A molecular cytogenetic study of intergenomic recombination and introgression of chromosomal segments in lilies (*Lilium*)

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Chapter 1
General Introduction

Lilies

The genus *Lilium* belongs to the Liliaceae family which comprises of about 80 species (Comber 1949), and thousands of cultivars (Leslie 1982-2005). The lily species are taxonomically classified into seven different sections based on various morphological and physiological characteristics. The seven sections are Lilium, Martagon, Pseudolirium, Archelirion, Sinomartagon, Leucolirion and Oxypetalum (Comber 1949; De Jong 1974).

In general, wild species within each section are relatively easy to cross and the hybrids are fertile (McRae 1990; Van Tuyl et al. 2002). Most of the cultivars, however, are interspecific hybrids within the sections (especially Leucolirion, Archelirion and Sinomartagon) and represent the most important cultivated groups which are:

1. The **Longiflorum** hybrids (**L**-genome) which originate from intra- or interspecific hybridization in the Leucolirion section, have trumpet-shaped, pure white flowers, a distinctive fragrance, year- round forcing ability and mostly outward-facing flowers (McRae 1990).
2. The **Asiatic** hybrids (**A**-genome) are derived from interspecific crosses among at least 12 species of the Sinomartagon section (Leslie 1982-2005). Cultivars of Asiatic hybrid lilies have a wide colour variation in their flower tepals (orange, white, yellow, pink, red, purple and salmon) and early to late flowering (Woodcock and Stern 1950). Some species in this section show resistance to *Fusarium* and viruses (McRae 1998).
3. The **Oriental** hybrids (**O**-genome) are nowadays the most important lily hybrid group. They result from hybridization among five species of the Archelirion section. Generally, Oriental hybrids are late-flowering, with big and showy flowers with a pleasant fragrance (McRae 1998). Most Oriental hybrids show a fair degree of resistance to *Botrytis elliptica* (Barba-Gonzalez et al. 2005). Fig. 1.1 shows Longflorium, Asiatic and Oriental hybrids.

Fig 1.1. Figures from different lily hybrids: (a) Longiflorum hybrids, (b) Oriental hybrids and (c) Asiatic hybrids

Fertilization barriers and hybrid sterility

The species of *Lilium* possess a wide range of ornamental traits as well as resistances to fungal and virus diseases. Interspecific hybridization is the most important method in *Lilium* breeding to produce cultivars from distantly related species (Van Tuyl and Lim 2003). Most of the lily cultivars have an interspecific hybrid origin, basically between diploids (2n = 24) and, more rarely, between tetraploid parents. Breeding of lilies for new cultivars is time consuming. They need 2 to 3 years from sowing of seeds to first flowering. Vegetative propagation takes another 3 - 5 years. Because of their heterozygous genome structure they are maintained as clones (Booy et al. 1998).

It is desirable to combine or introgress some important horticultural traits from species of different sections into a single cultivar. In *Lilium* it is very difficult to obtain F1 interspecific hybrids due to pre- and post fertilization barriers. Nearly all the F1-hybrids between Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) are sterile. This sterility is due to irregular chromosome paring between the parental genomes during meiosis (Asano 1982; Lim et al. 2000; Barba-Gonzalez et al. 2004). For successful interspecific hybridization, pre- and post-fertilization barriers can be overcome by the use of various *in vitro* pollination and embryo rescue techniques respectively (Van Tuyl et al. 1991). Successful interspecific crosses in *Lilium* are depicted in Fig. 1.2 as a crossing polygon (Lim et al. 2007) Somatic chromosome doubling of the F1 hybrids using colchicine or oryzalin can induce allotetraploids in which homologous pairing can restore fertility (Van Tuyl and De Jeu 1997). However, this method could not contribute much to introgression breeding due to the formation of the so-called “permanent hybrids” as their progenies never segregate for parental characters due to autosyndatic chromosome pairing (Ramanna and Jacobsen 2003; Van Tuyl and Lim 2003).

On the contrary, intergenomic recombination may occur in sexual polyploids induced through the formation of 2*n* gametes (Ramanna et al. 2003; Ramanna and Jacobsen 2003). Interestingly, some F1 LA and OA hybrids produce functional 2*n* gametes in reasonable frequencies. When such 2*n* gametes are used for generating back cross progenies they have the potential to generate genetic variation as has been shown in BC1 progenies of Oriental × Asiatic (Barba-Gonzalez et al. 2005a) and Longiflorum × Asiatic hybrids and cultivars (Zhou 2007; Zhou et al. 2008). 2*n* gametes occur occasionally in interspecific hybrids of *Lilium* (Van Tuyl et al. 1989) and, as has been demonstrated in other plants, they result from abnormal meiosis (Mok and Peloquin 1975; Ramanna 1979; Lim et al. 2001a). In lilies the BC1 progenies resulting from functional 2*n* gametes hybridize and sometimes their homoeologous chromosomes pairs and recombine at certain level (Lim et al. 2001a; Barba-Gonzalez et al. 2004; Zhou 2007). This may

result in the assembly of complementary characters within a single genotype in a back cross breeding program.

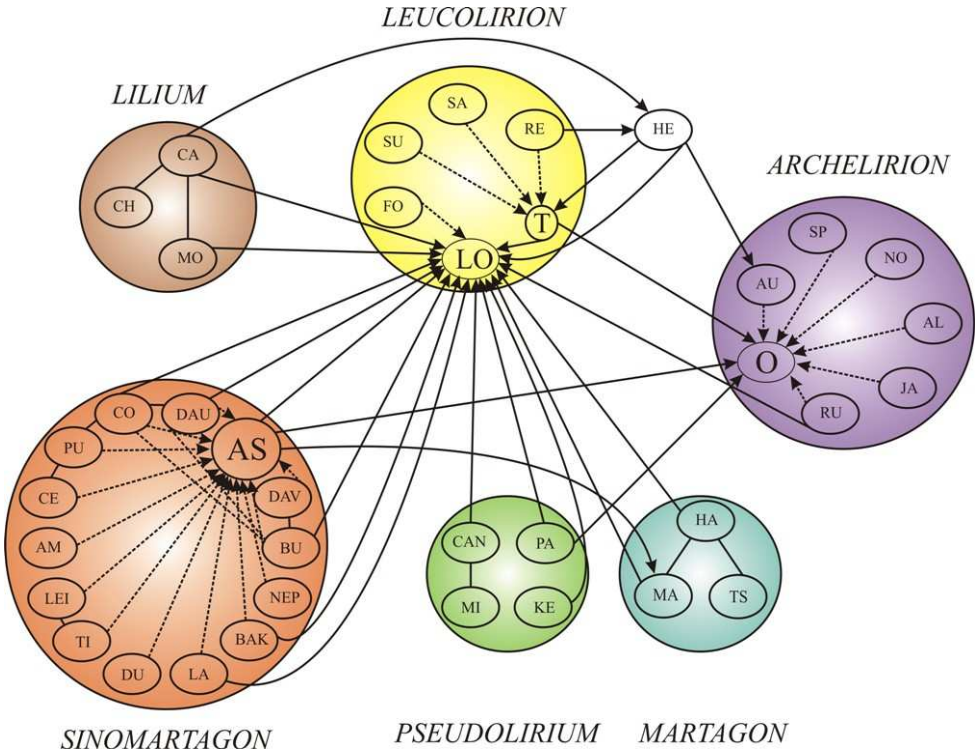

Fig. 1.2. Crossing polygon of the genus *Lilium* including all the successful crosses of species between different sections of the genus *Lilium* developed at Plant Research International, Wageningen University and Research Centre, The Netherlands. In this figure, the connection between the Asiatic, Trumpet, and Oriental hybrid groups (large ellipses) are shown by dotted lines. In successful crosses between species (small circles) of different sections (large circles) the arrows point towards the female parent. Abbreviations: AL: *L. alexandrae*; AM: *L. amabile*; **AS**: Asiatic hybrids; AU: *L. auratum*; BAK: *L. bakerianum*; BU: *L. bulbiferum;* CA: *L. candidum*; CAN: *L. canadense*; CE: *L. cernuum*; CH: *L. chalcedonicum*; CO: *L. concolor*; DAU: *L. dauricum;* DAV: *L. davidii*; DU: *L. duchartrei*; FO: *L. formosanum*; HA: *L. hansonii*; HE: *L. henryi*; JA: *L. japonicum*; KE: *L. kelloggii*, LA: *L. lankongense*; LEI: *L. leichtlinii*; **LO**: *L. longiflorum*; MA: *L. martagon*; MI: *L. michiganense*; MO: *L. monadelphum*; NEP: *L. nepalense*; NO: *L. nobilissimum*; **O**: Oriental hybrids; PA: *L. pardalinum*; PU: *L. pumilum*; RE: *L. regale*; RU: *L. rubellum*; SA: *L. sargentiae;* SP: *L. speciosum*; SU: *L. sulphureum*; **T**: trumpet hybrids, TI: *L. tigrinum*; TS: *L. tsingtauense*.

Introgression breeding and relevance of genomic *in situ* hybridization techniques in cytogenetic research

Intergenomic recombination is essential for introgression breeding in which specific traits from wild species are introduced into the cultivated crops. It has played an important role in the improvement of some major economically important crops like wheat (Jiang et al. 1994; Wang et al. 1996), rice (Multani et al. 1994; Khush 2005), potato (Tek at al. 2004), *Alstroemeria* (Kamstra et al. 1999a; 1999b and 2004) and sugarcane (Ram et al. 2001) among others. In

Lilium, the species or cultivars from different sections possess a number of valuable traits. The main goals of modern lily breeding are to combine the distinctive groups and realize introgression breeding. In order to combine some valuable horticultural traits from different alien species into a cultivar, interspecific hybridization and recurrent backcrossing are required.

Genomic *in situ* hybridization (GISH) is one of the most powerful and effective molecular cytogenetic techniques to detect homoeologous chromosomal recombination. After hybridization it allows the localization of genomic DNA (or DNA sequence) of one of the parents labeled with fluorescent dyes on chromosomes (Schwarzacher and Heslop-Harrison 2000). Thus, when DNA from one of the parents in an interspecific hybrid, is used as probe, it is possible to identify the chromosomes from that specific parent. This technique has been applied successfully in potato (Jacobsen et al. 1995), *Lolium-Festuca* (Kosmala et al. 2006), *Gasteria lutzii* × *Aloe aristata* (Takahashi et al. 1997), *Alstroemeria aurea* × *A. inodora* (Kamstra et al. 1999a; Ramanna et al. 2003), *Tulipa gesneriana* × *T. fosteriana* (Marasek and Okazaki 2008) among several others. GISH enables the parental chromosomes of interspecific lily hybrids to be distinguished, and the sites of any genomic recombination to be identified (Lim et al. 2001b; Barba-Gonzalez et al. 2005a; Zhou et al. 2008). The molecular cytogenetic techniques, FISH (Fluorescent *in situ* hybridization) and GISH have enabled researchers to investigate the genetic material of *Lilium* in detail. The karyotypes of *L. longiflorum* Thunb and *L. rubellum* Baker *via* chromosome banding and FISH have been constructed (Lim et al. 2000). GISH has been used extensively in lily to recognize the three genomes of *Lilium viz*. Longiflorum (L), Asiatic (A) and Oriental (O) genomes and to study the recombinant chromosomes in the BC1 and BC2 progenies of *L. longiflorum* × Asiatic (LA) and Oriental × Asiatic (OA) hybrids (Karlov et al. 1999; Lim et al. 2003; Barba-Gonzalez et al. 2004; 2005a and 2005b; Zhou et al. 2008). Furthermore, this technique is also employed to study the mechanisms of 2*n* gamete formation in interspecific hybrids of *L. longiflorum* × Asiatic and Oriental × Asiatic (Lim et al. 2001a; Barba-Gonzalez et al. 2004 and 2005b).

Inrogression breeding and ploidy manipulation

In crosses involving a diploid F1 hybrid that produces 2*n* gametes with a diploid cultivar that produces normal *n* gametes the progeny is expected to be triploid. In crosses involving both parents producing 2*n* gametes, tetraploid progeny is expected. Analyses of the progeny of LA hybrids producing functional 2*n* gametes, obtained by crosses with diploid Asiatic (A) cultivars, showed that most of the progeny obtained was triploid as expected and considerable amounts of intergenomic recombination had occurred (Lim et al. 2003; Zhou et al. 2008). Similarly, OA

hybrids producing functional 2*n* gametes backcrossed with diploid Asiatic cultivar also produced triploid progenies with a certain amount of intergenomic recombination (Barba-Gonzalez et al. 2005a).

Generally the allotriploid hybrids can not be used in breeding because of their sterility. However, it has been demonstrated that some triploid hybrids can produce aneuploid and euploid (*x*, 2*x* and 3*x*) gametes and have been used to produce progeny in many plant species (King et al. 1998 and 1999; Ramanna and Jacobsen 2003). Furthermore, triploid hybrids might have contributed to the origin of a majority of new polyploids in nature (Husband 2004). In case of *Lilium*, triploid ALA hybrids derived from 2*n* gametes, have been successfully used in crosses and it has been shown that the recombinant chromosomes can be transmitted to the progeny. Crosses of these triploids with diploids and tetraploids produced aneuploid or near diploid and pentaploid progenies, respectively (Lim et al. 2003). Zhou (2007) also used triploid LA hybrids obtained from functional 2*n* gametes and crossed with diploid Asiatic cultivar. Here in case of 3x - 2x and in reciprocal cross (2x - 3x) diploid progenies were obtained indicating the production of balanced *n* gametes by triploid cultivars. The advantages of polyploids for breeding need to be considered. It is well known that polyploids differ from their diploid progenitors in morphological, ecological, physiological and cytological characteristics (Soltis and Soltis 1999; 2000; Levin 2002; Knight et al. 2005). These genotypic and phenotypic differentiations are caused mainly by the increased cell size, gene dosage effect and allelic diversity (Ramsey and Schemske 2002). Thus, the creation of polyploid interspecific hybrids with the use of 2*n* gametes might be rewarded with a higher degree of genetic variation.

One of the most important advances that has been achieved recently is with the development of diploid backcross progenies from interspecific Longiflorum × Asiatic (LA hybrids) backcrossed to Asiatic parents (Khan et al. 2009b). It was found that some of the F1 LA hybrids produced not only 2*n* gametes but also *n* gametes. This provides unique opportunities for generating allotriploid (2n = 3x = 36) as well as diploid (2n = 2x = 24) BC1 progenies from backcrossing LA hybrids to Asiatic parents. The initial attempts indicated that it might be possible to generate fairly large numbers of diploid BC1 and BC2 progenies from LA hybrids. Introgression breeding at the diploid level is important due to three main advantages over the triploid BC1 progenies for introgression breeding: i) Mendelian inheritance of characters might be more clearly analysed. ii) Introgression can be accomplished at the diploid level. iii) Diploid BC1 progenies are expected to have better fertility than triploid BC1 progenies. So there is a possibility to carry out breeding in lilies at the diploid level.

