosome breakage and this may provide a mechanism behind the appearance of B chromosome following interspecific hybridization (Beukeboom 1994; Camacho et al. 2000; Jones and Houben 2003). It is not known whether rDNA sites of As are more vulnerable for breakage compared to other chromosome regions. It may be pointed out that such breakage may not result in a chromosome fragment that can survive on its own, a centromere is absolutely necessary. For this reason, it might be logical to assume that a chromosome arm that possesses a secondary constriction or nucleolus organizer, is probably more susceptible for breakage, or centromere misdivision. In tomato, the origin of an isochromosome of the short arm of chromosome 2 (2S) is instructive in this connection. Moens (1965) reported the occurrence of an isochromosome of 2S in *Lycopersicon esculentum* which had resulted from the misdivision of the centromere in a trisomic of chromosome 2. In addition to being heterochromatic, 2S also carries the nucleolus organizer. Although this isochromosome possessed a functional centromere, telomeres in addition to nucleolus organiser, it was not stable morphologically (Quiros 1976) but was transmitted to the progenies, accumulating as many as eight copies in some of the progenies. In a later study, the isochromosomes of 2S were shown to be highly unstable due to breakage-fusion-bridge cycle (Ramanna et al. 1985).

It has not, however, been established that the instability is due to the presence of rDNA sites on both arms of the isochromosome but it does provide an instance of instability in such newly produced chromosomes. In *Lilium* species, there are many 5S and 45S rDNA sites and some of them are in proximal positions (Lim et al. 2001b). One example is the 45S rDNA site on the short arm of chromosome 4 of Longiflorum. The isochromosome in the genotype, 074051-9 which showed a stable mitosis, might have originated from the short arm of chromosome 4 of Longiflorum. A critical further investigation of the behaviour of the newly arisen iso-chromosomes reported in this investigation might shed light on the probable modes of origins of B chromosomes.

Chapter 6

General discussion

The results presented in this thesis mainly focus on the analysis of chromosome behaviour in lily hybrids, including interspecific F1 hybrids as well as backcross progenies, using molecular cytogenetic techniques. It has been found that 1) there were no chromosome rearrangements in neopolyploids of *Lilium* hybrids (Chapter 2); 2) the intergenomic recombination, which has been found in sexual polyploidized backcross progenies, originated from chiasmata formation and crossing over during meiosis (Chapter 2 and 3); 3) meiotic abnormalities, such as non-homologous chromosome pairing involved in multivalents and (few) bivalents, were due to the existence of a reciprocal translocation in the paternal parent 'Connecticut King'; 4) chromosome breakage and anaphase bridging were found to be the cause of chromosome structure variation (Chapter 4); 4) isochromosomes were produced due to the irregularity of meiosis in the interspecific hybrids of lily (Chapter 5). Such results do not only contribute to fundamental research in allopolyploid evolution and speciation, but can also benefit plant breeding by solving problems in genetic mapping. In this Chapter, some topics namely:

1) Interspecific hybrids of lily: a model for molecular cytogenetic research
2) Chromosome rearrangements and its relevance to genetic mapping
3) Sexual polyploidization and its significance in polyploidy mapping
4) Meiotic abnormalities in lily interspecific hybrids
5) Crossing over and introgression breeding
6) Genomic shock, isochromosome formation and B chromosome origin during sexual polyploidization

will be discussed and future perspectives will be presented to draw more attention to the theoretical and practical aspects of homoeologous chromosome interaction.

Interspecific hybrids of lily: a model for molecular cytogenetic research

Conventional diploid lily cultivars are being replaced by recently produced polyploidy cultivars. The genus *Lilium* consists of about 80 species and has been classified into 7 botanical sections (Comber 1949; De Jong 1974). A noticeable feature is that interspecific crosses within each section are relatively easy and the resultant hybrids are generally fertile, while crosses between species from different sections are difficult because of the existence of pre- and post- fertilization barriers (Van Tuyl and Lim 2003). As a result, a number of hybrid groups which show distinct morphological characteristics have been bred through conventional crossing methods (McRae 1998). However, neopolyploids, derived from interspecific (between sections) hybridization and polyploidization, are playing a prominent role in lily breeding with the aim of combining desirable traits of different hybrid groups (Van Tuyl and Lim 2003). With the advance of technology, barriers of interspecific hybridization

have been overcome by using cut-style pollination and embryo rescue methods (Van Tuyl et al. 1991), while the hybrid sterility can also be restored by using mitotic and meiotic polyploidization (Ramanna and Jacobsen 2003; Van Tuyl and Lim 2003). This is also the reason that polyploid cultivars are becoming increasingly popular and most of the new registered cultivars are derived from interspecific hybridization between different hybrid groups.

The allopolyploid and interspecific hybrids of lily offer an excellent model for molecular cytogenetic research. Besides the large size of chromosomes, the divergent genomes in different hybrid groups, which is ideal for studying homoeologous genome interaction in interspecific hybrids and backcross progenies on the chromosome level facilitate the utilization of DNA *in situ* hybridization (GISH and FISH). Numerical examples have showed that the genomes of Longiflorum, Asiatic, Longiflorum, Oriental and Trumpet can be well distinguished simultaneously by GISH (Barba-Gonzalez et al. 2006a; Xie et al. 2010; Zhou et al. 2008b). Through an effort of more than 25 years in our group (Plant Breeding, Wageningen Univerisity), lily hybrids have been used to clarify several cytogenetic mechanisms. The first one is the reduced fertility in interspecific hybrids. The association failure at meiosis has been proven to be the main reason for the fertility reduction (Asano 1982; Lim et al. 2001a). The second one is the meiotic restitution mechanisms with relevance to the production of unreduced gametes. Through observations of pollen mother cells of F1 hybrids and analysis of genomic composition in backcross progenies, FDR has been proven to be the main mechanism that contributes to viable unreduced gametes in interspecific hybrids of lily (Barba-Gonzalez et al. 2006a; Lim et al. 2001a). In addition, a novel restitution mechanism -indeterminate meiotic restitution (IMR)- has also been identified (Lim et al. 2001a). The third one is the occurrence of chromosome rearrangements in neopolyploids. Other than translocation, the extensive inter-genomic exchanges existing in newly synthesized allopolyploids of lily have been shown to be derived from chiasmata formation and crossing over events, through meiotic and mitotic analysis (Xie et al. 2010). The last one to be mentioned is the origin of anaphase I bridging during meiosis of interspecific F1 hybrids. During meiosis of interspecific hybrids of lily, broken chromosomes at metaphase I, two types of bridges involving sister and non-sister chromatids as well as a putative ring chromosome have suggested that these bridges and fragments were the results of spontaneous chromosome breakage and fusion (U-type exchanges)(Chapter 4). In conclusion, the interaction of homoeologous chromosomes in interspecific hybrids of lily can be well studied using GISH and FISH, and gives more information to the allopolyploid origin, sexual polyploidization, chromosome structure variation and speciation in nature. In contrast, genomes in other crop hybrids (Tulip) are either too close to each other (homologous genomes), which makes it difficult to distinguish by GISH, or too distantly related which makes it non-homologous (wheat). Moreover, chromosomes in some genera are too small to be critically observed both in mitosis and meiosis (*Brassica*). Hence, interspecific hybrids of lily have become an ideal

model for molecular cytogenetic research when studying the interaction of homoeologous genomes in interspecific hybrids and allopolyploids. However, it should also be noticed that since the large genome, large probes (>2Kb) need to be used to get clear signals when analyzing lily with FISH.

Chromosome rearrangements and its relevance to genetic mapping

In genetic mapping, normally two crossing parents are involved to produce a segregating population. These crossing materials, although related, should produce enough detectable sequence polymorphism throughout the genome. These populations, however, might give complicated maps because of parental chromosome structural differences (Chapter 3), which is discussed in the following paragraph.