Innovative molecular breeding techniques for resistance breeding in lily: Molecular and physical mapping in *Lilium*

Cytological maps represent the location and order of markers along the chromosomes in relation to structures such as centromeres, telomeres, and (if any) secondary constrictions. Such maps are created by microscopic determination of the position of visible structures (or "markers") in fixed and stained chromosomes. These cytological maps are essential to relate genetic loci and molecular sequences to morphological features of chromosomes (Fransz et al. 2000; Cheng et al. 2001). Induced chromosome deletions and translocations that can be visualised cytologically have also been used for mapping genes in some cases, e.g., tomato (Khush and Rick 1968), wheat (Gill et al. 1996; Castilho et al. 1996; Sandhu and Gill 2002; Bhat et al. 2007), *Brassica* (Howell et al. 2002) and barley (Künzel et al. 2000).

Chromosome maps or, the so-called, cytomolecular maps, have been constructed in some of the plants with small as well as large chromosomes by FISH to study the genomic structure of different plant species. Examples are: *Arabidopsis* (Schmidt et al. 1995; Jackson et al. 1998), *Sorghum* (Islam-Faridi et al. 2002), legumes (Fuchs et al. 1998; Ohmido et al. 2007) and *Pinus* (Hizume et al. 2002; Islam-Faridi et al. 2007). Since the cloned DNA sequences can be directly localized on chromosomes, this method is becoming increasingly important in plant molecular cytogenetics (Jiang and Gill 2006). Unlike the FISH technique, genomic DNA *in situ* hybridization (GISH) can be most useful for analysing the process of intergenomic recombination as well as for the elucidation of chromosome organization. Species of the genus *Lilium* have the largest genomes among plants except *Fritillaria* (Bennett and Smith 1976; 1991) and have been extensively used for cytological analysis in the past (Stewart 1947; Noda 1991; Mather 1940; Brown and Zohary 1955; Fogwill 1958; Bennett and Stern 1975). Nevertheless, to our knowledge, no reliable molecular or cytogenetic maps are available for lily so far.

Integration of molecular and physical maps: FISH approach to integrate molecular and physical map in interspecific lily hybrids

At present, breeding programs aim at combining desirable traits within different plant species complexes and are mainly focused on introgression procedures (Kosmala et al. 2006 and 2007). The main focus area is the transfer of genes conferring a particular trait from a wild species into a cultivar. Markers associated with genes conferring particular characters can facilitate the marker-assisted selection program to obtain new cultivars with desired traits. For this purpose, genetic or molecular maps have been constructed in several crops and integrated in cytogenetic maps of respective genomes (Khrustaleva et al. 2005; Humphreys et al. 2005). Multiple

approaches have been used to develop integrated maps. Integrated maps are developed by combining the information from molecular maps and physical distribution of these markers in the genome through cytogenetic techniques. Such maps are constructed by insertion of large DNA clones like yeast artificial chromosomes (YAC) and bacterial artificial chromosomes (BAC) and comparison of these maps with genetic maps (Wu et al. 2003). Künzel at al. (2000) constructed integrated maps of barley based on translocation breakpoints as physical land marks for mapping of RFLP markers that were transformed into PCR markers. The other techniques used are *in situ* hybridization of BACs and YACs on plant chromosomes with recombination maps (Kulikova et al. 2001). Physical distribution of AFLP markers along the chromosomes of *Festuca pratensis* (King et al. 2002a), *Lolium-Festuca* hybrid (Skibinska et al. 2002) and *Allium* trihybrid (Khrustaleva et al. 2005) chromosomes has been carried out. The GISH-AFLP combined technology proved to be an effective strategy because it is relatively simple and a fast way to develop integrated maps.

One of the current developments in lily breeding is the introduction of molecular breeding techniques for the incorporation of disease resistances in commercially successful lily cultivars. *Fusarium*, *Botrytis* and virus (LMoV) resistance are the most important goals in lily breeding. Screening methods to determine resistance against these pathogens in several lily cultivars have been developed (Straathof and Van Tuyl 1994; Straathof and Löffler 1994). However, it takes a long time before resistance symptoms become visible, and therefore, faster selection methods are needed.

In lily molecular marker techniques have been used for the identification of cultivars and interspecific hybrids (Yamagishi, 1995; Yamagishi et al. 2002), identifying genetic diversity (Arzate-Fernández et al. 2005), genetic analysis of anthocyanin pigmentation (Abe et al. 2002) and for finding linked RAPD-markers to *Fusarium oxysporum* resistance in Asiatic hybrids (Straathof et al. 1996). Van Heusden et al. (2002) used AFLPs and a QTL approach to locate markers linked to LMoV virus on Asiatic genome. A total of 251 AFLP markers based on 100 descendents of a lily backcross population were used for the construction of a genetic map. Based on these approaches it was found that LMoV (TBV) resistance was clearly a monogenic trait and could reliably be mapped on linkage group 9. Despite the difficult screening for *Fusarium* resistance, four significant QTLs were mapped to linkage groups 1, 5, 13 and 16 respectively.

Diversity Array Technology (DArT), a new DNA marker technology was developed as hybridization based alternative for multilocus PCR approaches, which captures the value of the parallel nature of the microarray platform. With DArT simultaneous screening of several

thousands of loci in a single assay can be performed. It generates whole genome fingerprints by scoring the presence versus absence of DNA fragments in genomic representation generated from samples of genomic DNA. This technology was originally developed for rice (Jaccoud et al. 2001) and then extended to a range of other plants like *Arabidopsis thaliana* (Wittenberg et al. 2005), barley (Wenzl et al. 2004) and wheat (Akbari et al. 2006; Stodart et al. 2007). With this technique it is possible to detect which of the fragments are transferred to the progeny and which fragments might contain the genes carrying the particular trait. Markers linked to some specific traits can easily be converted into PCR markers to be used as a probe in cytogenetic techniques and finally visualized by chromosome painting (GISH/FISH).

The physical location of genes on chromosomes is of great interest as chromosome structure profoundly influences gene activity. However, linkage maps can not be superimposed on chromosomes as map distances are not proportional to physical distances (Khush and Rick 1968; Moore and Sherman 1975). The most direct means of location of genes on chromosomes is through the FISH technique, a powerful tool by which even single and low-copy DNA sequences can be localized on chromosomes (Guzzo et al. 2000; Wang et al. 2006). FISH mapping permits superimposition of markers from molecular linkage maps directly on chromosomes and thereby contribute to our understanding of the relationship between chromosome structures, gene activity and recombination frequency together with total DNA genomic *in situ* hybridization (GISH).

Scope of the thesis

The aims of this research were to analyse the nature and extent of intergenomic recombination and amount of genome introgression in interspecific lily hybrids (Longiflorum × Asiatic and Oriental × Asiatic) obtained from functional *n* and 2*n* gametes. For this purpose cytomolecular maps were constructed using GISH. Based on these results the potential use of *n* and 2*n* gametes in the introgression breeding has been discussed. Furthermore, DArT markers were developed and a genetic linkage map was constructed in a F1 population of Longiflorum × Asiatic hybrids. These linkage groups will be aligned with their respective chromosomes by using FISH technique.

To accomplish the aforementioned tasks, molecular cytogenetic techniques were employed to elucidate the back cross progenies of interspecific lily hybrids

In **Chapter 2** F1 Longiflorum × Asiatic (LA) hybrids were backcrossed with Asiatic parents to get BC1 population. Similarly BC2 progenies were obtained from a cross between BC1 with Asiatic parents in 3x - 2x and 2x - 2x cross combination. Genomic *in situ* hybridization (GISH)

was performed to study the intergenomic recombination, the amount of introgression of alien segments and karyotype composition. The prospect of using the diploid plants of these BC progenies of LA hybrids in introgression breeding has been evaluated. In **Chapter 3** chromosomal recombination maps were constructed for three genomes of lily (*Lilium*) using GISH analyses. For this purpose, the backcross (BC) progenies of two diploid ($2n = 2x = 24$) interspecific hybrids of lily, *viz.*, Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) were used. In all cases, it was possible to identify the homoeologous recombinant chromosomes as well as accurately count the number of crossover points (recombination sites). The distribution of these recombination sites among and within different chromosomes as well as in different genomes is described and their potential significance as a prerequisite for developing molecular cytogenetic maps and their usefulness in breeding is discussed. **Chapter 4** details the analysis of BC1 as well as sib-mated plants in different interspecific hybrids of lilies (LA and OA). These BC progenies were obtained by functional $2n$ gametes either from male or female parents. However, some F2 LA hybrids were obtained by sib-mating through $2n$ gametes donated from both parents. Flow cytometeric and actual chromosome counts were compared. GISH technique was employed to analyse the possible contribution of parental gametes and the extent of intergenomic recombination and amount of introgression of various genomes from different cross types. The scope of unilateral and bilateral sexual polyploidization was evaluated from 3x and 4x progeny plants. **Chapter 5** reports on the Diversity Array Technology (DArT) and the development of genetic linkage map in LA population based on DArT markers. So a large number of these DArT markers (of varying sizes) are available to map on certain chromosomes in interspecific *Lilium* hybrids with FISH. Different DArT markers (linked to some specific traits) were generated that could be mapped physically on lily chromosomes in different interspecific hybrids using FISH technique. In **Chapter 6** the importance of functional n and $2n$ gametes in introgression breeding, the transmission of recombinant chromosomes at different ploidy level and its implication for genetic variation is reviewed. Furthermore, construction of cytomolecular maps in three genomes of *Lilium* and the development of DArT genetic linkage maps in LA hybrids are discussed. The part is further extended to discuss the possible solution for integration and alignment of genetic linkage map to the cytogenetic maps based on cytogenetic techniques.

Chapter 2

Relevance of diploid BC progenies for intergenomic recombination and introgression breeding in Longiflorum × Asiatic hybrids of lilies (*Lilium* L.)

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Abstract

Ploidy level and intergenomic recombination was studied in interspecific hybrids between Longiflorum × Asiatic lilies (LA hybrid) backcross to Asiatic parents in order to assess the possibility for intergenomic recombination and introgression in lily (*Lilium*). By backcrossing the diploid (2n = 2x = 24) F1 interspecific hybrid between Longiflorum × Asiatic lilies to Asiatic parents, 104 BC1 progeny plants were produced. Among these, there were 27 diploids, 73 triploids (2n = 2x = 36) and 4 aneuploids (2x – 1, 2x + 2 or 2x + 3). In addition, by backcrossing triploid BC1 (LAA) plants to diploid Asiatic parents in 2x - 3x and reciprocal combinations, 14 diploid BC2 progenies were produced. Genomic *in situ* hybridization (GISH) was performed to study the intergenomic recombination and karyotype composition. Similarly, one BC2 LA diploid obtained from a cross between BC1 genotype with an Asiatic parent was also studied. GISH indicated extensive intergenomic recombination among the chromosomes in LA hybrids. A large number of Longiflorum chromosomes were transmitted to the BC1 progenies from LA hybrids. However, few Longiflorum chromosomes were transmitted from the BC1 diploid (LA) and triploid (LAA) plants to the BC2 progenies. The occurrence of diploid plants in the BC progenies of LA hybrids has opened the prospects of analytic breeding in lilies. In this approach, the selection of superior genotypes with introgression can be carried out at the diploid level and polyploid forms are synthesized from superior diploid parents. The advantages of analytic breeding are evident: a) a maximum level of heterozygosity can be attained in the synthetic polyploids and b) introgression can be achieved with a minimum of linkage drag and c) the diploid BC progenies can be used for mapping. Based on GISH results the potential use of diploid BC progenies and application in introgression breeding in lily allopolyploids are discussed.

Introduction

The process of introgression involves the transfer of an alien DNA segments with specific traits into cultivated crops. For the successful development of new cultivars, introgression of traits from a wild relative in to cultivated crop is an important phenomenon and it has been realized in many crops such as bread wheat (Hegde and Waines 2004; Schoenenberger et al. 2006), rice (Multani et al. 1994, Khush 2005), *Festuca-Lolium* (King et al. 1998; Humphreys et al. 2005; Kosmala et al. 2006), potato (Tek et al. 2004), Alstroemeria (Kamstra et al. 1999a; 1999b, 2004), sugarcane (Bakshi et al. 2001) and tomato (Canady et al. 2007). The formation of fertile hybrids and backcross individuals are necessary for the successful introgression between the species. Similarly, genetic difference, ploidy level, and hybrids sterility are limiting factors during the process of introgression (Stebbins 1958; Rieseberg and Wendel 1993). The introgressions are mainly restricted to species that were cross compatible or closely related species. However, introgression of chromosomal segments is hindered in crops which were incompatible to cross due to crossing barriers.

Introgression of important trait for crop improvement via interspecific hybridization at the diploid level implies that the genotypes used in this approach necessarily produce haploid (*n*) gametes so that they give rise to diploids in the subsequent generations (Zeldin and McCown 2004). In the hybrids of species that possess very closely related or similar genomes (the case for autopolyploids) it is not a problem to have diploid forms that can produce functional *n* gametes due to normal segregation of chromosomes during meiosis (Weiss and Maluszynska 2000; Lopez-Lavalle and Orjeda 2002; Gu et al. 2005; Hoya et al. 2007; Zhang et al. 2007). However, when distantly related species are to be used as parents in the improvement of the cultivars at the diploid level, there can be several obstacles. Apart from the crossability barriers, which can be usually overcome through the use of *in vitro* embryo or ovule rescue methods (Van Tuyl et al. 1991; Lim et al. 2001; Barba-Gonzalez et al. 2004), the F1 hybrid sterility caused by the lack of chromosome pairing during meiosis and the failure of normal viable gamete formation can be a great impediment. In some cases, numerically unreduced gametes, or 2*n* gametes, can occur and thus the fertility might be restored. In this case, however, the progenies that are produced will be polyploid. In rare cases, even the distant hybrids can possess the ability to undergo normal meiosis and produce haploid gametes, as for example in hybrids of *Festuca* × *Lolium* (Zwierzylowski et al. 1998; Thomas et al. 2003); *Allium cepa* × *A. fistolium* (Khrustaleva and Kik 2000) and *Lycopersicon esculentum* × *Solanum lycopersicoides* (Chetelat et al. 1997). In such cases it is possible to produce diploid backcross progenies and carryout introgression without changing the ploidy level. If diploid (distant) interspecific hybrids that can produce both

n as well as 2*n* gametes become available, there might be an opportunity for selection of traits at diploid level. Once the selection has been made at the diploid level, then the unilateral sexual polyploids can be achieved by using 2*n* gametes to reach the optimum ploidy level, e.g. triploid in lilies (Van Tuyl and Lim 2003; Zhou et al. 2008) and tetraploids in potato (Jacobsen et al. 1991).