Changes in chromosome composition have been considered as a cause of ambiguities in genetic mapping with molecular markers. Such changes consists of translocations, deletions, duplications and inversions. Each of these events involves breakage of DNA double helices in the genome at two different locations, followed by a reunion of the broken ends to produce a new chromosomal arrangement of genes, and causes gene order variation compared to the original order. These alterations of gene order will have certain consequences in genetic mapping when parents with chromosome rearrangements are involved in crossing to generate segregating populations. In general, structure variations cause reduced fertility in gametes, which lead to skewed populations (Fig. 6.1; Fig. 6.2). Different types of chromosome rearrangements give rise to various mapping problems. First, inversions, both pericentric and paracentric, lead to suppressed recombination between the inverted and non-inverted genomic regions (Loren H 2001; Noor et al. 2001; Rieseberg 2001a; Schaeffer and Anderson 2005). Molecular mapping studies have highlighted that loci within inversions can be in strong linkage disequilibrium with each other for two reasons: a) chromosome pairing of the inverted region is commonly hampered and an inversion loop is formed when the size of the inverted segment is not big enough. b) even if inverted segments paired together and a single crossover happened in the inverted region, pericentric inversions would produce sterile gametes with duplication and deletion while paracentric inversions give rise to anaphase bridging, which would also result into unviable gametes (Fig. 6.1). Second, reciprocal translocations give pseudolinkage when progenies result from material with a reciprocal translocation is used for genetic mapping. During meiosis of a plant with a reciprocal translocation, quadrivalents are normally formed at metaphase I. Chiasma formation and crossing over will be suppressed in the interstitial area (between centromere and translocation breakpoints) because such exchanges between non-sister chromatids will lead to gametes with duplication and deletion (Fig. 6.2, see unviable gametes from alternate segregation).
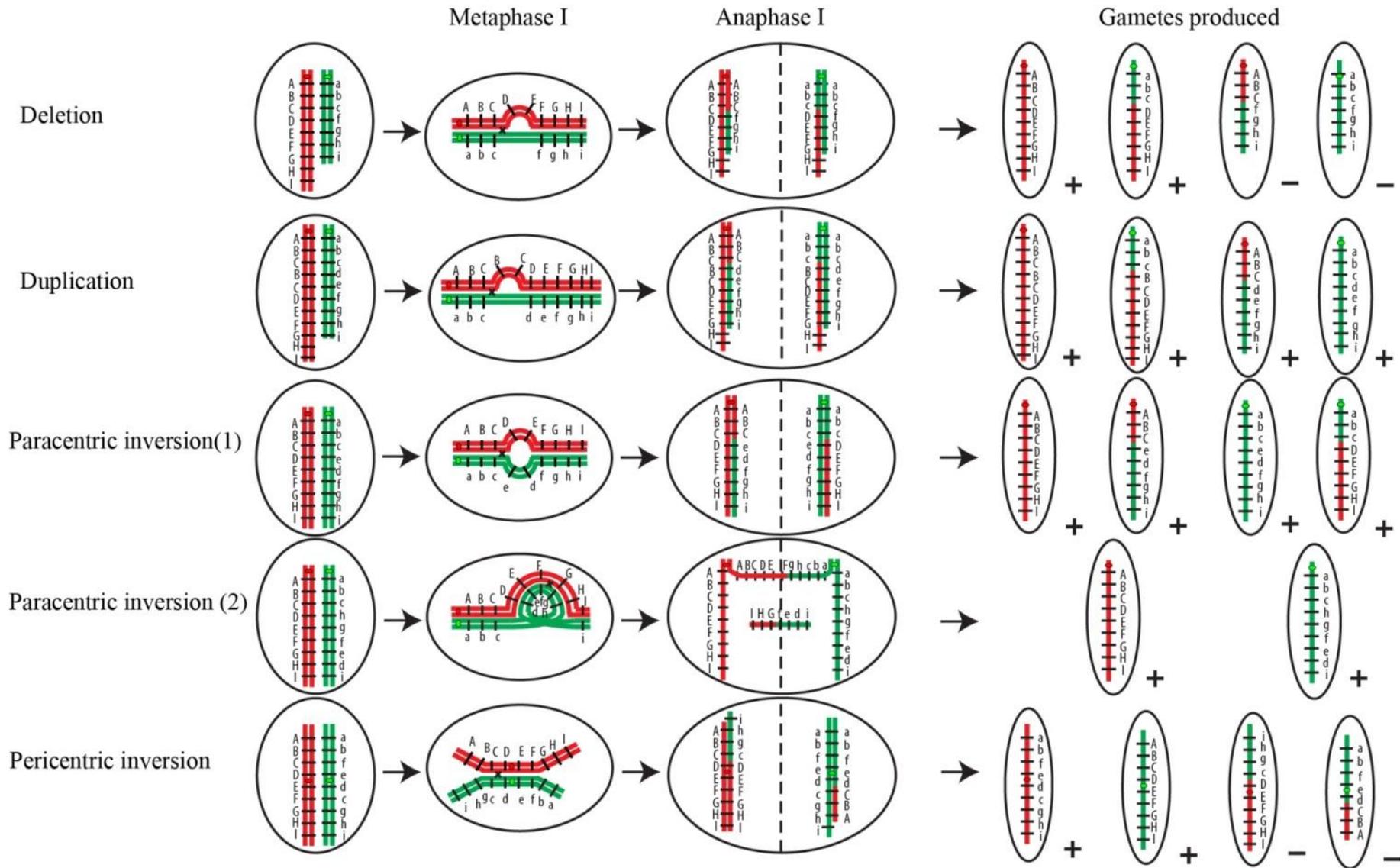
Fig. 6.1. The meiosis process of a chromosome with a deletion, a duplication and an inversion and their relevance to genetic mapping. Note: "+" stands for gametes that are viable, "-" stands for gametes that are unviable.
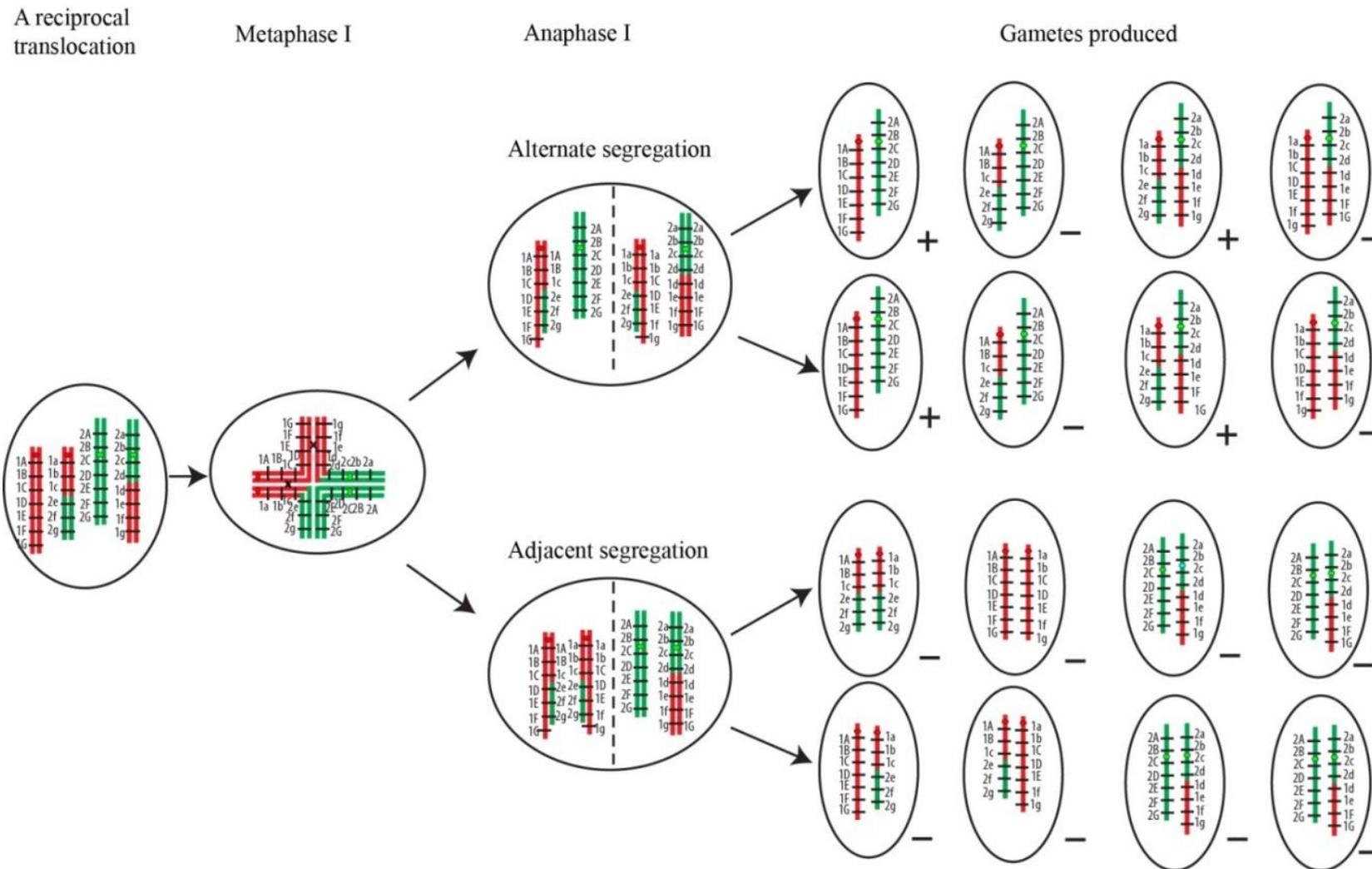
Fig. 6.2. The meiosis process of chromosomes with a reciprocal translocation and their relevance to genetic mapping. Gametes from adjacent segregation of a quadrivalent from a reciprocal translocation are generally unviable, while gametes from alternate segregation are balanced and viable if crossovers happened in the translocated chromosome segments, as well as the other chromosome arms. Any single crossover between non-sister chromatids in the interstitial area will lead to the production of duplication-deficiency gametes. Note: "+" stands for gametes that are viable, "-" stands for gametes that are unviable.