There are three important groups of cultivated lilies (*Lilium*), *viz*., Longiflorum, Asiatic and Oriental, which were predominantly diploid (2n = 2x = 24) until recently (Lim et al. 2000). During the last few decades, polyploids have been replacing the diploid forms due to their superior characteristics of the former. In *Lilium*, because the species or cultivars, especially those of different sections, possess more valuable traits the main goals of modern lily breeding are to combine the three distinctive groups and realize introgression breeding. In order to combine some valuable horticultural traits from different alien species into a cultivar, interspecific hybridization and recurrent backcrossing are required. These resulting polyploid cultivars have originated from hybridization of cultivars that belong to different taxonomic sections of lilies such as Longiflorum × Asiatic (LA) [Lim et al. 2001a; Zhou et al. 2008] or Oriental × Asiatic (OA) [Barba-Gonzalez et al. 2004; 2005b]. Because the three groups possess clearly differentiated genomes, their hybrids have given rise to typical allotriploid (ALA and AOA) or allotetraploid (LLAA) cultivars (Barba-Gonzalez et al. 2005a; Zhou 2007; Zhou et al. 2008). These polyploids are difficult to use as parents in breeding because the triploids are mostly sterile and the allotetraploids obtained after mitotic chromosome doubling are the so-called permanent hybrids. A salient feature of some of the diploid LA hybrids was that they produced not only 2*n* gametes but also *n* gametes (Zhou 2007). This provides unique opportunities for generating allotriploid (2n = 3x = 36) as well as diploid (2n = 2x = 24) BC1 progenies from backcrossing LA hybrids to Asiatic parents. In addition, diploid BC2 progenies were also generated from backcrossing allotriploid (ALA or LAA) to diploid Asiatic parents, i.e., 3x - 2x or reciprocal crosses and 2x - 2x crosses. These initial attempts indicated that it might be possible to generate fairly large numbers of diploid BC1 and BC2 progenies from LA hybrids and their backcross progenies. With the aim of evaluating the prospects of using diploid backcross progenies in introgression breeding, quite a number of diploid BC progenies from LA hybrids were produced and the genome composition of the diploid BC1 and BC2 progenies were analysed by GISH. Based on these results the potential application and significance of these hybrids is discussed with particular reference to introgression breeding and intergenomic recombination in lilies at diploid level.

Materials and methods

Plant material

Most of the diploid F1 Longiflorum × Asiatic hybrids (2n = 2x = 24), and allotriploids i.e., LAA and ALA (2n = 3x = 36) cultivars were supplied by Dutch lily breeding companies: De Jong Lilies BV, Royal Van Zanten BV, Testcentrum BV, Vletter and Den Haan BV and World Breeding BV. One F1 LA hybrids i.e. 024004-5 was developed at the department of Plant Breeding, Wageningen UR. As the two groups which belong to two different taxonomic sections, the cultivars of different sections could be hybridized, or backcrossed, only through special techniques (Van Tuyl et al. 1991; Lim et al. 2001a; Barba-Gonzalez et al. 2004). For backcrossing, the F1 LA hybrids were used both as female and male parent and crossed with 8 different Asiatic parents to get LAA or ALA progenies (Table 2.1). Furthermore, the triploid genotypes from the breeders (which were BC1s) were backcrossed to diploid Asiatic parents by using both 2x - 3x or reciprocal combinations (Table 2.2). One BC2 diploid (074373-1) was developed from a backcross BC1 diploid (044538-2) with an Asiatic parent. All the plant material is being maintained vegetatively at Wageningen UR, Plant Breeding, Wageningen, The Netherlands.

Pollen germination

Pollen was collected on day of anthesis and cultured for 5-20 hrs at 25°C in an artificial agar medium containing 100g/l sucrose, 5g/l of bacteriological agar, 20mg/l of boric acid and 200mg/l of calcium nitrate. The percentage of germinating pollen was determined based on the growth of pollen tube under an anatomical microscope.

Flow cytometry

Flow cytometry was done to evaluate the ploidy level of the BC1 and BC2 progenies. The germinating embryos were transferred into the propagation medium and allowed to grow until leaves developed. One leaf or scale was collected from each seedling for testing ploidy level as described by Van Tuyl and Boon (1997).

Mitotic chromosome preparation

For mitotic metaphase chromosome analysis, root tips were collected early in the morning, incubated in 0.7 mM cycloheximide solution for 4 - 6 h and then fixed in ethanol-acetic acid (3:1) solution for 12 to 24 h and stored at 4°C until use. The root tips were washed in distilled water and incubated in a pectolytic enzyme mixture containing 0.2% (*w/v*) pectyolase Y23, 0.2%

(*w/v*) cytohelicase and 0.2% (*w/v*) cellulase RS in 10 mM citrate buffer (pH 4.5) at 37°C for about 1 h. Squash preparations were made in a drop of 45% acetic acid and frozen in liquid nitrogen. The cover slips were removed by using a razor blade. The slides were then dehydrated in absolute ethanol and air dried.

Genomic in situ hybridization (GISH)

Total genomic DNA was extracted with the CTAB method (Rogers and Bendich 1988). Genomic DNA of Longiflorum cultivar 'White Fox' was used as probe in GISH and labelled with digoxigenin-11-dUTP by nick translation (Roche Diagnostics GmbH, Mannheim, Germany). The GISH procedure was performed as described by Lim et al. (2003) and Zhou et al. (2008). Briefly, the hybridization mixture contained 50% (v/v) deionized formamide, 10% (w/v) sodium dextran sulfate, 2× SSC, 0.25% (w/v) sodium dodecyl sulfate, 1-1.5ng/µl Digoxigenin labelled DNA from Longiflorum cultivars and 25 - 100ng/µl block DNA of the Asiatic cultivar. DNA was denatured by heating the hybridization mixture at 70°C for 10 min followed by incubation in ice for 5 min. The hybridization mixture (40µl) was then applied on each slide. The chromosome preparations were denatured at 80°C for 5 min immediately prior to incubation. After overnight hybridization in a humid chamber at 37°C the slides were washed at room temperature in 2× SSC for 15 min followed by stringent washing with 0.1× SSC for 30 min at 42°C. Digoxigenin labelled DNA was detected with antidigoxigenin-fluorescein raised in sheep (Boehringer, Mannheim, Germany) and amplified with fluorescein anti-sheep immunoglobulin raised in rabbit (Vector Laboratories). Preparations were analysed using a Zeiss Axiophot epifluorescence microscope and photographed with Canon digital camera. For each plant, the total number of chromosomes and the number of recombinant points were determined.

Chromosome identification, measurement and determination of genome contribution

For karyotyping in both genomes (L and A), chromosomes are arranged in sequence of decreasing short arm length according to Stewart (1947). Some of the chromosomes in the somatic karyotype could be identified on the basis of total length and arm ratios (e.g., 1, 2, 3 10, 11 and 12). In other cases, however, the differences in the lengths of short arms were used for identification. By identifying recombination sites analysed by GISH, the same chromosome could be accurately identified in 5-10 cells and compared with the previous and the succeeding chromosomes in the karyotype. Furthermore, the centromeric index [short-arm length/(short arm length + long arm length)] and the relative chromosome length index (individual chromosome length/total length of all chromosomes) were determined for all genotypes (Barthes and Ricroch

2001). For the measurements and determination of genome contribution, images of mitotic metaphase chromosomes from each genotype were collected and were measured (micrometres). The contribution of the amount of both L and A genomes (in terms of percentage) in BC progenies was determined using the computer program MicroMeasure (Reeves and Tear 2000; http://www.colostate.edu/Depts/Biology/MicroMeasure)

Results

Production of BC1and BC2 progenies from LA hybrids

In order to assess the possibility to produce relatively large number of diploid backcross progenies from the LA hybrids or from its BC1 progenies a large number of crosses were made. For this purpose, one BC1 LA hybrid, several F1 LA hybrids and allotriploid BC1 (LAA) genotypes were backcrossed to diploid Asiatic parents and the ploidy levels of these progenies were determined through flow cytometry (data not presented) as well as by cytological chromosome counting (Tables 2.1 and 2.2).

Not all LA hybrids were successful as male and female parents. Viable pollen grains (5-30%) were produced in some cases (e.g., F1 hybrids 041502, 041519, 041557) and these could be used successfully as male parents (Table 2.1). Other genotypes could only be used as female parent. By crossing LA hybrids both as female and male parent, a total of 104 BC1 progenies were obtained of which 27 (26%) were diploid, 73 (70.2 %) were triploid and 4 were aneuploid (3.8%) with 2x – 1, 2x + 2 and 2x + 3 respectively. This indicated the occurrence of haploid (*n*), diploid (2*n*) and aneuploid gametes in the LA hybrids (Table 2.1). Analysis of the different BC1 types produced by each genotype indicated that three genotypes produced only *n* eggs. All other produced both *n* and 2*n* eggs although in the majority of cases 2*n* eggs are produced. Similarly, there was one genotype that produced only *n* pollen (041557); one that produced both *n* and 2*n* pollen (041519) and one genotype produced only 2*n* pollen (041502). The observations indicated that it was possible to produce reasonable numbers of diploid BC1 progenies along with a large number of triploid progenies from LA hybrids despite possessing highly differentiated genomes and abnormal meiosis in these hybrids.

Apart from producing BC1 progenies from diploid LA hybrids, an attempt was also made to obtain diploid progenies by backcrossing diploid (LAA) and allotriploid (LAA and ALA) BC1 plants with different diploid Asiatic parents. From the flow cytometric determination of the ploidy levels of the progenies of 2x - 3x and reciprocal crosses, it was established that 90.33% of the BC2 plants showed diploid chromosome numbers and this was confirmed cytologically (Table 2.2; Fig. 2.1c). Obviously, despite being allotriploids, they produced balanced haploid

gametes both male and female, giving rise to diploid BC2 progenies on back crossing. Similarly a cross between 2x - 2x also gave a diploid progeny which showed that normal diploid BC1 LA hybrid also produced normal *n* gametes.

Table 2.1. The frequencies of diploid (2n = 2x = 24), or aneuploid, and triploid (2n = 3x = 36) BC1 progenies resulting from crosses between different genotypes of LA hybrids and the Asiatic parents.

Cross	Genotype Code	Parents		No. of plants analysed	Ploidy level**		
		Female	Male		2x	3x	Others
LA A*	044511	041543	Mont Blanc	2	2		
LA A	044512	041558	Mont Blanc	1		1	
LA A	044518	041543	Pollyanna	1		1	
LA A	044525	041556	Mont Blanc	1		1	
LA A	044535	041562	Pollyanna	1		1	
LA A	044536	041563	Pollyanna	1	1		
LA A	044538	041560	Pollyanna	4	4		
LA A	044539	041558	Pollyanna	5		5	
LA A	044541	041549	Pollyanna	1		1	
LA A	044567	041548	Pollyanna	1		1	
LA A	044571	041557	Mont Blanc	4		4	
LA A	044611	041559	Pollyanna	1		1	
LA A	044624	041565	Pollyanna	1		1	
LA A	044630	041502	Mont Blanc	1		1	
LA A	062035	041560	Sarina	2		2	
LA A	062071	041560	Montreux	4	1	3	
LA A	062074	041560	Montreux	5	2	3	
LA A	065051	024004-5	061095	4	2	1	1(2x-1)
LA A	066828	041543	051072	5	1	4	
LA A	066960	041543	Lanzarote	18	3	14	1(2x+2)
LA A	066963	041543	Gironde	7	2	5	
LA A	066994	041560	Alaska	13	6	5	2(2x+3)
LA A	066995	041560	031040	1		1	
A LA*	044595	Pollyanna	041519	3	1	2	
A LA	044601	Mont Blanc	041502	9		9	
A LA	044602	Mont Blanc	041557	2	2		
A LA	044608	Vivaldi	041502	6		6	
Total				**104**	**27**	**73**	**4**

* = Denotes only the direction of the crosses but not the ploidy level. LAA indicates that the hybrid LA was the female parent and ALA indicates that LA hybrid was the male parent; **** =** Estimated cytologically.

Genome composition and the extent of crossing-over in BC progenies

Through GISH analysis, the genome composition was determined in 24 diploid BC1 (Table 2.3) and five BC2 plants (Table 2.4). Among BC1 progenies, 20 had the expected 24 chromosomes and 4 were aneuploid (2x – 1, 2x + 2 or 2x + 3). Because the Asiatic parent was used for backcrossing, the number of A genome chromosomes (chromosomes of which the centromere was of Asiatic genome) predominated in the BC1 progenies and varied from 15 to 23 (Table 2.3). On the other hand, the chromosomes with the centromere of the L genome varied from only one (044602-2) to nine (066960-2 and 066963-2) (Table 2.3).

There were obviously four type of chromosomes; two non-recombinant chromosomes, L and A and two recombinant chromosomes L/A and A/L. Those with a centromere of Asiatic chromosome with a recombinant segment of Longiflorum, are indicated as A/L, and *vice versa*, i.e., L/A (Table 2.3 and Fig. 1A, B and C). In all, there were 71 L/A and 86 A/L types of recombinant chromosomes found in the total of progenies analysed. Taking the total length of both A and L chromosomes in the BC1 progenies, the percentages of each genome present in the BC1 progenies was estimated (Table 2.3). The percentage of L genome deviated from the expected (25%) and it varied from 3.3% (044602-2) to as high as 32.5% (066960-15), nearly a 10 fold variation. In order to estimate the number and types of recombinant chromosomes and the lengths of recombinant segments, the karyotypes of some of the BC1 progenies were determined as shown in Fig. 2.2. It was found that a considerable amount of Longiflorum genome was transmitted from LA hybrids to BC1 progenies, however, the BC2 progenies possessed hardly any of the alien genome or segments of L genome (Fig. 2.1c).

Genome comparison and the extent of recombination in BC2 progeny obtained from BC1 diploid plants

A total of 17 flowers from three diploid BC1 LAA genotypes were pollinated using Asiatic parents to get BC2 (LAA × AA) progenies. From the 17 pollinated flowers only one embryo could be rescued (Table 2.2). Ploidy level of the germinated seedling was determined by flow cytometry and the total number of chromosomes, intergenomic recombination and the amount of L- genome transferred from BC1 to BC2 level was confirmed by GISH. In total 24 chromosomes were identified making this individual diploid (2n = 2x = 24).

With GISH it was found that a total of 10 break points distributed amongst 6 chromosomes, two of them containing Longiflorum centromeres (Fig. 2.3b and Fig. 2.4). BC2 generation is expected to contain 12.5% of the donor genome (L) rather than 25%. A comparison in the genomic composition, number of recombinant chromosomes and break points of both the BC1 and its progeny (BC2) has been given (Table 2.5; Fig 2.4). The BC1 showed 28.5% of L-genome with 28 break points with 7 chromosomes showing Longiflorum centromeres. Even though it showed more than the expected percentage of the L- genome, its progeny shows only 9% which is lower than the expected 12.5%.

Table 2.2. The ploidy level of BC2 progenies resulting from crossing triploid BC1 plants with diploid Asiatic parents.

Interploid Crosses	Parents		No. of plants analysed	No. of diploid plants**
	Female	Male		
2x - 3x (BC2)				
044529	Pollyanna	041513	3	3
044530	Mont Blanc	041513	5	5
3x - 2x (BC2)				
044519	041513	Pollyanna	3	3
044524	041513	Mont Blanc	1	1
044634	041552	Pollyanna	3	2
2x - 2x (BC2)				
074373-1	044538-2	051073	1	1
Total			**16**	**15**

** = Estimated cytologically.

Discussion

Nearly 30% of the 104 BC1 progenies produced from LA hybrids in this investigation turned out to be diploid or near diploid (i.e. aneuploid with almost diploid chromosome count). This shows that there is a prospect for producing sufficient number of diploid BC1 progenies with homoelogous recombinant chromosomes suitable for introgression breeding in LA hybrids. An attractive feature of these progenies is that it might be possible to monitor the segregation of distinctive genetic traits that distinguish the two parental species. The inheritance patterns of some of the desirable horticultural traits in these progenies might provide an insight into how such traits can be utilized in breeding lilies. Because the generation time of lilies is long (two or more years) the segregation of traits has not yet been analysed in the present study. Nevertheless, a cursory look at the karyotypes of BC progenies (Fig. 2.2 and 2.4) does indicate that there is a potential for genetic variation and consequently a possibility for selection at the diploid level. From the point of view of breeding allopolyploid crops, the results of the present investigation are relevant to other ornamental. Just as in *Lilium*, an important group of tulips, the so called "Darwin Hybrids", have originated from interspecific hybrids between *Tulipa gesneriana* × *T. fosteriana* (Marasek at al. 2006; Marasek and Okazaki 2008). Despite having genetically differentiated genomes, these F1 hybrids have been shown to produce both *n* and 2*n* gametes so that both diploid (2n = 2x =24) and triploid (2n = 3x =36) BC1 progenies can be obtained by back crossing with one of the parent i.e. *T. gesneriana* (Marasek and Okazaki 2008).

Similarly, occurrence of both *n* and 2*n* gametes has also been reported in other ornamentals like *Alstroemeria aurea* × *A. inodora* (Kamstra at al. 1999b). This indicates that there are prospects of using introgression breeding at diploid level in more ornamental crops other than lily. The potential for genetic variation and consequently the possibility for selection

at the diploid level have been practiced in some other crops of economic importance like *Lycopersicon esculentum* × *Solanum lycopersicoides* (Chetelat et al. 1997) and *Festuca* × *Lolium* (Zwierzylowski et al. 1998; Thomas et al. 2003).

Table 2.3. Genome composition of BC1 (diploid and aneuploid) LA hybrids and the number of recombinant chromosomes among different genotypes.

Cross	Genotype Code	Ploidy level	Genome composition		Genome percentage		No of recombinant chromosomes
			$L^{(L/A)}$	$A^{(A/L)}$	L (%)	A (%)	
LA A	044511-1	2x	3(1)	21(2)	12.55	87.45	3
LA A	044538-2	2x	7(6)	17(4)	25.37	74.62	10
LA A	044538-3	2x	7(6)	17(3)	28.6	71.4	9
LA A	044538-3	2x	4(3)	20(4)	23.03	76.96	7
LA A	044538-4	2x	5(2)	19(5)	24.32	75.67	7
LA A	062704-2	2x	4(2)	20(4)	25.6	74.63	6
LA A	062704-5	2x	6(4)	18(4)	16	84	8
LA A	065051-3	2x	3(3)	21(2)	8.81	91.18	5
LA A	065051-5	2x-1	3(2)	20(3)	17.80	82.20	5
LA A	065051-6	2x	7(6)	17(3)	27.5	72.5	9
LA A	066828-5	2x	5(4)	19(2)	15.2	84.8	6
LA A	066960-2	2x	9(5)	15	26.88	73.11	5
LA A	066960-15	2x+2	7(2)	19(1)	32.52	67.47	3
LA A	066960-17	2x	8(4)	16(3)	31.88	68.11	7
LA A	066960-22	2x	5(5)	19(4)	13.9	86	9
LA A	066963-2	2x	9(5)	15	28.9	71.09	5
LA A	066963-7	2x	7(3)	17	25.2	74.8	3
LA A	066994-1	2x	6(3)	18(4)	27.81	72.18	7
LA A	066994-2	2x	4(4)	20(5)	29.28	70.72	9
LA A	066994-5	2x	7(5)	17(4)	21	79	9
LA A	066994-6	2x+3	7(4)	20(4)	35.9	64.1	8
LA A	066994-7	2x+3	7(3)	20(4)	24.37	75.63	7
LA A	066994-10	2x	6(3)	18(4)	26.2	73.8	7
AL A	044602-2	2x	1(1)	23(2)	3.34	96.66	3

Three important aspects of homoeoleogous recombination and introgression breeding using *n* gametes are, a) conventional breeding at diploid level for improved quality and high yield traits b) the maximization of heterozygosity in the polyploids that might be produced from the selected diploid genotypes and c) introgression of desirable traits from on species to the other. For good reasons, it has been argued that a higher degree of heterozygosity can be achieved at the polyploid rather than at the diploid level (Bingham 1980; Sanford 1983). The reason being that polyploids have a greater probability of possessing three or more different alleles at the same locus whereas it is restricted to only two in diploids. The importance of poly-allelic loci for inducing vigour and fertility in autopolyploids has been demonstrated in the case of *Medicago sativa* (Busbice and Wilsie 1966). This aspect can be verified by using diploid BC progenies for breeding and selection followed by polyploidization in lilies.

Table 2.4. Genome composition of BC2 (diploid and aneuploid) LA hybrids and the number of recombinant chromosomes among different genotypes analysed by GISH.

Cross	Genotype Code	Ploidy level	Genome composition		No of recombinant chromosomes
			$L^{(L/A)}$	$A^{(A/L)}$	
A LAA	044529-2	2x	1(1)	23	1
A LAA	044529-3	2x	-	24	0
A LAA	044529-4	2x		24	0
A LAA	044530-1	2x	-	24(1)	1
LAA A	044634-1	2x+1	1	24(2)	2
LAA A	077373-1	2x	2(2)	22(4)	6

The traditional method of introgressing traits into allopolyploids was through the re-synthesis of the crop by using the putative parental species as has been done in *Ipomea* spp. (Orjeda et al. 1991), *Aegilops-Triticum* (Feldman et al. 1997; Ozkan et al. 2001), *Spartina* spp. (Baumel et al. 2002), *Musa* Spp. (Ortiz 1997), and *Brassica* (Lukens et al. 2006; Nicolas et al. 2007). Hybridization of such re-synthesized allopolyploid with elite cultivars adds the entire genomes of the alien species rather than only the chromosome segments that possess the desirable traits.

Table 2.5. Relative Asiatic to Longiflorum genomic composition of a BC2 individual compared to its mother (BC1).

Backcross	Genotype Code	Ploidy level	Genome composition		Genome %		Number of recombinant chromosomes	No. of break points
			$A^{(A/L)}$	$L^{(L/A)}$	A%	L%		
BC1	044538-2	2x	17(4)	7(6)	71.5	28.5	10	28
BC2	074373-1	2x	22(4)	2(2)	91	9	6	10

The addition of entire genomes, obviously, adds many undesirable traits along with the desirable ones into the cultivars enhancing so called, linkage drag (Hospital 2001). One effective way of preventing linkage drag is to add specific recombinant segments that possess desirable traits (Servin et al. 2004). An important requirement for this is that, a) homoeologous recombination must be accomplished in the hybrids of the putative parental species and b) the recombinant chromosomes must be recovered in the progenies of the F1 hybrids. These two requirements are most ideally achieved in the case of LA hybrids studied here and a wide range of recombinant chromosomes with varying numbers and lengths of recombinant segments have been recovered in the BC1 progenies (Table 2.2; Fig. 2.1a, b and Fig. 2.3).

Besides, it has also been possible to produce BC2 progenies from the diploid and triploid BC1 genotypes (Table 2.2). In a previous study it was found that in the progenies of 3x - 2x crosses of Longiflorum × Asiatic and 3x - 2x and reciprocal crosses of Oriental × Asiatic hybrids lilies, predominantly near diploid (i.e., aneuploid) or diploid progenies occurred indicating that

triploid BC1 plants produced both balanced *n* as well as near haploid (e.g., n + 1 or n + 2 etc.) gametes (Lim et al. 2003; Barba-Gonzalez et al. 2006a). Furthermore, diploid progenies were also obtained when allotriploid *Lilium lankongense* and Asiatic hybrids and *L. longiflorum* and Asiatic hybrids (LAA) were backcrossed with diploid and tetraploid Asitaic hybrids (Proscevičius et al. 2007). This was also an indication of balanced *n* gametes production in allotriploid interspecific lily hybrids. Similarly, present investigation shows that most of the BC2 progenies obtained after 2x - 3x and in reciprocal crosses produced mostly diploid progenies. However, there is no convincing explanation for the occurrence of balanced *n* gametes in triploids used in the present study. This appears to be genotype dependent that needs to be confirmed by further analysis. Therefore, more progenies from this cross have to be anlysed. A notable feature of the BC2 progenies obtained from 2x - 2x, 2x - 3x and its reciprocal cross (3x - 2x) is that they transmit very short recombinant segments or very few alien chromosomes to the progenies (Fig. 2.1c and 2.3).

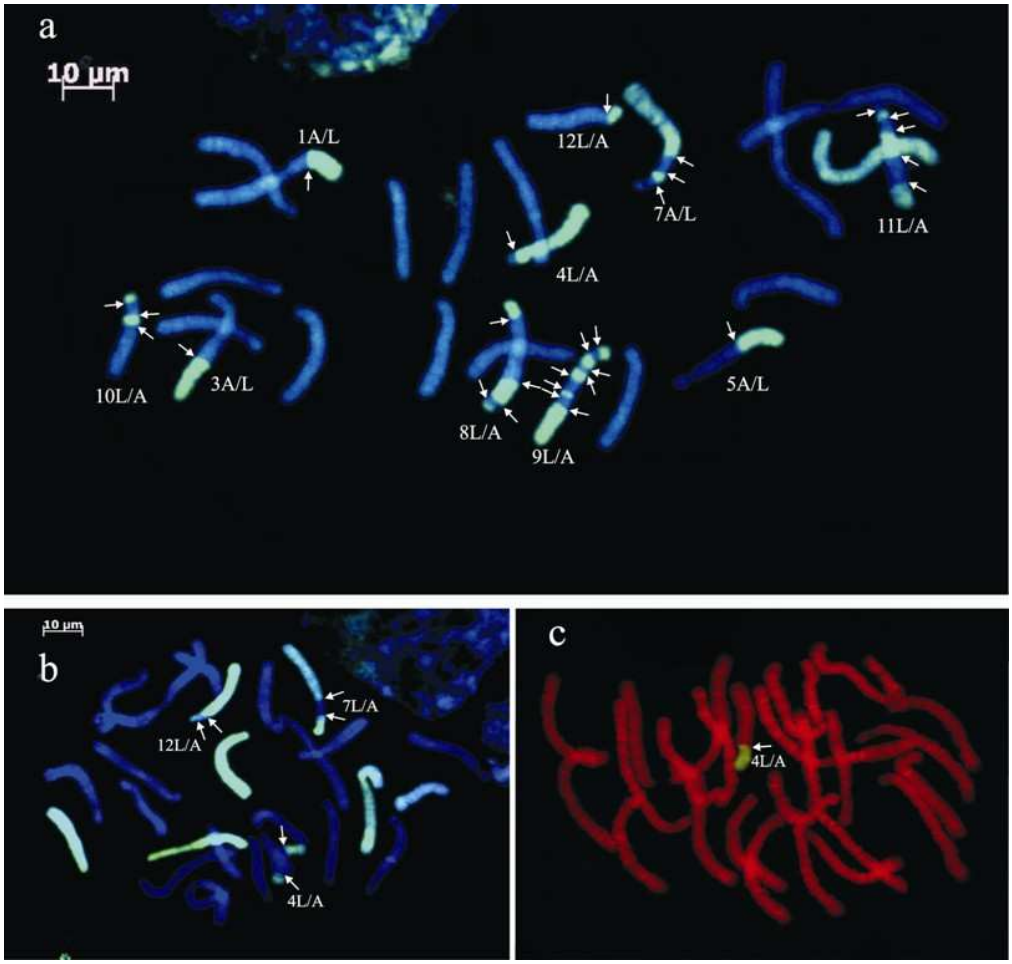

Fig. 2.1. Chromosome identification and identification of recombination sites with GISH. (**a** and **b**) BC1 LA diploids (044538-2; 066963-7) with Longiflorum (green) and Asiatic (blue) genomes. (**c**) BC2 LA diploid (044529-2) with Longiflorum (green) and Asiatic (red) genome.

These might be especially favourable in avoiding linkage drag. The scarcity of L genome chromosomes or segments in these BC2 progenies might have resulted from a process of selection of gametes during fertilization or embryo development or due to non-inclusion of L genome chromosomes during gamete formation. This implies that introgression of some desirable Longiflorum traits into an Asiatic cultivar could be realized at BC2 level. Similar results have been found in *Festuca-Lolium* intergeneric hybrids where freezing tolerance genes were successfully introgressed into *Lolium multiflorum* from *Festuca pratensis* in a back cross breeding program (Kosmala et al. 2006).

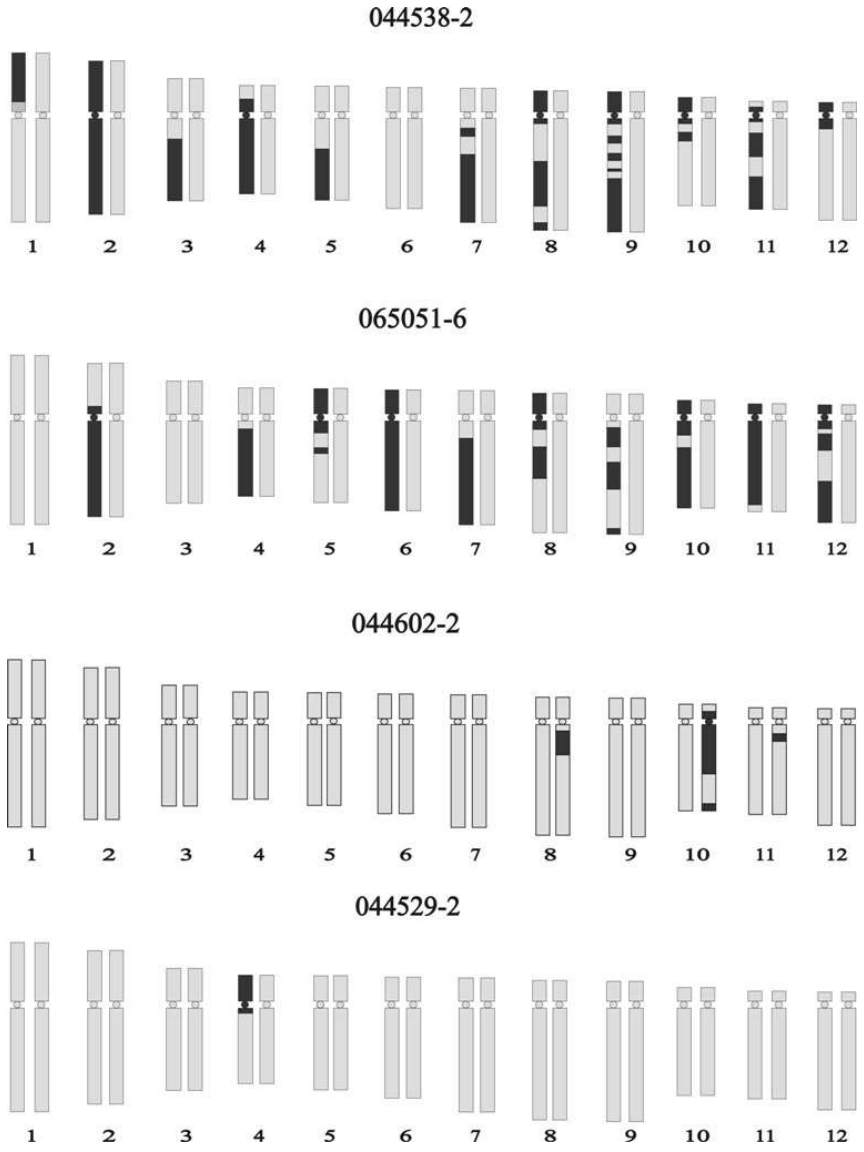

Fig. 2.2. Diagrammatic representation of recombinant chromosomes in BC diploid LA hybrid. 044538-2 and 065051-6 (BC1 LAA type hybrids); 044602-2 (BC1 ALA hybrid) and 044529-2 (A LAA BC2 hybrid). In this Fig. the black colour represents the Longiflorum genome while grey colour represents the Asiatic one.