Two translocated chromosomes usually form 'pseudolinkage' (Albrecht and Chetelat 2009; Farré et al. 2010; Kamphuis et al. 2007). Meanwhile, since normal and translocated segments lead to reduced crossover interference, distance between markers on normal and translocated chromosome fragments will be wrongly estimated, marker order is ambiguous along the merged linkage groups and higher stringencies (increase the LODs) do not result in a division into two balanced chromosomes (Albrecht and Chetelat 2009). Third, the existence of duplication leads to erroneous location of markers in the linkage group associated with the chromosome with duplication (Fig. 6.1). Furthermore, chromosomes with two or more types of structure variation make the genetic maps even more complicated. In conclusion, chromosome rearrangements not only cause reduced fertility, but also lead to errors when estimating genetic distances between markers.

Sexual polyploidization and its significance in polyploidy mapping

When interspecific crosses are made between distantly related species, the resulting hybrids are generally sterile. This hybrid sterility is explained to be due to the failure of chromosome association and the forthcoming error-disjoining during meiosis because of the parental divergence (Asano 1982). However, there is still a wide genetic variation, with some individuals possessing a low fertility. These outstanding genotypes normally produce unreduced (2n) gametes, as well as fewer n gametes (Ramanna and Jacobsen 2003). The process of restoring fertility through unreduced gametes is termed as sexual (meiotic) polyploidization, as a comparison with asexual (mitotic) polyploidization. The production of unreduced gametes has been reported in many interspecific hybrids, such as *Lilium* (Van Tuyl et al. 1989), *Alstroemeria* (Kamstra et al. 1999), *Allium* (Khrustaleva and Kik 1998) and others. One of the main advantages of sexual polyploidization, compared with its counterpart, is the occurrence of intergenomic recombination during the production of unreduced gametes, which will lead to segregation and diversity in the next generation (Ramanna and Jacobsen 2003). The segregation in the resulting population provides a possibility for genetic mapping.

When detecting intergenomic alteration in sexual polyploidized allopolyploids, molecular cytogenetic techniques (GISH and FISH) are more powerful compared with molecular markers. The detecting efficiency of the two methods with respect to unreduced (2n) gametes is compared in Fig. 6.3. GISH can simultaneously detect intergenomic recombination, characterize the crossing over events and trace the origin of non-sister chromatid exchanges when combined with meiosis observation. However, molecular markers with multi-locus analysis in crossing progenies cannot detect reciprocal crossing over, and quantification of allele number by the intensity of bands is not always accurate (Gaeta and Pires 2010; Nicolas et al. 2007). For example, in four types of segregated products between homoeologous chromosomes with a single or two strand double crossover during FDR meiosis (Fig. 6.3), GISH can detect all the intergenomic recombinations, whereas molecular markers can only detect two types of them. In the recombinant chromosomes derived from different crossing

over events during FDR meiosis in the present study, only half of them can be detected by molecular markers (Fig. 6.3), which significantly underestimates the real occurrence of crossover. For SDR originating allopolyploids, underestimation will also occur in case of recombination in a three strand double crossing over (Fig. 6.3). In conclusion, progeny analysis of genetic mapping in polyploids resulted from unreduced gametes will considerably underestimate the real crossing over events during meiosis.