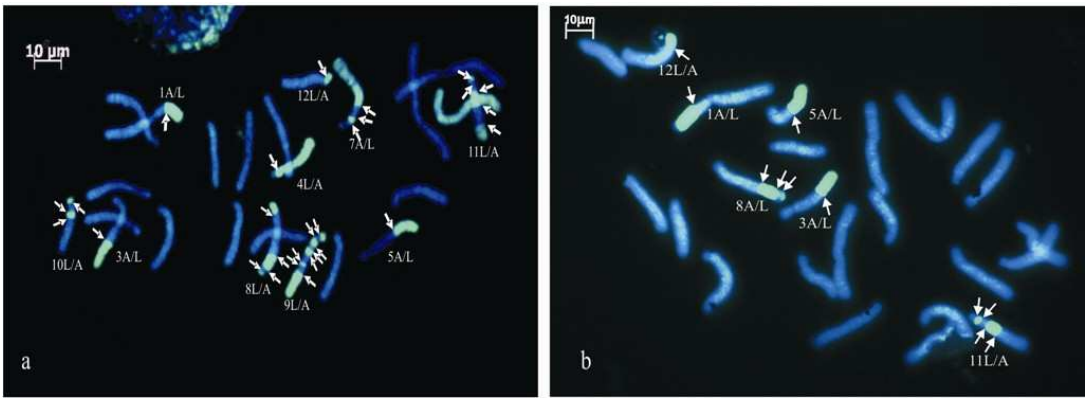

Fig. 2.3 (a and **b).** GISH picture represents the diploid BC1 and BC2 LA hybrids. **a-** BC1 LAA (044538-2) and **b-** BC2 LAAA (074373-1). Logiflorum DNA is detected with FITC signal (green) and the Asiatic DNA is counterstained with DAPI (blue). The arrows indicate recombinant segments.

Besides their relevance to introgression breeding, the diploid BC progenies can be useful for molecular mapping of useful traits as exampled in other crops like banana (Fauré et al. 1993; Raboin et al. 2005), wheat (Dubcovsky et al. 1996), alfalfa (Barcaccia et al. 1999) and cotton (Desai et al. 2008). Especially when quantitative traits are to be transferred, diploids might more conducive for tagging the quantitative loci that might be used in marker assisted selection (Dirlewanger et al. 2004). Diploid BC1 ALA and LAA plants can be backcrossed with the recurrent Asiatic parents to get the genes of interest in backcross progenies. Finally, the concept of hybrid vigour resulting from the degree of heterozygosity of the polyploid progenies can be assessed through the use of molecular methods and the advantages of analytic breeding can be determined.

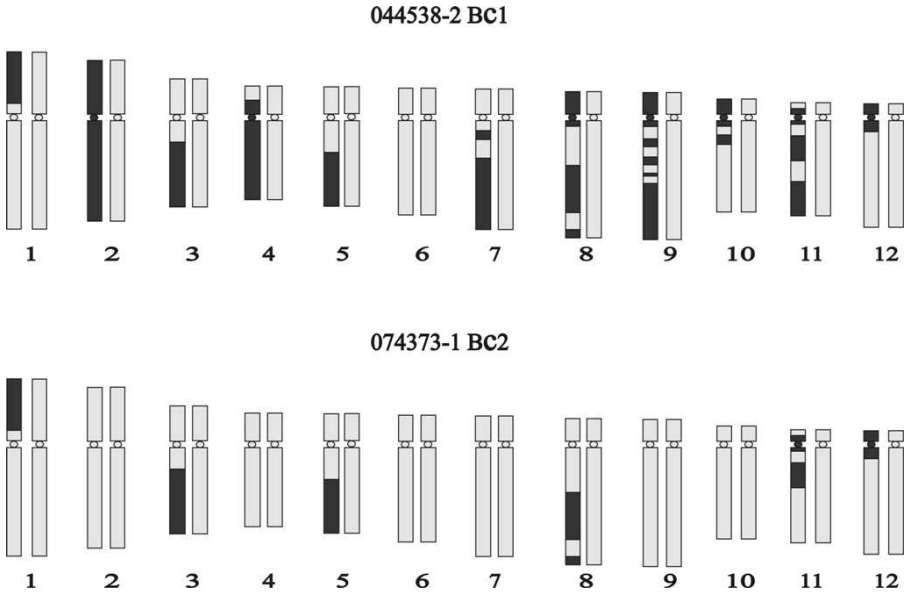

Fig. 2.4. Diagrammatic representation of the genome comparison between BC1 LAA (044538-2) and its progeny plant i.e. BC2 LAAA (074373-1). In this Fig. the black colour represents the Longiflorum genome while grey colour represents the Asiatic one.

Chapter 3

Construction of chromosomal recombination maps of three genomes of lilies (*Lilium*) based on GISH analysis

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Abstract

Chromosomal recombination maps were constructed for three genomes of lily (*Lilium*) using GISH analyses. For this purpose, the backcross (BC) progenies of two diploid (2n = 2x = 24) interspecific hybrids of lily, *viz.*, Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) were used. Mostly the BC progenies of LA hybrids consisted of both triploid (2n = 3x = 36) and diploid (2n = 2x = 24) with some aneuploid genotypes and those of OA hybrids consisted of triploid (2n = 3x = 36) and some aneuploid genotypes. In all cases, it was possible to identify the homoeologous recombinant chromosomes as well as accurately count the number of crossover points, which are called "recombination sites". Recombination sites were estimated in the BC progeny of 71 LA and 41 OA genotypes. In the case of BC progenies of LA hybrids, 248 recombination sites were cytologically localized on 12 different chromosomes of each genome (i.e., L and A). Similarly, 116 recombinant sites were localized on the 12 chromosomes each from the BC progenies of OA hybrids (O and A genomes). Cytological maps were constructed on the basis of the percentages of distances (in micrometres) of the recombination sites from the centromeres. Since an Asiatic parent was involved in both hybrids, *viz*., LA and OA, two maps were constructed for A genome which were indicated as Asiatic (L) and Asiatic (O). The other two maps were Longiflorum (A) and Oriental (A). Remarkably, the recombination sites were highly unevenly distributed among the different chromosomes of all four maps. Because the recombination sites can be unequivocally identified through GISH, they serve as reliable land marks and pave the way for assigning molecular markers or desirable genes to chromosomes of *Lilium* and also monitor introgression of alien segments.

Introduction

Cytological maps represent the location and order of markers along the chromosomes in relation to structures such as centromeres, telomeres, secondary constrictions (if any), and knobs among others. Such maps are created by microscopic determination of the position of visible structures (or "markers") in fixed and stained chromosomes. These cytological maps are essential to relate genetic loci and molecular sequences to morphological features of chromosomes (Fransz et al. 2000; Cheng et al. 2001). In fact, cytological maps have been most useful in relating and orienting genetic linkage groups on individual chromosomes in crops such as, maize (Burnham 1962), tomato (Khush and Rick 1968), and rice (Khush et al. 1984; Singh et al. 1996). Besides chromosome markers, the discovery of differential staining techniques, such as Giemsa C-banding, paved the way for using chromosome markers more extensively (Linde-Laursen 1988; Gill et al. 1991; Pedrosa et al. 2002). In addition, induced chromosome deletions and translocations that can be visualised cytologically have also been used for mapping genes in some cases, e.g., tomato (Khush and Rick 1968), wheat (Gill et al. 1996; Castilho et al. 1996; Sandhu and Gill 2002; Bhat et al. 2007), *Brassica* (Howell et al. 2002), and barley (Künzel et al. 2000).

Fluorescent *in situ* hybridization (FISH), a molecular cytogenetic technique, has opened up possibilities for localizing large numbers of cloned DNA sequences directly on chromosomes for mapping purposes. Nevertheless, chromosome maps or, the so-called, cytomolecular maps, have been constructed in some of the plants with small as well as large chromosomes by FISH. Examples are: *Arabidopsis* (Schmidt et al. 1995; Jackson et al. 1998), *Sorghum* (Islam-Faridi et al. 2002), legumes (Fuchs et al. 1998; Ohmido et al. 2007) and *Pinus* species (Hizume et al. 2002; Islam-Faridi et al. 2007). Since the cloned DNA sequences can be directly localized on chromosomes, this method is becoming increasingly important in plant molecular cytogenetics (Jiang and Gill 2006). In addition, although FISH is useful for the construction of physical maps and for the elucidation of molecular organization of chromosomes, it is less suitable for unravelling the process of crossing-over.

Unlike the FISH technique, genomic DNA *in situ* hybridization (GISH) can be most useful for analysing the process of intergenomic recombination as well as for the elucidation of chromosome organization. But GISH is restricted only to distant hybrids and their progenies for unravelling intergenomic recombination. This is because the parental genomes need to be sufficiently differentiated, as is the case for distant hybrids, to be able to distinguish the parental genomes in the hybrids. Using GISH, inter-genomic recombination has been demonstrated to occur in the progenies of some of the intergeneric and interspecific hybrids such as, *Gasteria* ×

Aloe (Takahashi et al. 1997), *Festuca* × *Lolium* (King et al. 2002a; Kosmala et al. 2006; Kosmala et al. 2007), *Alstroemeria aurea* × *Alstroemeria inodora* (Kamstra et al. 1999a), *Alstroemeria inodora* × *Alstroemeria pelegrina* (Ramanna et al. 2003), *Allium cepa* × [*Allium fistulosum* × *Allium roylei*] (Khrustaleva and Kik 1998), *Lilium longiflorum* × Asiatic hybrids (Karlov et al. 1999), Oriental × Asiatic lily hybrids (Barba-Gonzalez et al. 2004) and *Tulipa gesneriana* × *Tulipa fosteriana* (Marasek et al. 2006). In the case of distant hybrids, various strategies are required for backcrossing and analysing the progenies so that the number and positions of crossover points can be estimated. For example, in the case of *Festuca* × *Lolium*, triploid hybrids were successfully backcrossed to the *Lolium* parent using a disomic substitution for chromosome 3 of *F. pratensis* in *L. perenne* complement for physical mapping as well as establishing a 1:1 relation between chiasma formation and crossing-over (King et al. 2002a; 2002b). In the case of *Allium*, the diploid hybrid, *A. roylei* × *A. fistulosum*, produced haploid (*n*) gametes that could be successfully used to cross with *A. cepa* and to obtain the so-called trispecific hybrid that was used for GISH analysis (Khrustaleva and Kik 1998). An advantage of using a trispecific hybrid for GISH analysis was that the recombinant segments of the *A. roylei* × *A. fistulosum* could be directly visualised in the background of the chromosome complement of the *A cepa* parent. Using such genotypes, the integrated recombination and physical maps of two chromosomes (chromosome 5 and 8) of the interspecific parent (i.e., *A. roylei* × *A. fistulosum*) were constructed (Khrustaleva et al. 2005). However, in these approaches the cytogenetic map has been constructed for only one or two chromosomes of these plant species. As a rare exception, the intergeneric hybrid of *Gasteria* × *Aloe* produced haploid gametes that were used for the production of diploid backcross (BC) progenies so that the number and positions of recombinant break points were accurately estimated (Takahashi et al. 1997). Apart from these, some interspecific hybrids of *Alstroemeria* and *Lilium* were reported to produce either *n* or 2*n* gametes (Kamstra et al. 1999a; Ramanna et al. 2003; Lim et al. 2001b; Barba-Gonzalez et al. 2004; Khan et al. 2009b; Zhou et al. 2008). In the case of lilies, by using interspecific hybrids that produced both *n* and 2*n* gametes, it was possible to produce BC1 progenies that were diploids as well triploids. Such progenies were used to identify a large number of homoeologous recombination break points, referred to as '*recombination sites*' through GISH analysis.

The species of the genus *Lilium* have probably the largest genomes among plants (Bennett and Smith 1976; 1991) and have been extensively used for cytological analysis in the past. For example, basic studies on chromosome identification and karyotype analysis (Stewart 1947; Noda 1991), chiasma formation and crossing-over (Mather 1940; Brown and Zohary 1955; Fogwill 1958), and time and duration of female meiosis (Bennett and Stern 1975) were

conducted. Nevertheless, to our knowledge, no maps of any type are available for the genomes of lilies so far. In the present study, we describe cytological maps of three complete genomes of lilies based on the recombination sites identified through GISH in the BC progeny populations of two interspecific hybrids. These involve hybrids between three main groups of diploid (2n = 2x = 24) cultivars, *viz*., Asiatic, Longiflorum and Oriental lilies which belong to three different taxonomic sections (Lim et al. 2000). The genomes are completely differentiated and suitable for GISH analysis (Lim et al. 2001a; Barba-Gonzalez et al. 2004). The distribution of crossover sites among different chromosomes within each as well as in different genomes is described below and their significance is discussed.

Materials and methods

Plant material

Diploid (2n = 2x = 24) cultivars of three groups of lilies (*Lilium*), Longiflorum (L), Asiatic (A) and Oriental (O), were used for producing F1 hybrids and the BC progenies. Because all the cultivars of the three groups are interspecific hybrids of closely related species of complex origin, the species names are not mentioned. As the three groups belong to three different taxonomic sections, the cultivars of different sections could be hybridized, or backcrossed, only through special techniques like cut-style pollination and embryo rescue (Lim et al. 2001b; Barba-Gonzalez et al. 2004). The two types of hybrids used were Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA). For backcrossing, the LA hybrids were used either as female or male parents and crossed with 12 different Asiatic parents. An important feature of some of the LA hybrids was that they produced both *n* (haploid) and 2*n* gametes in both types of spore mother cells (Zhou 2007). Therefore, the BC1 progenies resulting from LA × AA (LAA) or from the reciprocal AA × LA (ALA), consisted of both diploid or near diploid (2n = 2x = 24) and triploid (2n = 3x = 36) progenies (Table 3.1). Beside BC1, five BC2 genotypes were also selected, three from reciprocal interploidy crosses between A and LAA and two from ALA × LA (Table 3.1). Similarly five BC3 plants were also analyzed for chromosomal recombination mapping in LA hybrids (Table 3.1). In the case of OA hybrids, only those genotypes that produced 2*n* pollen were used for backcrossing with the Asiatic cultivars as female parents (i.e., AOA). In all cases the progenies were triploid with two exceptions (Table 3.2). Similarly, ten BC2 progeny plant of OA hybrids resulted from a cross (AOA × AA) were also analysed (Table 3.2). All the plant material is maintained vegetatively at Wageningen UR Plant Breeding, Wageningen.

Mitotic chromsosome preparation and Genomic in situ hybridization (GISH)

Total genomic DNA was extracted with the CTAB method (Rogers and Bendich 1988). Genomic DNA of Longiflorum cultivar 'White Fox' and genomic DNA of Oriental cultivar 'Sorbonne' were used as probes and labeled with either digoxigenin-11-dUTP or biotin-16-dUTP by a standard nick translation protocol (Roche Diagnostics GmbH, Mannheim, Germany). Digoxigenin-labeled DNA was detected with antidigoxigenin-fluorescein raised in sheep (Boehringer, Mannheim, Germany) and amplified with fluorescein anti-sheep immunoglobulin raised in rabbit (Vector Laboratories, Burlingame, California). Biotin-labeled DNA was detected with CY-3-conjugated streptavidin and amplified with biotinylated goat-antistreptavidine (Vector Laboratories). The rest of the procedure for mitotic chromosome spreads and GISH were performed as described in Chapter 2.

Chromosome identification

In all the three genomes the chromosomes are arranged in sequence of decreasing short arm length according to Stewart (1947) taking into account the position of 45S rDNA hybridization signals in LL and OA hybrids (Lim et al. 2001b; Barba-Gonzalez et al. 2005a). Some of the chromosomes in the somatic karyotype could be identified on the basis of total length and arm ratios (e.g., 1, 2, 3 10, 11 and 12). In other cases, however, the differences in the lengths of short arms were used for identification. By using recombination sites as markers, the same chromosome could be accurately identified in 5-10 cells and compared with the previous and the succeeding chromosomes in the karyotype. Furthermore, the centromeric index [short arm length/(short arm length + long arm length)] and the relative chromosome length index (individual chromosome length/total length of all chromosomes) were determined for all genotypes (Barthes and Ricroch 2001) and used as an additional identification tool. The differences in size of the genomes of L, O and A are less than 5% (Van Tuyl and Boon 1997), so that will have no influence on the calculations.

Measurements and mapping

Images of mitotic metaphase chromosomes from each genotype were collected and were measured in micrometres using the computer program MicroMeasure (Reeves and Tears 2000). The centromere of each chromosome was taken as the starting point and recombination sites identified by GISH were used as markers for recombination mapping. Recombination sites were identified and their distances from the centromeres were measured and expressed as a percentage of the arm length (both short arm and long arm). After compiling the recombination data, the

recombination distribution was determined on each chromosome based on its length in relation to the size of the whole genome in micrometres. To determine the number of expected recombination sites (based on random distribution) on each chromosome the length of that chromosome was measured, divided by the total length of the genome and multiplied by the total number of observed recombination sites in the whole genome. The calculated expected values were compared with the observed ones for each chromosome.

Statistical analysis

We applied a χ^2 test to check whether the number of observed recombination sites significantly deviates from the expected number of recombination sites among the chromosomes. Furthermore, by considering the distances between the centromeres and the recombination sites as "*fragments*" their distribution in the four cytological maps was tested against the expected distribution to verify whether the distribution of these recombination sites is exponential (Haldane 1919).

Results

In order to construct cytological maps, as a first step, the genome composition and recombinant chromosomes were identified in 71 BC progenies (BC) of LA hybrids and 41 BC progenies of OA hybrids. Subsequently, the frequencies and distribution of recombination sites in different chromosomes of three genomes were determined.

Genome constitution and recombinant chromosomes

In the BC1 progenies of LA hybrids there were triploid as well as diploid plants together with a few aneuploids (Table 3.1). Among triploids, 35 were derived from the LAA type of crosses and 12 from the reciprocal crosses (i.e., ALA). In both cases, the chromosome constitution was predominantly 12 Longiflorum and 24 Asiatic (Table 3.1). This was consistent with the expectation that either the 2*n* egg or the 2*n* pollen from the LA hybrid had contributed 12 L + 12 A chromosomes to the BC1 progenies. There were, however, some deviations from the expected numbers which resulted from indeterminate meiotic nuclear restitution during meiosis (Lim et al. 2001a). The recombinant chromosomes could be clearly distinguished from the non-recombinant chromosomes and there were two distinct types. Chromosomes with a Longiflorum centromere possessing an Asiatic recombinant segment were indicated as L/A whereas chromosomes with

an Asiatic centromere possessing a Longiflorum recombinant segment were indicated as A/L (Fig. 3.1a and b; Table 3.1).

Table 3.1. Genome composition, recombinant chromosomes and recombination sites in the BC progenies of LA hybrids.

Cross	Genotype code	Ploidy level	Genome composition		No. of recombinant chromosomes	No. of recombination sites
			$L^{(L/A)}$	$A^{(A/L)}$		
LAA	041551	3x	12(1)	24(1)	2	2
LAA	041552	3x	12(2)	24(2)	4	4
LAA	041553	3x	12(1)	24(2)	3	3
LAA	041554	3x	12(1)	24	1	1
LAA	041555	3x+1	12(1)	25	1	1
LAA	041569	3x	12(1)	24(1)	2	2
LAA	041571	3x	12(2)	24(2)	4	4
LAA	041572	3x+1	13(2)	24(1)	3	3
LAA	041573	3x	12(1)	24(1)	2	2
LAA	041574	3x	12(1)	24(1)	2	2
LAA	041575	3x	12(1)	24(1)	2	2
LAA	041578	3x	12(1)	24	1	1
LAA	041580	3x	12(3)	24(2)	5	5
LAA	041581	3x	12	24(3)	3	3
LAA	041583	3x	12(1)	24(1)	2	2
LAA	044525-1	3x	12(1)	24(1)	2	2
LAA	044539-1	3x	12(1)	24(1)	2	2
LAA	044571-1	3x	12(1)	24(2)	3	4
LAA	062035-1	3x	12 (4)	24 (2)	6	9
LAA	062035-2	3x	12(3)	24(3)	6	7
LAA	062071-1	3x	12(7)	24(5)	12	23
LAA	062071-2	3x	13(9)	23(5)	14	42
LAA	062074-1	3x	12(7)	24(7)	14	29
LAA	062074-3	3x	12(6)	24(6)	12	20
LAA	066960-6	3x	11(4)	25(3)	7	11
LAA	066960-8	3x	12(4)	24	4	6
LAA	066960-9	3x	12(4)	24	4	8
LAA	066960-12	3x	12(4)	24(2)	6	8
LAA	066960-13	3x	12(3)	24(4)	7	9
LAA	066960-14	3x	12(1)	24(2)	3	5
LAA	066960-20	3x	11(4)	25(4)	8	11
LAA	066994-3	3x	13(11)	23(9)	20	49
LAA	066994-11	3x	12(8)	24(5)	13	23
LAA	066995-1	3x	12(5)	24(3)	8	8
LAA	066963-8	3x	12(3)	24(1)	4	4
A LA	044595-1	3x	10(2)	26(3)	5	5
A LA	044601-1	3x	12(1)	24(2)	3	3
A LA	044601-2	3x	10(2)	26(4)	6	6
A LA	044601-3	3x-1	11	24(1)	1	1
A LA	044601-4	3x	12(1)	24(1)	2	2

$L^{(L/A)}$ chromosome having a Longiflorum centromere with Asiatic recombination sites
$A^{(A/L)}$ chromosome having an Asiatic centromere with Longiflorum recombination sites

Table 3.1. cont. Genome composition, recombinant chromosomes and recombination sites in the BC progenies of LA hybrids.

Cross	Genotype code	Ploidy level	Genome composition		No. of recombinant chromosomes	No. of recombination sites
			$L^{(L/A)}$	$A^{(A/L)}$		
A LA	044601-5	3x-1	11(1)	24	1	1
A LA	044601-6	3x	12(2)	24(1)	3	3
A LA	044601-7	3x	12	24(3)	3	3
A LA	044601-8	3x+1	13(2)	24(1)	3	3
A LA	044638-1	3x-1	11(1)	24(1)	2	2
A LA	044638-2	3x	10	26(2)	2	2
A LA	044638-3	3x	10(1)	26(3)	4	4
LAA	044511-1	2x	3(1)	21(2)	3	3
LAA	044538-1	2x	7(7)	17(4)	11	23
LAA	044538-3	2x	4(2)	20(4)	6	10
LAA	044538-4	2x	5(2)	19(5)	7	14
LAA	062074-2	2x	5(2)	19(3)	5	6
LAA	066828-5	2x	4(3)	20(3)	6	8
LAA	066960-2	2x	9(5)	15	5	5
LAA	066960-5	2x	9(4)	15(2)	6	9
LAA	066960-15	2x+2	9(3)	17	3	3
LAA	066960-17	2x	9(4)	15(1)	5	5
LAA	066963-2	2x	9(6)	15(3)	9	12
LAA	066966-2	2x	6(3)	18(3)	6	10
A LA	044602-2	2x	1(1)	23(2)	3	7
A LAA*	044529-2	2x	1(1)	23	1	1
A LAA*	044530-1	2x	0	24(1)	1	1
LAA A*	044634-1	2x+1	1	24(2)	2	2
ALA LA*	066836-13	5x	18(5)	24(4)	9	13
ALA LA*	066836-45	5x-4	20(5)	36(1)	6	7
L ALA LA†	044501-1	3x+1	23(1)	14(1)	2	2
L ALA LA†	044501-2	3x	23(2)	13(4)	6	6
A ALA LA†	044506-4	3x	9(2)	27(1)	3	4
A ALA LA†	044507-2	3x	12(3)	24(1)	4	7
A ALA LA†	044507-5	3x	9(1)	27(3)	4	11
A ALA LA†	044507-6	3x	11(3)	25(1)	4	5

$L^{(L/A)}$ chromosome having a Longiflorum centromere with Asiatic recombination sites
$A^{(A/L)}$ chromosome having an Asiatic centromere with Longiflorum recombination sites
* BC2 LA hybrids; †BC3 LA hybrids

The number of these two types of recombinant chromosomes varied in different BC1 genotypes, and the total ranged from 1 to 20 (Table 3.1). This variation was expected to occur in view of the disturbed homoeologous chromosome pairing during meiosis in the LA hybrids which showed near absence to complete pairing, forming 12 bivalents (Lim et al. 2001a; Zhou 2007). A notable feature was that the 2*n* gametes had transmitted two parental genomes of the LA hybrid to the BC1 progeny and the number of recombinant chromosomes that occurred was a direct reflection of the amount of crossovers which had occurred during the meiotic division that preceded restitution nucleus formation. In the case of 14 diploid and two near diploid BC1 progenies (Table 3.1), the number of recombinant chromosomes also varied among different

genotypes (Table 3.1; Fig. 3.1b) but the range was more narrower (3 - 11) as compared with their triploid counterparts.

This could be explained by the fact that when normal *n* (haploid) gametes arise due to normal meiosis, both chromosome and chromatid segregations occurred. As a result, the number of recombinant and non-recombinant chromatids segregated randomly to four haploid spores and, therefore, the number of recombinant chromatids in each *n* gamete were expected to range only from 0 - 12. On the other hand, in restitutional meiosis leading to 2*n* gametes (and triploid BC1 progenies) only chromatid segregation had occurred (chromosome segregation at anaphase I was avoided) and a large proportion of recombinant chromatids were included in one or the other of the two spores that resulted from each cell. Thus, 2*n* gametes were able to transmit more recombinant chromosomes to the progenies as compared to *n* gametes.

The 31 BC1 progenies of OA hybrids (Table 3.2) were triploid with two exceptions. One was an aneuploid (3x + 1) and the other a tetraploid, which originated from 2*n* gametes of both parents. Nevertheless, the OA hybrid had contributed 12 O + 12 A chromosomes to the progeny in all cases (Table 3.2). The number of recombinant chromosomes varied from 0 to 9 among different genotypes (Table 3.2). Chromosomes with an Oriental centromere with a recombinant segment from an Asiatic were indicated as O/A and those with an Asiatic centromere with a recombinant segment from an Oriental as A/O (Table 3.2; Fig. 3.1c). As compared with the number of recombinant chromosomes in the triploid progenies of LA hybrids (maximum of 20), there were fewer recombinant chromosomes (maximum of 9) in the case of BC1 progenies of OA hybrids. This implied that in LA hybrids certain genotypes had much higher levels of homoeologous chromosome pairing and recombination as compared with those of OA hybrids (Barba-Gonzalez et al. 2005b). Some additional recombination sites have been found in both BC2 and BC3 progenies of LA and OA hybrids. As in BC1 progenies, a higher rate of recombination sites was found in BC2 and BC3 progenies of LA hybrids with 2-13 recombination sites per genotype as compared with BC2 progenies of OA hybrids with 1-5 recombination sites per genotype (Table 3.1 and 3.2).

Table 3.2. Genome composition, recombinant chromosomes and recombination sites in the BC progenies of OA hybrids.