Similarly, underestimation of crossing over events also takes place in autopolyploid genetic mapping. Currently, most polyploidy mapping is based on disomic inheritance (1:1 and 3:1 segregation) and maps were based on the scoring of allele number and/or dosage using dominant markers (Luo et al. 2001; Zhang et al. 2006), while trisomic inheritance (trivalent formation during meiosis), tetrasomic inheritance (quadrivalent formation during meiosis), as well as intermediate inheritance were ignored. However, multivalent formation, like trivalents and quadrivalents, is quite a normal phenomenon in polyploids, especially in autopolyploids (Kamiri et al. 2011; Stift et al. 2008). At anaphase I, segregants including two or more chromosomes resemble random segregation in FDR-like meiosis in the second meiotic division, and crossing over between non-sister chromatids in the same segregant also form reciprocal and non-reciprocal products. Recombinant sites can be detected in the former, on the contrary, the latter cannot be detected, which will lead to the underestimation of crossing over and errors in locating the exact positions of markers in the linkage groups (Fig. 6.3). Since reciprocal recombination is impossible to be detected with molecular markers, polyploidy with the formation of multivalent should be avoided when generating a mapping population. Hence, analytic breeding is proposed and genetic mapping can be done at diploid level and the ploidy level can be raised by mitotic or meiotic polyploidization.
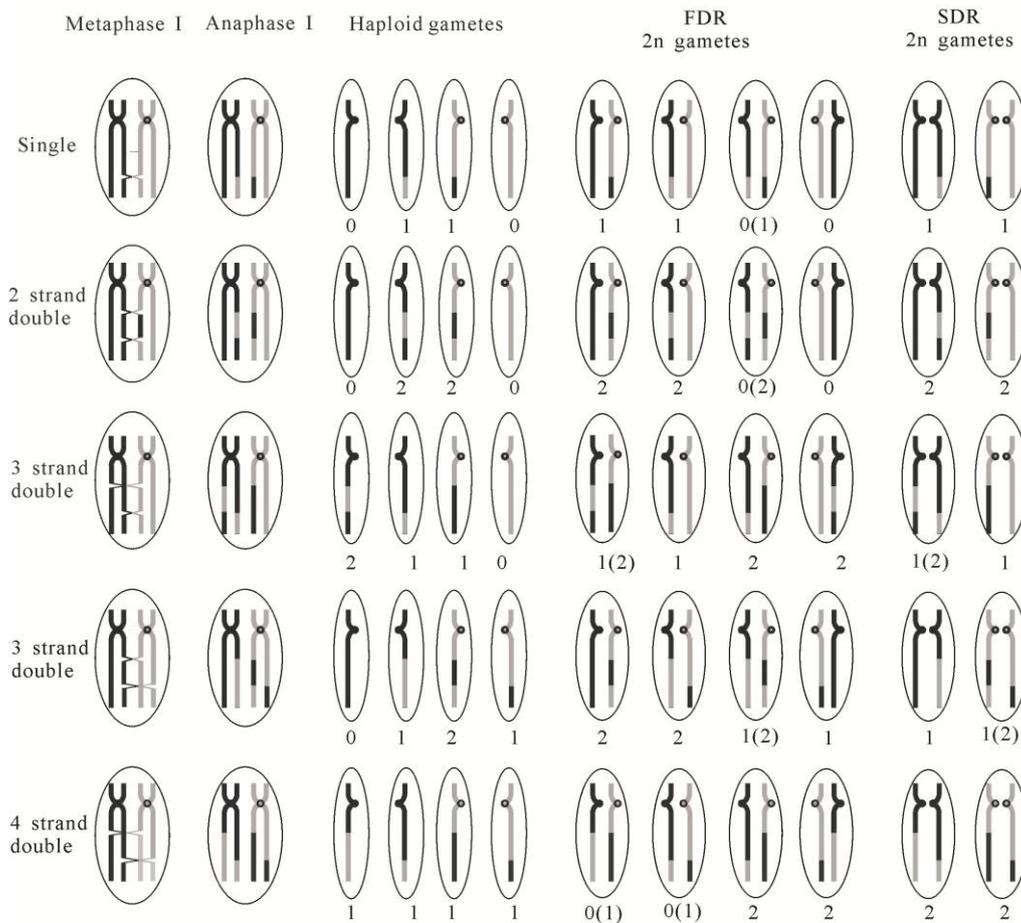
Fig. 6.3. A comparison of detecting efficiency of crossing over events in the unreduced gametes resulted from a FDR or SDR meiosis. Numbers stand for recombination sites detected by molecular markers. In some cases, GISH is more accurate when detecting reciprocal products (in brackets)

Meiotic abnormalities in lily hybrids

Different types of meiotic irregularities has been found during meiosis of interspecific hybrids of lily. Homoeologous chromosome pairing as well as univalents is one of the main features at metaphase I during meiosis of lily hybrids. Bivalent numbers ranging from 0 to 12 as well as univalents are present. These bivalents predominantly involve homoeologous chromosome pairing, while the univalents are chromosomes with failed association (Chapter 3; Barba-Gonzalez et al. 2005a; Lim et al. 2001a; Zhou et al. 2008a). Except homoeologous bivalents and univalents, abnormal pairing is also observed in some of the genotypes of lily hybrids (Chapter 3). Multivalents and non-homologous bivalents have been found in two of LA hybrids, and it has been proven that a reciprocal translocation exists in the paternal parent 'Connecticut King' (Chapter 3). Another abnormality is the chromosome disjoining at anaphase stage in the first division. Bivalents divided into two half-bivalents normally, whereas two chromatids of a univalent segregated and moved to different poles (Chapter 3; Lim et al. 2001a; Zhou et al. 2008a). Chromosome breakage also contributes to the irregularity of meiosis. At both metaphase and anaphase I stages, broken chromosomes have

been detected with GISH and FISH, and later on a U-type reunion led to the formation of anaphase bridges and fragments (Chapter 4). Finally, microspores with different chromosome numbers have also been detected after meiosis (Zhou et al. 2008a). In conclusion, intersectional lily hybrids show a range of abnormalities during different stages of meiosis.

Some other kinds of meiotic abnormalities in interspecific lily hybrids have also been reflected and emphasized by progeny analysis. The first evidence is the polyploidized backcross progenies. The resultant progenies from crosses involving interspecific lily hybrids were predominant triploids, indicating the functional gametes were unreduced gametes and the mechanism has been identified as first division restitution (FDR) and indeterminate meiotic restitution (IMR) (Lim et al. 2001a). The second feature in backcross progenies of lily is aneuploidy. When analyzing the genomic composition of these triploid lily hybrids, a small proportion of aneuploids has been found. The last character of the backcross progeny is the presence of isochromosomes. In a few genotypes, resulting from some interspecific hybrids of LA lilies, isochromosomes with different sizes were detected, and these newly-generated small aberrant chromosomes were derived from the fusion of the two short arms of the missing chromosomes during meiosis, respectively (Chapter 5).

Crossing over and introgression breeding

The role of crossing over during evolution and speciation has long been realized and studied in flowering plants. Crossing over, which is one of the key features that distinguish meiosis from mitosis, not only facilitates the proper segregation of homologous chromosome in the first meiotic division, but also generates novel combinations of alleles via homologous chromosome exchanges. This process, in addition to maintaining the ploidy level during sexual reproduction, contributes to genetic diversity, which is essential for introgression breeding.

Crossing over between homoeologous chromatids has been proven to be less frequent as compared with crossing over between homologous non-sister chromatids. In monosomic additions of tomato, a homologous bivalent (II) together with a univalent was the main meiotic configuration, GISH has revealed that the number of rod bivalents (stands for single crossing over) was much higher compared with that of ring bivalents (stands for other types of crossing over which probably lead to chromosomes with two or more recombinant sites), indicating single crossing over was the predominant type of exchange between homologous chromosomes. While in the substitution line of tomato SL-8, reduction of homoeologous recombination has been revealed by the considerable decrease of ring bivalent formation (Ji and Chetelat 2003). Similarly, results from several studies of homeologous recombination between chromosomes of wheat and related species have showed the absence of multiple crossovers (Dubcovsky et al. 1995; Lukaszewski 1995, 2000; Luo et al. 1996; Luo et al. 2000).

Homoeologous crossing over has been proven to occur with different frequencies in different species hybrids. Although different types of crossing over events have been checked

during meiosis in the interspecific hybrids of lily (Chapter 3), the number of chromosomes in the half-bivalents with two or more recombinant sites is low, compared with those with one recombinant site. Since different crossover events have certain segregation patterns (such as single crossover produces two recombinant chromosomes each with one recombinant site, with the exception of multiple crossover), the 637 pairs of half-bivalents in pollen mother cells in Chapter 3 showed 1191 recombinant chromosomes in total. 1102 chromosomes, which occupied 92.5%, possessed one recombinant site and 89 chromosomes (7.5%) with two recombinant sites. Although chromosomes with more than two recombinant sites did occur during meiosis, the frequency is relatively low compared with other species hybrids. In polyploid cotton (*Gossypium*), the frequency of intergenomic recombination events possessed one, two, three or more recombinant sites were 70.3%, 20.6% and 9.1% respectively (Salmon et al. 2010). Similarly, in an alien substitution line of tomato, in which chromosome with two breakpoints took up around 15% of the total recombinant chromosomes (Tam et al. 2011), the percentage of chromosomes with more than 1 recombinant site in lily is considerably low. There are three potential reasons for the low frequency of chromosomes with two or more recombinant sites in lily hybrids: 1) the genomes of the lily parents are more divergent compared those in cotton and as a result, complicated crossing overs with multiple recombinant sites on each chromosome are suppressed; 2) gene conversion, which usually gives rise to two or more recombinant sites in genetic mapping and can be detected by mRNA sequencing, occurs frequently in cotton; or 3) since the limited resolution of GISH, such gene conversions or small introgressed chromosome segments cannot be detected by molecular cytogenetic methods, which gives an underestimation of recombinant sites on chromosomes.