Cross	Genotype code	Ploidy level	Genome composition		No. of recombinant chromosomes	No. of recombination sites
			$O^{(O/A)}$	$A^{(A/O)}$		
AOA	022538-1	3x	12(4)	24(3)	7	9
AOA	022538-3	3x	12(4)	24(2)	6	7
AOA	022538-7	3x	13(3)	23(3)	6	12
AOA	022538-8	3x	12(2)	24(2)	4	11
AOA	022538-9	3x	13(2)	23(2)	4	8
AOA	022538-15	3x	11(1)	25(3)	4	5
AOA	022538-16	3x	12(5)	24(4)	9	18
AOA	022538-17	3x	12(1)	24(1)	2	2
AOA	022605-2	3x	12(1)	24(1)	2	5
AOA	022605-3	4x	12(4)	36(3)	7	9
AOA	022605-5	3x	12(1)	24(1)	2	5
AOA	022605-8	3x	12(2)	24(1)	3	3
AOA	022605-9	3x	12(4)	24(3)	7	8
AOA	022605-10	4x	12	36	0	0
AOA	022605-11	3x	12(1)	24(1)	2	2
AOA	022605-12	3x	12(1)	24(1)	2	2
AOA	022605-18	3x	12(2)	24(2)	4	4
AOA	022605-20	3x	12(4)	24(2)	6	6
AOA	022605-21	3x	12(4)	24(2)	6	6
AOA	022605-22	3x	12(1)	24(1)	2	2
AOA	022605-24	3x	12(3)	24(2)	5	6
AOA	022605-25	3x	12(3)	24(2)	5	5
AOA	022605-27	3x	12(3)	24(2)	5	2
AOA	022605-28	3x	12(1)	24	1	1
AOA	022605-30	3x+1	12(2)	25(2)	4	8
AOA	022605-34	3x	12(2)	24(2)	2	4
AOA	022605-37	3x	12(1)	24	1	1
AOA	022605-38	3x	12(1)	24(1)	2	2
AOA	022605-39	3x	12(2)	24(2)	4	3
AOA	022605-40	3x	12(2)	24(2)	4	4
A OA	022605-46	3x	12(3)	24(3)	6	8
AOA A*	042616-2	2x+5	5(1)	24(2)	3	5
AOA A*	042620-3	3x-1	11(1)	24(1)	2	2
AOA A*	042620-14	3x-1	11(2)	24(1)	3	4
AOA A*	042627-1	2x+1	1	24(1)	1	1
AOA A*	042627-2	2x+8	8(1)	24(1)	2	2
AOA A*	042627-3	2x+7	7(1)	24(1)	2	2
AOA A*	042627-4	2x+6	6(1)	24(1)	2	2
AOA A*	042627-5	2x+5	5(1)	24(2)	3	4
AOA A*	042627-6	2x+5	5(1)	24(2)	3	4
AOA A*	042627-7	2x+8	8(2)	24(2)	4	4

$O^{(O/A)}$ chromosome having an Oriental centromere with an Asiatic recombination sites
$A^{(A/O)}$ chromosome having an Asiatic centromere with an Oriental recombination sites
*BC2 OA hybrids

Recombination sites and their distribution

One of the advantages of GISH analysis was that a direct and reliable assessment of the recombination sites on individual chromosomes could be made in the BC progenies of both LA and OA hybrids. The non-recombinant and recombinant chromosomes of both genomes could be identified unambiguously and the number of recombination sites on individual chromosomes could be counted (Fig. 3.1). The number of recombination sites on individual chromosomes varied from one to eight (Fig. 3.1a inset). The total number of recombination sites per genotype also varied from 1 to 49 in LAA and ALA and from 1 to 18 in AOA (Tables 3.1 and 3.2). The identification of two types of recombinant chromosomes and the recombination sites in the progenies of each of the hybrids, i.e., L/A, A/L in LAA or ALA, and O/A, A/O in AOA progenies, enabled a simultaneous mapping of recombination sites on all the 12 individual chromosomes of the constituent genomes of both LA and OA hybrids. A total of 248 recombination sites were mapped on the L and A genomes and a total of 116 recombination sites were mapped on the O and A genomes.

From the analysis of two hybrids, four maps were constructed as the Asiatic parent was common to both hybrids this resulted in two maps, Asiatic (L) and Asiatic (O). The remaining two were Longiflorum (A) and Oriental (A). For the construction of maps, the distances (micrometres) between the centromere and recombination site were expressed as percentages of the total length of the respective chromosome arms (Fig. 3.2). In some cases, two or more recombination sites on individual chromosomes mapped very close to each other and these were indicated by forked bars in the maps.

A remarkable feature was that the recombination sites were unevenly distributed on different chromosomes in all genomes (Table 3.3). In general, the number of recombination sites was not proportional to the size of the chromosomes. For example, the two largest chromosomes (chromosomes 1 and 2) possessed hardly any sites in both Asiatic (O) as well as Oriental (A) maps (Fig. 3.2c, and 3.2d). A similar tendency, though not as pronounced, was also evident in the case of Asiatic (L) and Longiflorum (A) maps (Fig. 3.2a and 3.2b). In contrast, large numbers of recombination sites were concentrated on the long arms of chromosomes 7, 8, 9, 10, 11 and 12 in Longiflorum (A) and Asiatic (L) genomes. Although a similar concentration of recombination sites was evident in the case of Oriental (A) and Asiatic (O) genomes, the trend was less pronounced. It should be pointed out, however, that the numbers of recombination sites mapped in the latter (OA) are less than half of those mapped in the case of the progenies of LA hybrids. In general, very few recombination sites were found on the short arms of Oriental (A) and Asiatic (O) genomes but in the case of Longiflorum (A) and Asiatic (L) genomes sites were

present on the short arms in several cases. Besides the uneven distribution of recombination sites among chromosomes, there were large gaps or even total absence of recombination sites in some cases, e.g., chromosome 6 of Asiatic (O) (Fig. 3.2d).

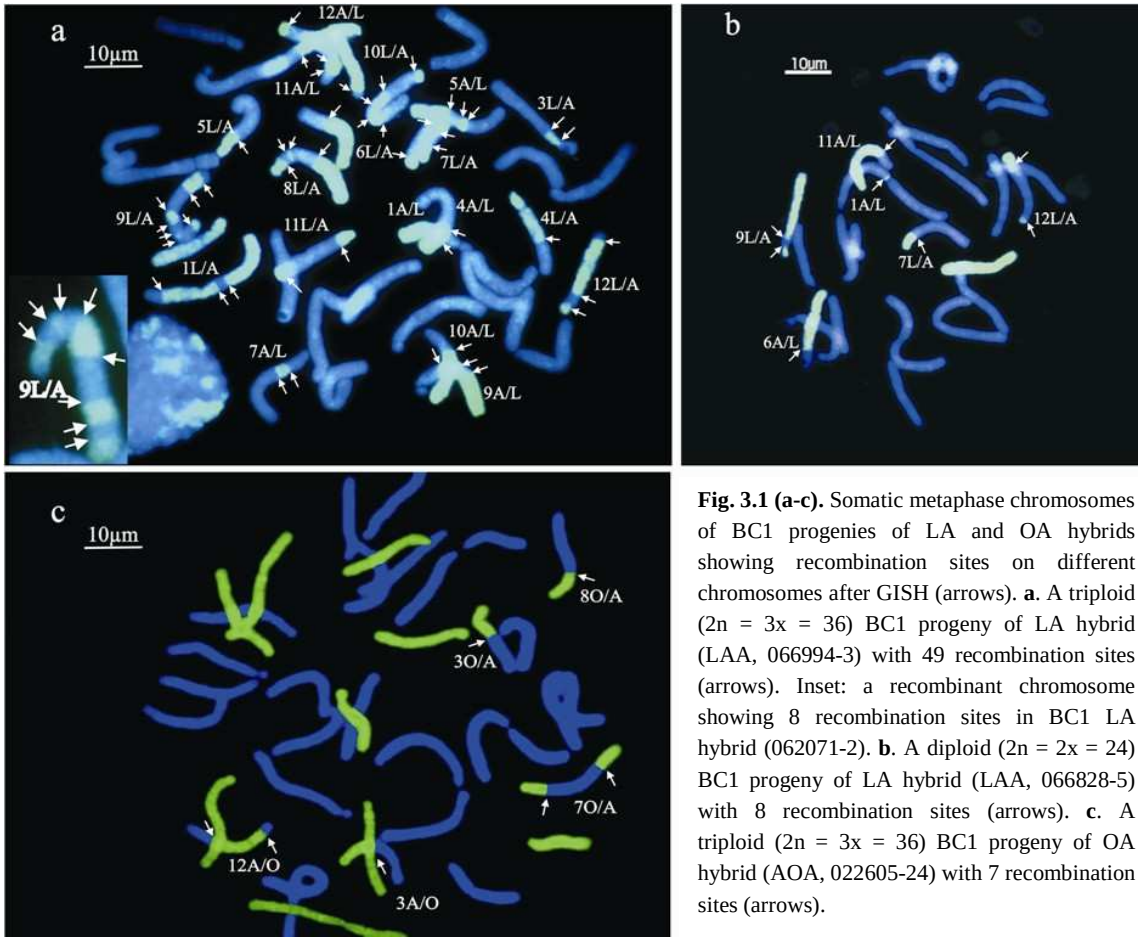

Fig. 3.1 (a-c). Somatic metaphase chromosomes of BC1 progenies of LA and OA hybrids showing recombination sites on different chromosomes after GISH (arrows). **a**. A triploid (2n = 3x = 36) BC1 progeny of LA hybrid (LAA, 066994-3) with 49 recombination sites (arrows). Inset: a recombinant chromosome showing 8 recombination sites in BC1 LA hybrid (062071-2). **b**. A diploid (2n = 2x = 24) BC1 progeny of LA hybrid (LAA, 066828-5) with 8 recombination sites (arrows). **c**. A triploid (2n = 3x = 36) BC1 progeny of OA hybrid (AOA, 022605-24) with 7 recombination sites (arrows).

The distribution of recombination sites on all 12 chromosomes of four cytological maps [(Longiflorum (A), Asiatic (L), Oriental (A) and Asiatic (O)] of three different genomes showed significant deviation from the expected number of recombination sites per chromosome at P= 0.025, <0.001, and 0.005 respectively. The contribution of individual chromosomes has been estimated and those chromosomes that contributed much to the χ^2 test are indicated in bold type (Table 3.3). In order to statistically verify whether the distribution of recombination sites on different chromosomes significantly deviated from expectation, the distances between the centromere and the recombination site in each case was considered as a *fragment* in the statistical analysis. If it is assumed that recombination sites occur along the chromosome according to a Poisson distribution (Haldane 1919), then the distribution of the fragment sizes should be exponential. So we fitted an exponential distribution from these fragment sizes and then tested

the empirical distribution of the fragment sizes against the expected exponential distribution at 95% confidence limits. There were clear deviations in all four maps (Fig 3.3).

Discussion

The cytological maps constructed in the present investigation show that the entire genomes of lilies can be mapped through GISH. Inevitably, the BC progenies from distant hybrids could be used for mapping without which the constituent genomes and the recombinant sites could not be distinguished through GISH. Thus, it should be recognized that these maps are constructed from progenies derived from distant species hybrids, such as LA and OA, which show a failure of normal chromosome pairing during meiosis. Therefore, the question arises whether these maps are comparable with those that are constructed from the progeny analyses of intra-specific hybrids which are normally used in other plant species for mapping. In the case of BC1 progenies of LA hybrids there is convincing evidence that they have originated from the functioning of both *n* and 2*n* gametes following normal chromosome pairing in the spore mother cells at least in some cases. This is evident from the occurrence of a maximum of up to 20 recombinant chromosomes and 49 recombination sites in a genotype (Fig. 3.1a; Table 3.1) and indicates normal levels of chromosome pairing in some of the LA hybrids that contributed 2*n* gametes. Based on the argument that each recombination site represents a chiasma that was formed in the spore mother cell, one should expect that 49 recombination sites in the genotype 066994-3 (Table 3.1) were the result of nearly as many chiasmata that had occurred in the megaspore mother cell.

A comparable number of chiasmata were counted in the pollen mother cells of some six *Lilium* species and four hybrid derivatives (Fogwill 1958). However, it should be mentioned that the present cytological maps only indicate the pattern of recombination in the hybrids of species and provides an insight into the nature of differentiation.

There are some notable advantages of using 2*n* gametes rather than *n* gametes for mapping of recombination sites. i) In most cases, distant hybrids do not produce *n* gametes because of abnormal meiosis, as in the case for OA hybrids, and the only method of mapping in such cases is through the use of 2*n* gametes. ii) A large number of recombinant chromosomes and recombination sites can be recovered in the BC progenies when 2*n* gametes are used instead of *n* gametes. This is because almost always, (only) chromatid segregation occurs during meiosis that leads to restitution gametes. As a result, the probability of inclusion of crossover chromatids in each of the two unreduced spores that result from such meiosis is increased.

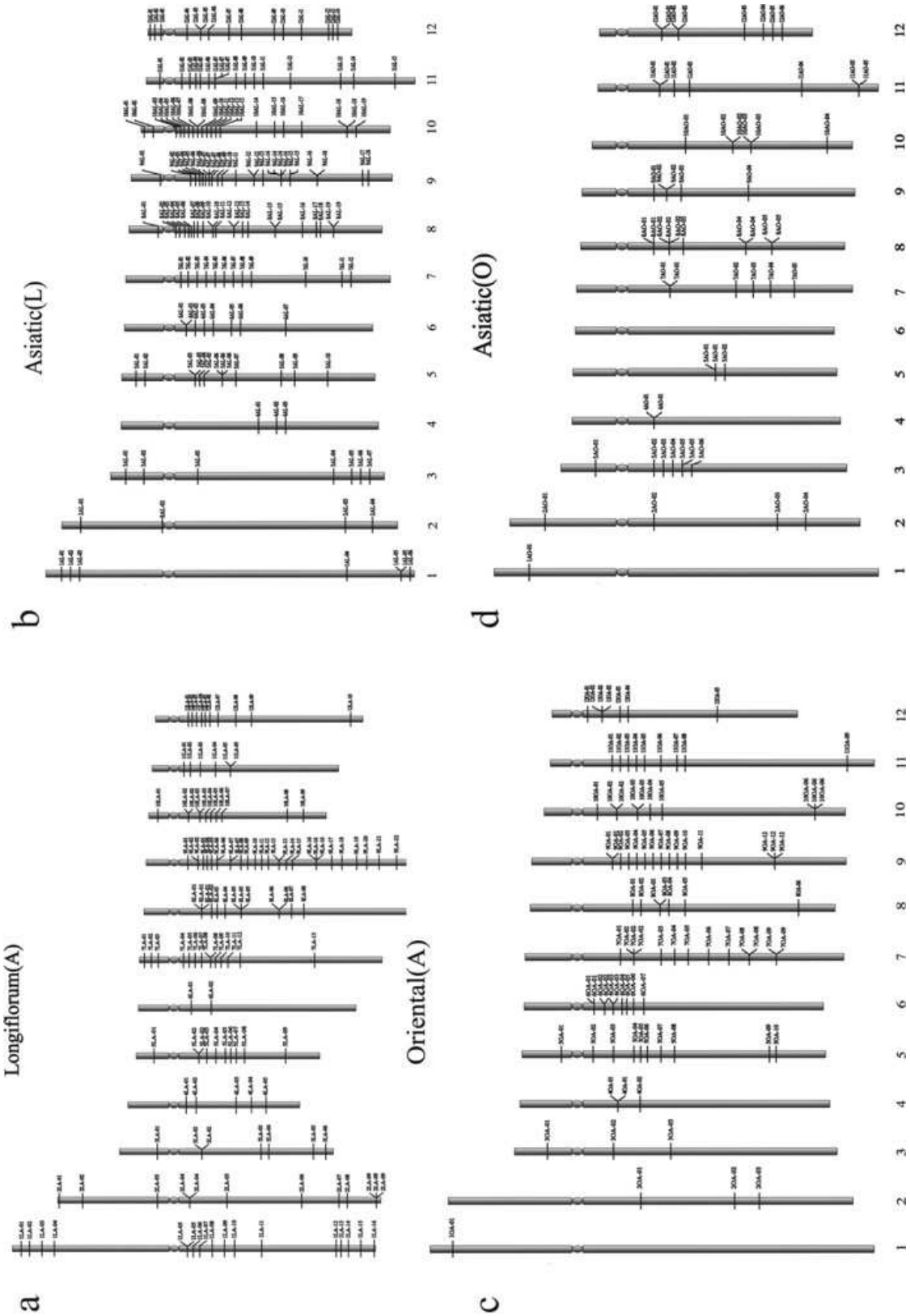

Fig. 3.2 (a-d). Four chromosomal recombination maps resulting from the analysis of BC progenies of LA and OA hybrids. The genomes are indicated as Longiflorum (A), Asiatic (L), Oriental (A) and Asiatic (O)- the recombination partner in each is given in parenthesis