Genomic shock, isochromosome formation and B chromosome origin during sexual polyploidization

Genomes facing stress will suffer genomic shock which, on a chromosomal level, leads to structure remodeling (McClintock 1984). All kinds of structure remodeling (structure variation) experience a process that involves double strand breaks (DSBs, chromosome breakage in cytogenetics) and error-reunions. DSBs can happen at centromere (centric fission), in interstitial or terminal regions on a chromosome, and error-reunion of broken chromosomes give rise to the production of structure variation. A simple example is that chromosome breakage followed by the fusion of broken arms from different chromosomes leads to the generation of so-called Robertsonian translocation in humans (Perry et al. 2004). In view of this, genomic shock is the driver of chromosome breakage, which causes erroneous repair in plants. It is not surprising that interspecific hybridization leads to spontaneous chromosome breakage, which has been detected in Chapter 4. As a second step, error-reunion leads to various types of chromosome rearrangements, including chromosomal inversions, deletions, translocations, and duplication (Britt 1999).

Another feature caused by interspecific hybridization is the occurrence of univalents during meiosis in interspecific hybrids. Univalents, which arose from association failure, have been found in many interspecific hybrids, and are considered to be the main reason of the reduced fertility (Asano 1982; Lee et al. 2011; Lukaszewski 2010; Sears 1950). Meiotic univalents not only randomly move to one pole when segregating at anaphase I, but also have a tendency to misdivide at the centromere (Lukaszewski 2010). Centromere misdivision gives rise to centric translocation, production of telocentric and isochromosomes, which have been found in maize and wheat (Kaszas et al. 2002; Lukaszewski 2010). Chapter 5 reported the production of isochromosomes, which were derived from centric fission and fusion during meiosis of the maternal parent. Meanwhile, chromosome breakage has been found not only in univalents, but also in bivalents (Chapter 4). As a result, it is still not known whether the newly-generated isochromosomes in the backcross progenies are the result of centric breakage and fusion from either an univalent or a bivalent.

B chromosomes, which extensively exist in many flowering plants, are probably derived from aberrant chromosomes. It is already well accepted that B chromosomes originate from meiotic errors in which interspecific hybridization provides an ideal platform, and this type of chromosomes are deduced to be escaped from standard chromosomes (Jones and Houben 2003). However, what should be noticed is that the origin of B chromosomes is not a one-step process, which has been shown by Dhar et al. (Dhar et al. 2002) in *Plantago*. Combined with the fact that most of the species are involved in at least one round polyploidization, it can be concluded that B chromosomes arose in the process of speciation of polyploids in interspecific hybrids, which has been shown by the production of small aberrant chromosomes during sexual polyploidization of lily hybrids.

Conclusions and future perspectives

As presented in this thesis, it has been shown that not only intergenomic recombination which is derived from crossovers, but also chromosome rearrangements causes genetic variation in backcross progenies of lily. Moreover, chromosome breakage and fusion lead to the production of chromosome bridges at anaphase I stage during meiosis and the generation of small aberrant chromosomes in the backcross progenies. However, to apply these results in practical breeding, the following research should also be done in the future:

Although crossover events have been studied in this thesis, it is necessary to study it on the level of individual chromosomes. Since recombination sites on different chromosomes are highly uneven (Khan et al. 2009a), it is a precondition to make an accurate identification of individual chromosomes. Traditional methods to identify chromosomes are based on chromosome length, arm length, arm length ratio and so on, which makes it difficult to recognize chromosomes with short arms since most lily chromosomes are morphological similar (Noda 1978; Stewart 1947). Later on, a few efforts were made to distinguish chromosomes with different banding techniques (Smyth et al. 1989; Von Kalm and Smyth

1980) and FISH with different repetitive probes, but no substantial progress has been made (Lim et al. 2001b; Sultana et al. 2011; Sultana et al. 2010). FISH with 45s and 5s can only recognize a few chromosomes and short single copy probes (up to 2kb) or microsatellite motif probes were unsuccessful in lily because no signals could be detected (unpublished results). In view of this, it is necessary to develop techniques, such as bacteria artificial chromosomes (BACs) with repetitive sequences, to identify individual chromosomes in lily.

Manipulation of crossovers in other plants has provided a promising way for lily breeding. Due to its significance, crossovers have been studied in model organisms, such as yeast (*Saccharomyces*), in detail and researchers are trying to control meiotic recombination (Phadnis et al. 2011). As mentioned before, the frequency of intergenomic recombination in lily hybrids is relatively low, as compared with other crops. Increase of crossovers though control of double strand breaks and the repair can cause more intergenomic recombination and hence, speed up the introgression breeding.

The occurrence of small aberrant chromosomes in lily hybrids opens a new window for lily breeding. As mentioned in Chapter 5, the new generated isochromosomes are the fusion of the short arms from the missing chromosomes. Because of the structure variation of chromosomes, phenotypic variation caused by such chromosomes can be expected in the progenies and the function of these isochromosomes can be studied in the future. The dosage effect of the genes on the isochromosomes (duplicated arms) has a potential to create breeding materials with outstanding phenotypes. Moreover, these genotypes are quite unique because isochromosomes are only present in a few backcross progenies from a certain interspecific LA hybrid. Beside the length relationship between isochromosomes and the missing chromosomes, rDNA sites are also present on the isochromosome in one of the three genotypes. If the isochromosomes are stable during the meiosis and can pass to next generations, they have a potential to be used as markers for selection in breeding, cultivar identification and protection of breeder's right in the future.

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Summary

Lily (*Lilium*) has become one of the top bulbous crops for the cut flower industry in the past two decades. The genus *Lilium* comprises of approximately 80 species, which have been classified into seven sections. Each section possesses distinctive phenotypic characters, such as flower color, flower shape and resistances to diseases and pests. Crosses between species in the same section are relatively easy and the resulting hybrids are in general fertile, while interspecific crosses between species from different sections are rather difficult and the resulting hybrids are in general sterile. As a result, different hybrid groups have been bred in the 20th century. Within these different hybrid groups, Longiflorum (L), Asiatic (A) and Oriental (O), which are derived from the section Leucolirion, Sinomartagon and Archelirion respectively, are of commercial importance and hence, are the most widely cultivated lilies worldwide.

Lily hybrids provide an ideal model for molecular cytogenetic research. With the development of techniques of overcoming pre- and post- crossing barriers of interspecific crosses, as well as the application of asexual and sexual polyploidization to restore the fertility of F1 lily hybrids, combining of desirable traits from different hybrid groups has become feasible. As a result, interspecific hybridization and polyploidization have been widely used in the breeding of new cultivars of lily. These cultivars, as well as other breeding materials from interspecific crosses, facilitate the application of molecular cytogenetic analysis due to three reasons: 1) the chromosomes of lily are large enough for cytological observations; 2) genomes of different hybrid groups are homoeologous; and 3) these homoeologous genomes can be simultaneously distinguished by DNA in situ hybridization. Using these lily hybrids combined with genomic in situ hybridization (GISH) and florescence in situ hybridization (FISH), the interaction of homoeologous genomes can be studied though meiotic observation of the F1 hybrids. Meanwhile, chromosome sequential variation with relevance to crossover and chromosome rearrangements can also be observed.

For this purpose, interspecific crosses between the *Lilium longiflorum* cultivar 'White Fox' and the Asiatic cultivar 'Connecticut King' were made, and some of these F1 hybrids, which show a relatively high fertility with the production of unreduced gametes, were backcrossed with an Asiatic cultivar . The meiosis of the interspecific hybrids, as well as the sexual polyploidized progenies, were analysed by GISH and FISH. In addition, one population of sexual polyploidized AOA hybrids was also analysed for the genome composition. Results showed that there was no evidence that lily allopolyploids possess any noticeable

Summary

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chromosome rearrangements. The equal segregation of reciprocal and non-reciprocal recombinant product showed that the intergenomic recombination in the sexual polyploidized progenies was indeed from a natural process-chiasmata formation and crossovers and hence, should not be considered as translocations as was suggested in literature for intergenomic recombination. This conclusion was further confirmed by meiotic observation of the interspecific F1 hybrids.

Detailed meiotic observations were carried out in interspecific hybrids between Longiflorum × Asiatic groups of lilies (*Lilium*) which were used as parents to generate sexual polyploids with intergenomic recombination. Bivalents involving two homoeologous chromosomes, as well as unpaired univalents were the main configurations at metaphase I. However, in two genotypes, multivalents and bivalents both involving non-homologous pairing of two Asiatic chromosomes were observed. This indicated the presence of a duplication which was common to two non-homologous chromosomes in the hybrids. It is deduced that there was a reciprocal translocation in the Asiatic parent cv. 'Connecticut King' and these two genotypes resulted from duplication-deficiency gametes. Results from Anaphase I showed that chiasma formation involving non-sister chromatids gave rise to two strand single, two strand double, three strand double, four strand double and multiple exchanges. It is also noticeable that there was a high frequency of multiple crossovers in the genotypes with duplication, indicating a reduced crossover interference in multivalents. Beside the normal crossovers, also chromosome bridges at anaphase I of meiosis were observed. GISH and FISH painting showed that these bridges involve not only non-sister chromatids but also sister-chromatids. The bridges, without any differentiation along their length, were always accompanied by fragments with a variable size. These results indicated that the bridges, together with the accompanying fragments, were derived from U-type exchanges. Other than homologous recombination (HR), nonhomologous end joining (NHEJ) probably led to the production of bridges when repairing the double strand breaks (DSBs) during meiosis.

Progenies from unilateral polyploidization of crosses between LA hybrids and Asiatic cultivars were predominant triploids. However, three exceptional plants, which possessed 35 normal chromosomes and a small aberrant chromosome instead of the expected normal number of 36, were observed. In all three cases the small aberrant chromosomes were isochromosomes which had obviously originated during the first backcross generation, and the length of the arms of these aberrant chromosomes were always related with the length of

the short arm of the missing chromosome. Furthermore, one of these three chromosomes possessed 45S rDNA hybridization sites in the proximal positions, which resembles the short arm of the missing chromosome (chromosome 4 of L genome). Combined with the results of chromosome breakage during meiosis, centric breakage and fusion is a putative mechanism of the production of these isochromosomes. Meanwhile, two small, supernumerary or B chromosomes were detected as extra chromosomes in a tetraploid plant derived from chromosome doubling of an intersectional hybrid (2n=2x=24) between a cultivar of the Longiflorum (L) and the Trumpet (T) group. When this tetraploid LLTT hybrid was crossed with a triploid LLO hybrid (O=Oriental), the B chromosome was transmitted to 73.4% of the progenies. Based on GISH and FISH characterization it was shown that the B chromosome found consisted of two identical arms, with 5S rDNA hybridizing to the majority of it, which were flanked by normal telomeres, suggesting that this is an isochromosome.

The results of current investigations are of practical implication for a number of reasons. Firstly, the behavior of homoeologous chromosomes during meiotic processes in lily hybrids was studied in detail, and it can be used to explain the profound genetic changes in the early generations during hybrid speciation. Secondly, some problems that go unnoticed in genetic mapping can be predicted and well explained by the occurrence of chromosome rearrangements in the parents which are used to produce the segregation population and thirdly, the discovery of U-type exchanges during meiosis and de novo isochromosomes in the backcross progenies supplies an alternative mechanism for the origin of B chromosomes.

Samenvatting

Lelie (*Lilium*) is in de afgelopen twee decennia één van de belangrijkste bolgewassen geworden voor de snijbloemen sector. Het genus *Lilium* bestaat uit ongeveer 80 soorten die in zeven secties zijn onderverdeeld. De secties onderscheiden zich in fenotypische eigenschappen zoals bloemkleur, bloemvorm en resistentie tegen ziektes en plagen. Terwijl kruisingen binnen dezelfde sectie relatief gemakkelijk zijn en de resulterende hybriden fertiel, zijn interspecifieke kruisingen tussen soorten uit verschillende secties niet eenvoudig en zijn de resulterende hybriden vaak steriel. Ten gevolge hiervan zijn in de 20^e eeuw verschillende hybride groepen ontstaan. Deze hybride groepen zijn Longiflorums (L), Aziaten (A) en Orientals (O) welke zijn ontstaan uit respectievelijk de secties Leucolirion, Sinomartagon en Archelirion. Dit zijn wereldwijd de meest geteelde en geproduceerde lelies.

Lelie hybriden zijn een ideaal modelsysteem voor moleculair cytogenetisch onderzoek. Door de ontwikkeling van technieken om pre- en post-fertilisatie barrières bij interspecifieke kruisingen te overbruggen en de toepassing van aseksuele en seksuele polyploïdisatie om de fertiliteit van F1 lelie hybriden te herstellen, is het mogelijk geworden om gunstige eigenschappen van verschillende hybride groepen te combineren. Hierdoor is interspecifieke hybridisatie en polyploïdisatie breed toepasbaar geworden in de veredeling van nieuwe lelie cultivars.

Deze cultivars en ander veredelingsmateriaal uit interspecifieke kruisingen faciliteren de toepassing van moleculair cytogenetisch onderzoek om drie redenen: 1) lelie chromosomen zijn groot genoeg voor cytogenetische observaties; 2) de genomen van verschillende hybride groepen zijn homoeoloog; en 3) deze homoeologe genomen kunnen worden onderscheiden door DNA in situ hybridisatie. Door gebruik van deze lelie hybriden in combinatie met genomische *in situ* hybridisatie (GISH) en fluorescentie *in situ* hybridisatie (FISH), kunnen de interacties tussen de homoeologe genomen worden bestudeerd tijdens de meiose van de F1 hybriden. Tegelijkertijd, kan chromosoom variatie in relatie tot overkruisingen en chromosoom reorganisaties worden waargenomen .

Voor dit doel zijn interspecifieke kruisingen tussen *Lilium longiflorum* cultivar 'White Fox' en de Aziatische cultivar 'Connecticut King' gemaakt waarvan sommige F1 hybriden, die een relatief hoge fertiliteit hebben in de productie van ongereduceerde gameten, werden teruggekruist met een Aziatische cultivar. De meiose van de interspecifieke hybriden en hun seksueel gepolyploïdiseerde nakomelingen zijn vervolgens geanalyseerd met GISH en FISH.

Daarnaast is ook de genoom samenstelling van een populatie van seksueel gepolyploïdiseerde AOA hybriden geanalyseerd. Resultaten laten zien dat er in allopolyploïde lelies geen aanwijzingen zijn voor chromosoom translocaties. De gelijke uitsplitsing van reciproke en niet-reciproke recombinanten laat zien dat de intergenomische recombinatie in seksueel gepolyploïdiseerde nakomelingen inderdaad het resultaat is van normale chiasmata formatie en overkruising en als zodanig niet beschouwd moeten worden als translocatie zoals gesuggereerd in de literatuur over intergenomische recombinatie. Dit wordt verder bevestigd door meiotische analyse van de interspecifieke F1 hybriden.

Gedetailleerde meiose observaties zijn uitgevoerd in interspecifieke hybriden tussen Longiflorum × Aziaat cultivar groepen welke zijn gebruikt als ouders om seksueel gepolyploïdiseerde planten met intergenomische recombinatie te genereren. Combinaties van zowel bivalenten met twee homoeologe chromosomen, als ongepaarde univalenten waren de meest voorkomende configuraties. Echter twee genotypen bevatten multivalenten en bivalenten van niet homologe Aziaat chromosomen. Dit is een aanwijzing voor de aanwezigheid van een duplicatie tussen twee niet homologe chromosomen in deze hybriden. Een reciproke translocatie in de Aziatische ouder 'Connecticut King' moet hieraan ten grondslag hebben gelegen en de twee afwijkende genotypen zijn uit duplicatie deficiënte gameten ontstaan.

Resultaten van anafase I laten zien dat chiasma formatie met niet-zuster chromatiden resulteert in dubbel strengs enkel, dubbel strengs dubbel, drie strengs dubbel, vier strengs dubbel en meervoudige overkruisingen. Opmerkelijk was de hoge frequentie van meervoudige overkruisingen in de genotypen met de duplicatie wat een indicatie is voor het wegvallen van recombinatie onderdrukking in multivalenten. Naast de normale overkruisingen werden ook chromosoom bruggen in de anafase I waargenomen. GISH en FISH laten zien dat deze bruggen ontstaan tussen zowel niet zuster chromatiden als zuster chromatiden. De chromosoom bruggen bestaan uit gelijke delen terwijl de bijbehorende fragmenten verschillende lengtes hebben. Deze resultaten wijzen erop dat de chromosoom bruggen en de bijbehorende fragmenten zijn ontstaan door zgn. U-type chromosoom uitwisselingen. Naast homologe recombinatie (HR), hebben niet homologe uiteinde verbindingen (in het Engels: Non Homologous End Joining) waarschijnlijk geleid tot het ontstaan van de chromosoom bruggen bij de reparatie van dubbel strengs breuken (DSB) tijdens de meiose.

Nakomelingen van eenzijdige polyploïdisatie in kruisingen van LA hybriden en Aziatische cultivars waren hoofdzakelijk triploïd. Echter in drie bijzondere planten is het chromosoom

aantal 35 met daarnaast een klein afwijkend chromosoom in plaats van het normale aantal van 36 chromosomen. In alle drie de gevallen waren de kleine afwijkende chromosomen iso-chromosomen die blijkbaar waren ontstaan tijdens de eerste generatie terug kruising en de lengte van het afwijkende chromosoom was altijd gecorreleerd met de lengte van de korte arm van het missende chromosoom. Eén van de drie iso-chromosomen liet bovendien 45S rDNA hybridizatie zien in de proximale posities die vergelijkbaar zijn aan de korte arm van het missende chromosoom (chromosoom 4 van het L genoom).

Samen met chromosoom breuken tijdens de meiose zijn centromeer breuken en fusies een mogelijk mechanisme voor het ontstaan van iso-chromosomen. In een tetraploïde plant die was ontwikkeld door chromosoom verdubbeling van een interspecifieke hybride (2n=2x=24) uit een Longiflorum cultivar met een Trompet veredelingslijn werden twee B chromosomen gedetecteerd bovenop het normale aantal chromosomen in een tetraploïd. Wanneer deze tetraploïde LLTT hybride werd gekruist met een triploïde LLO hybride (O=Oriental) werd in 73.4% van de nakomelingen een B chromosoom doorgegeven. Met GISH en FISH is aangetoond dat de gevonden B chromosomen bestaan uit twee identieke armen, met 5S rDNA hybridisatie signalen op het grootste deel van het chromosoom aan beide kanten geflankeerd door normale telomeren die erop duiden dat dit een iso-chromosoom is.

De resultaten van deze studie zijn van praktische waarde vanwege een aantal verschillende redenen. Ten eerste, het gedrag van homoeologe chromosomen tijdens de meiose in lelie hybriden is in detail bestudeerd en kan worden gebruikt voor de verklaring van de grote genetische veranderingen in de eerste generaties tijdens hybride soortvorming. Ten tweede, sommige problemen die onopgemerkt blijven in het genetisch karteren kunnen worden voorspeld en ook verklaard worden uit het voorkomen van chromosoom translocaties in de ouders die gebruikt zijn voor de uitsplitsende populatie. Ten derde, de ontdekking van U-type uitwisselingen tijdens meiose en het ontstaan van iso-chromosomen in terugkruisingspopulaties bieden een alternatief mechanisme voor de herkomst van B chromosomen.

摘要

百合系百合科百合属植物的统称,是世界上最重要的球根类切花之一,其亦可用于庭院绿化,盆栽,并具有重要的食用及药用价值。百合属由约 80 个野生种组成,广泛分布于北半球温带地区。依其生物学性状,杂交亲和性及 DNA 保守序列,百合属可再分为 7 个组。由于组内种间杂交亲和性较高且杂种可育而组间杂交不亲和且杂种高度不育,经过数十年的实践,育种者育成了 9 大百合杂种系,各系具有差别明显的农艺性状。在 9 大杂种系中,分别来源于 Leucolirion,Sinomartagon 及 Archelirion 组的麝香百合杂种系(Longiflorum,L),亚洲百合杂种系(Asiatic,A)及东方百合杂种系(Oriental,O)最具有商业价值,在世界范围内广泛用于切花生产。

百合远缘杂种及其后代是优良的分子细胞遗传学分析材料。二十世纪八十年代起,众多新技术成功用于克服百合远缘杂交不亲和,杂种胚败育,杂种一代高度不育等问题,这为百合组间渐渗育种提供了可能。截至目前,远缘杂交及多倍化已经在百合新品种培育中广泛应用。该多倍体新品种和众多的中间育种材料均为分子细胞遗传学分析提供了理想的材料。首先,百合巨大的染色体使得其成为经典细胞遗传学研究中的模式植物;其次,百合品种不同杂种系间形成了近同源基因组;最后,这些近同源基因组可以利用基因组原位杂交进行清楚的鉴别及区分。因此,DNA 原位杂交结合百合远缘杂种后代进行减数分裂过程中近同源染色体互作及行为分析能为染色体序列变异如交换,染色体重排等提供最直接的证据。

本论文的试验材料包括麝香百合与亚洲百合杂种 F1 代(LA)群体,有性加倍的 LA × AA 回交一代群体,有性加倍的 AA × OA 杂交后代群体,及父母本均为无性加倍来源的 LLO × LLTT 杂交后代群体。对以上百合杂种后代的基因组原位杂交分析结果显示百合异源多倍体内不存在任何形式的染色体重排,而有性加倍来源的 LA 及 OA 杂种后代广泛存在基因组间重组。通过对相互重组产物及非相互重组产物在杂种后代的分离统计及杂种 F1 代减数分裂分析显示,该重组来源于减数分裂过程中近同源染色体正常的联会,交叉及交换,因此不应被视为易位。

对麝香百合与亚洲百合杂种 F1 代的减数分裂的详细分析显示,在第一次分裂中期,两条近同源染色体组成的二价体及联会失败的单价体是最主要的联会形式。此外,在两个基因型内,四价体,三价体,及少数二价体都涉及了来自亚洲百合基因组的两条非同源染色体配对。这说明该染色体间存在一个同源重复,而对此的一个解释是其父本材料中存在一个相互易位,而该基因型来自其父本材料所产生的重复-缺失配子。第一次分裂后期显示非姐妹染色单体联会产生各种形式的交叉交换形式,如单交换,双线双交换,三线双交换,四线双交换,复合交换。需要指出的是,在存在非同源联会的基因型中复合交换的概率明显比其他基因型高,这可能是因为在多价体联会中交叉干涉降低所致。除却正常的交换外,在不同的基因型的花粉母细胞内存在不同

比率的染色体后期 I 桥，GISH 和 FISH 结果显示此染色体桥不仅涉及到姐妹染色单体而且涉及到非姐妹染色单体。此外，该种染色体桥的出现均伴随着不同大小的染色体片断。以上证据表明该染色体桥来自于姐妹染色单体或非姐妹染色单体间的 U 型交换。和 DNA 双链断裂及同源重组修复导致的交叉交换不同，U 型交换可能来源于 DNA 双链断裂和非同源末端连接。

虽然当 LA 百合杂种 F1 代与其父本回交时，单向有性加倍通常导致三倍体后代，但是少数的非整倍体基因型同样存在。在众多的回交一代中，三个非整倍体植株具 35 条正常的染色体外加一条畸形小染色体。此三条小染色体虽然大小不一，但均为等臂染色体且均来自母本材料。由于细胞学证据表明母本材料中染色体不存在任何异常，此畸形小染色体产生于母本的减数分裂过程。对比发现此小染色体臂长均与其对应基因型所缺失的正常染色体短臂长度相同。此外，在基因型 074051-9 中，畸形小染色体着丝粒附近的两臂上和其缺失的正常染色体靠近着丝粒位置的短臂上均有一个 45S rDNA 位点。因此，此畸形小染色体分别来自减数分裂过程中所缺失的正常染色体两条短臂的末端融合。同时，两条 B 染色体发现被于一个异源四倍体 LLTT 杂种中。当该材料以父本与一个异源三倍体 LLO 杂交后，73.4%的杂种后代均具有 B 染色体。GISH 和 FISH 结果表明此种 B 染色体亦为等臂染色体，除却正常的端粒结构外，整条染色体均为 5S rDNA 重复序列。

本论文结果具有以下应用价值：1）近同源染色体在减数分裂过程中的互作及行为可以为杂种物种形成的早期世代提供直接证据；2）减数分裂过程异常可以解释遗传图谱构建过程中一系列问题；3）减数分裂过程中的 U 型交换及回交后代中畸形小染色体为 B 染色体起源提供了另一种可能。

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淡看世事去如烟，铭记恩情存如血

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Curriculum Vitae

Songlin Xie was born in Taikang, Henan Province, P. R. China on 26th of July (lunar calendar), 1982. He has studied his bachelor with a major of Horticulture at Northwest A&F University from 2000 to 2004. In the following two years, he studied the subject of Landscaping and Garden Plants in the same university for a master's degree. In 2006, he was selected to continue his research on Germplasm of Ornamental Plants as a PhD. After 4 and half years, he got his doctors degree in December of 2010. Meanwhile, he started his PhD in Wageningen University and Research Centre when he came to Netherlands in November of 2007 (initially as a visiting PhD). This thesis is the results of his work carried out from the end of 2007 up to December of 2011 to obtain his second PhD degree.

不积跬步，无以至千里；不积小流，无以成江海。

——《荀子·劝学》

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Van Tuyl, Jaap M., Paul Arens, M. S. Ramanna, Arwa Shahin, Nadeem Khan, **Songlin Xie**, Agnieszka Marasek-Ciolakowska, Ki-Byung Lim And Rodrigo Barba-Gonzalez 2010. Lilium. Chapter In : Kole, C. Wealth of Wild Species : Genetic, Genomic and Breeding Resources Volume 9 - Plantation and Ornamental Crops. Springer-Verlag Series, in press.

Xie Songlin, Wang Xianzhi, Niu Lixin, Zhang Yanlong. 2010. Overcoming cross barriers and obtaining crossing hybrids between different hybrid groups of lily(Lilium). Acta Botanica Boreali-Occidentalia Sinica. 30(8): 1573-1578.

Wang Xianzhi, Zhang Yanlong, Niu Lixin, Wu Yunfeng, **Xie Songlin**. 2008. Identification of three kinds of viruses, and the preliminary evaluation on the resistance to the virus in six wild Lilium species from Qin-ba Mountains under the nursery field. Scientia Agricultura Sinica, 41(11): 3618-3625.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School **EXPERIMENTAL PLANT SCIENCES**

1) Start-up phase	date
► **First presentation of your project**	
A molecular cytogenetic research on lily hybrids	Nov 09, 2009
► **Writing or rewriting a project proposal**	
A molecular cytogenetic research on lily hybrids	Mar 2008
► **Writing a review or book chapter**	
Lilium: in Wild Crop Relatives: Genomic and Breeding Resources, Plantation and Ornamental Crops, Springer-Verlag Berlin Heidelberg 2011	Sep 2009
A molecular cytogenetic analysis of introgression in the backcross progenies of Lilium species hybrids	Sep 2011
The Significance of Polyploidy for Bulbous Ornamentals: A Molecular Cytogenetic Assessment	Oct 2011
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13,5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS Theme 4 Symposium 'Genome Biology', Wageningen University	Dec 12, 2008
EPS Theme 4 Symposium 'Genome Biology', Wageningen University	Dec 09 Dec, 2011
EPS Theme 1 Symposium Developmental Biology of Plants, Wageningen University	Jan 19, 2012
EPS Theme 2 and the Willie Commelin Scholten Day Interactions between plants and biotic agents, Wageningen University	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Science' Lunteren	Apr 07-08, 2008
ALW meeting 'Experimental Plant Science' Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Science' Lunteren	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
Plant breeding research day (WICC, Wageningen)	Jun 17, 2008
Plant breeding research day (WICC, Wageningen)	Mar 03, 2009
Chinese annual review of flower bulbs (Xining, China)	Jul 17-20, 2010
EPS Mini symposium 'Plant Breeding in the genomic era'	Nov 25, 2011
Mechanism and function of active DNA demethylation in Arabidopsis, Prof. Jiankang Zhu	Nov 03, 2008
Plant Breeding seminar series	Nov 2007-May 2012
► **Seminar plus**	
► **International symposia and congresses**	
23rd Eucapia Symposium - Section Ornamentals "Colourful Breeding and Genetics" (Leiden, Netherlands)	Aug 31-Sep 04, 2009
18th International Chromosome Conference (Manchester, UK)	Aug 29-Sep 02, 2011
XI International Symposium on Flower Bulbs and Herbaceous Perennials (Antalya, Turkey)	Mar 28-Apr 01, 2012
► **Presentations**	
Oral presentation: Biodiversity and ornamental plant breeding in China	Aug 31-Sep 04, 2009
Poster at 23rd EUCARPIA Symposium - Section Ornamentals "Colourful Breeding and Genetics in Leiden, Netherlands	Aug 31-Sep 04, 2009
Oral presentation (Lily company meeting, including Mak Breeding BV, World Breeding BV, De Jong lelies BV et al.)	Dec 12, 2007
Oral presentation: The creation of lily hybrids among three hybrid groups and its potential utilization	Jul 17, 2010
Oral presentation (lily company meeting, idem)	Dec 13, 2011
Oral presentation: Meiotic U-type exchanges lead to the production of anaphase I bridges and isochromosome formation	Dec 09, 2011
Oral presentation: GISH investigation of crossover events in *Lilium* hybrids	Mar 29, 2012
► **IAB interview**	
► **Excursions**	
Visit to Iribov for flow cytometry analysis and tissue culture	May 14, 2008
Visit to Lily and Zantedeschia companies	May 18, 2009
Visit to Lily companies (Van den Bos Flowerbulbs) and lily show in Keukenhof	May 14, 2012
Subtotal Scientific Exposure	*19,9 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Advances of research on germplasm of horticultural plants	Sep 2006-Dec 2006
Basic statistics	Dec 13-15 & 20-21, 2011
► **Journal club**	
Literature discussion plant breeding	2008-2012
► **Individual research training**	
Subtotal In-Depth Studies	*7,5 credits**

4) Personal development	date
► **Skill training courses**	
Acdemic Writing II	Sep 2011-Feb 2012
Information Literacy including EndNote Introduction	Nov 01-02, 2011
Advanced course guide to scientific artwork	Nov 07-08, 2011
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**43.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

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