Table 3.3. Chromosome length, expected and observed recombination sites per chromosome and their distribution in three genomes of *Lilium.*

Chromosome #	Total length of chromosome (µm)	Distance to centromere (µm)		No. of expected breaking points	No. of observed breaking points	% of expected values	$(O-E)^2/E$
		Long arm	Short arm				
1[L/A]	36.76	20.6	16.15	14.81	16	108.03	0.09
2	33.08	21.58	11.5	13.38	9	67.27	1.43
3	21.2	15.64	5.26	8.39	6	71.51	0.68
4	18.25	13.9	4.33	7.22	5	69.25	0.68
5	18.56	15.16	3.4	7.34	9	68.11	0.37
6	21.5	18.26	3.25	8.51	2	23.50	**4.98**
7	24	21	3	9.5	13	136.84	1.28
8	27.06	24.41	2.65	10.71	8	74.69	0.68
9	26.75	24.4	2.36	10.58	22	198.48	**12.32**
10	17.2	15.16	2.03	6.8	9	132.35	0.71
11	18.2	16.41	1.78	7.2	5	69.44	0.67
12	20.36	19	1.36	8.05	10	111.80	0.47
							24.40*
1[A/L]	33.5	22.5	11	15.45	6	38.83	**5.78**
2	30.43	20.83	9.6	13.93	4	28.71	**7.07**
3	24.66	19.66	5	11.9	7	58.82	2.01
4	23.09	19.06	4.03	10.57	3	28.38	**5.42**
5	22.79	18.83	3.96	10.43	10	94.6	0.01
6	22.33	18.6	3.73	10.22	7	68.5	1.01
7	23.75	20.25	3.5	10.87	12	110.4	0.11
8	22.71	19.41	3.3	10.4	19	182.7	**7.11**
9	23.41	20.41	3	10.71	18	168.06	4.96
10	22.45	20.25	2.2	10.27	19	185	**7.42**
11	24.26	22.53	1.73	11.1	15	135.13	1.37
12	18.19	16.66	1.53	8.32	14	168.27	3.87
							46.14*
1[O/A]	39.86	26.8	13.06	8.36	1	11.96	**6.47**
2	36.59	25.23	11.36	7.69	3	39.01	2.86
3	28.76	23.46	5.3	6.05	3	49.58	1.53
4	27.46	22.7	4.76	5.77	2	34.66	2.46
5	26.96	22.33	4.63	5.67	10	176.36	3.30
6	26.56	22.16	4.4	5.58	7	123.45	0.36
7	28.33	24.03	4.3	5.96	9	151.00	1.55
8	27.06	23.26	3.8	5.69	6	105.44	0.01
9	27.83	24.23	3.6	5.85	12	205.12	**6.46**
10	26.84	24.21	2.63	5.64	6	106.38	0.02
11	29.22	27.26	1.96	6.14	9	146.57	1.33
12	21.51	19.75	1.76	4.52	5	110.61	0.05
							26.44*

[L/A] Longiflorum chromosome with an Asiatic recombination sites; [A/L] Asiatic chromosome with a Longiflorum recombination sites
[O/A] Oriental chromosome with an Asiatic recombination sites; [A/O] Asiatic chromosome with an Oriental recombination sites: * Contribute significantly at P= 0.025, <0.001 and 0.005. Chromosomes that contributed much to the χ^2 test are indicated by bold type

Table 3.3. cont. Chromosome length, expected and observed recombination sites per chromosome and their distribution in three genomes of *Lilium.*

Chromosome #	Total length of chromosome (µm)	Distance to centromere (µm)		No. of expected breaking points	No. of observed breaking points	% of expected values	$(O-E)^2/E$
		Long arm	Short arm				
1[A/O]	33.5	22.5	11	4.8	1	25	**3.00**
2	30.43	20.83	9.6	4.36	4	91.75	0.02
3	24.66	19.66	5	3.53	6	181.26	1.72
4	23.09	19.06	4.03	3.31	1	30.2	1.61
5	22.79	18.83	3.96	3.36	2	59.52	0.10
6	22.33	18.6	3.73	3.2	0	0	**3.2**
7	23.75	20.25	3.5	3.4	5	156.25	0.63
8	22.71	19.41	3.3	3.25	5	153.84	0.94
9	23.41	20.41	3	3.35	4	119.4	0.12
10	22.45	20.25	2.2	3.22	4	124.22	0.18
11	24.26	22.53	1.73	3.48	5	143.67	0.66
12	18.19	16.66	1.53	2.61	6	229.8	**4.40**
							16.22

[A/O] Asiatic chromosome with an Oriental recombination sites: Chromosomes that contributed much to the χ^2 test are indicated by bold type

On the contrary, when *n* gametes originate through normal meiosis, both chromosome and chromatid segregation occur and the four chromatids of each tetrad (of a bivalent) are distributed to four haploid spores. The differences in the number of recombinant chromosomes and recombination sites observed in the case of triploid and diploid progenies of LA hybrids (Table 3.1) clearly illustrate the efficiency of 2*n* gametes. iii) Whereas *n* gametes are more likely subjected to selection during the process of fertilization or subsequent development, 2*n* gametes might be less susceptible for selection because they include both parental genomes nearly intact and are genetically more homogeneous.

Various methods have been used for the construction of cytological, cytogenetic, or cytomolecular maps (Khrustaleva et al. 2005). Maps constructed by using recombination sites have three important advantages. i) They serve as permanent cytological land marks on chromosomes that can be used for mapping molecular markers or genes of interest because of vegetative propagation in lilies. ii) They mark crossing-over sites in the genomes. iii). They give a clear picture of whole genomic structure rather than concentrating on only one or two chromosomes which might not be the representative for the whole genome. The numbers of crossovers on different chromosomes are disproportionately distributed on all the four maps. The largest chromosomes have very few recombination sites (e.g., chromosomes 1 and 2, Figs. 3.2c and 3.2d) whereas smaller chromosomes possess far more sites as in chromosomes 10 (Fig. 3.2a), 8 and 9 (Fig. 3.2b).

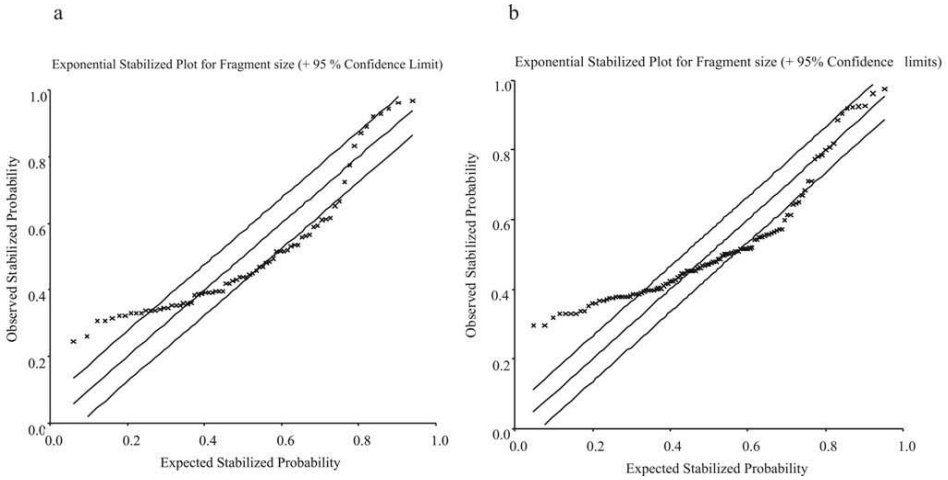

Fig. 3.3. Q-Q plots for the exponential distribution of "fragments" (distance between the centromere and the recombination sites) for observed and expected probabilities at 95% confidence limits among different chromosomes of two genomes i.e. a. Longiflorum (A) and b. Oriental (A).

With regards to the positions of recombination sites, there is no regular pattern that can be consistently assigned to any set of chromosomes. There are some instances of clustering of recombination sites in the proximal parts for a number of chromosomes i.e., near the centromere of chromosome 1, 7, 10 and 12 (Fig. 3.1a), chromosome 6, 7 (Fig. 3.2b) 6 and 12 (Fig. 3.2c) and 8 (Fig. 3.2d). But a similar pattern is not present in other chromosomes. There are fewer instances of clustering of recombination sites in the distal regions of the Longiflorum (A) genome i.e., chromosomes 1, 2 and 9 (Fig. 3.2a), and the Asiatic (L) genome, i.e., chromosome 1, 3 and 12 (Fig. 3.2b). The results are contrary to those for the wheat genome in which the recombination was low in proximal chromosomal regions and very high towards the distal ends (Werner et al. 1992; Akhunov et al. 2003). The present study reveals that there is an increase in recombination events just next to the centromere in most cases (Fig. 3.2). Previously, it was also found that recombination increases with relative distance from the centromere in various large as well as small genome crops like maize (Lukaszewski and Curtis 1993; Anderson et al. 2004). Similar behavior has been found in rice (Wu et al. 2003; Kao et al. 2006). A common feature is that all these species have less recombination just next to the telomere. However, in barley, it was found that the extreme distal regions of several chromosome arms have areas of increased recombination too (Künzel et al. 2000). Our investigation shows a gradient of recombination from one chromosome to another within a genome and also from one part to another on the same chromosome. A detailed cytological analysis of lily species genomes shows that distal recombination is not a general rule in *Lilium*.

One important feature that is common to all four cytological maps is that there are large gaps where there are no recombination sites at all, for instance, the chromosome 6 of Asiatic (O)

(Fig. 3.2d). An explanation for the occurrence of such gaps has to be deferred until more data becomes available. The most likely explanation for such gaps is the occurrence of structural differences between homoeologous chromosomes, especially heterozygous paracentric inversions. Indeed, paracentric inversions do occur in *Lilium* (Brown and Zohary 1955). If a chiasma occurs in the inverted segment, it leads to a dicentric bridge which might eliminate the chromatids concerned. The large numbers of gaps that are observed in the maps do indicate that constituent genomes of the two hybrids might be heterozygous for many paracentric inversions. This, however, needs to be confirmed.

Reduced recombination has been found between Oriental (A) and Asiatic (O) cytological maps. This might be attributed to larger genome divergence between A and O as compared with the L and A genomes in *Lilium*. We therefore conclude that there is more recombination between genomes in L and A than between those in O and A. Furthermore, chromosome pairing and crossing-over are genetically controlled and are thus genotype dependent. An attractive feature of the cytological maps is that large numbers of recombination sites become available as physical land marks on individual chromosomes. Using such sites or land marks, molecular markers, like AFLPs and RFLPs can be assigned to specific positions on individual chromosomes as has been done in the case of a *Festuca-Lolium* substitution line (King et al. 2002a) and *Allium cepa* × (*A. roylei* × *A. fistulosum*) interspecific crosses (Khrustaleva et al. 2005). Mapping of individual chromosome in these two afore mentioned cases shows that proper integration of molecular maps with respective chromosomes can only be accomplished when reliable cytological markers are available. Moreover, molecular maps reported for several crop plants have either under- or overestimated the map lengths depending upon the type of markers used, or the type of software used for the analysis of the data (King et al. 2002b). In order to overcome such pitfalls, cytological maps of genomes such as the ones reported here can pave the way for the construction of more meaningful integrated maps.

Chapter 4

Relevance of unilateral and bilateral sexual polyploidization in relation to intergenomic recombination and introgression in *Lilium* species hybrids

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Abstract

Sexual polyploids were induced in diploid (2n = 2x = 24) interspecific F1 hybrids of Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) *Lilium* hybrids by backcrossing to Asiatic (AA) parents as well as by sib-mating of the F1 LA hybrids. The BC1 progenies were triploid, with few exceptions and the progenies from sib-mating were tetraploid or near tetraploids. Genomic *in situ* hybridization (GISH) technique was applied to assess the intergenomic recombination in the BC1 populations of LA and OA hybrids obtained after unilateral sexual polyploidization. In case of LA hybrids, LA × AA and in reciprocal cross (AA × LA) a total of 63 plants were analysed. These plants were originated through the functioning of 2*n* gametes either as 2*n* eggs or 2*n* pollen. Similarly 53 plants from BC1 population of OA hybrids were also analysed. Most of these BC1 progenies of OA hybrids comprised of triploid plants which originated through functional 2*n* pollen from genotype (952400-1) of diploid OA hybrid. In both type of crosses, a majority of the progenies had originated through First Division Restitution (FDR) mechanism of functional 2*n* gamete either with or without a cross over. However, there were nine genotypes in LA × AA and its reciprocal cross where Indeterminate Meiotic Restitution (IMR) was the mechanism of 2*n* gamete formation. Similarly four genotypes were found in OA hybrids which originated from IMR 2*n* gamete. Based on GISH analyses the total amount of introgression of Longiflorum and Oriental genome into Asiatic genome was determined. Most of the LA hybrids exhibited recombination and the amount of recombination was higher in LA hybrids as compared to OA hybrids. Intergenomic recombination was also estimated in the progeny of sib-mated LA hybrid. In this case both parents had contributed gametes with the somatic number of chromosomes (i.e., 2*n* - 2*n*). This population consisted of 23 plants and originated through bilateral sexual polyploidization. GISH technique was applied on 16 plants of this population. Based on these results the nature of interspecific lily hybrids obtained from uni- and bilateral sexual polyploidization leading to allotriploid and allotetraploid formation in interspecific lily hybrids is discussed in the context of introgression and mapping.

Introduction

Sexual production of polyploids involve the fusion of two gametes, one or both of them contributing the somatic or unreduced number of chromosomes to the progeny. Depending on the parental contribution, the sexual polyploidization is either unilateral or bilateral (Mendiburu and Peloquin 1976). Normal meiosis in germ cells (pollen- or embryo sac mother cells) results in the formation of four haploid (*n*) gametes following regular chromosomes association and disjunction. On the other hand, 2*n* gametes originate due to deviating meiosis in plants. The process that leads to 2*n* gamete formation is called meiotic nuclear restitution that occurs either during micro- or megasporogenesis. Depending on the particular meiotic stages at which nuclear restitution occurs, different restitution mechanisms have been recognized, *viz*., first division restitution (FDR), second division restitution (SDR) (Mok and Peloquin 1972; Ramanna 1979) indeterminate meiotic restitution (IMR) (Lim et al. 2001a), and post meiotic restitution (PMR) (Bastiaanssen et al. 1998).

The formation of 2*n* gametes is a common phenomenon in angiosperms (de Wet 1980) which are thought to play a most important role in the origin and evolution of polyploid series (Harlan and de Wet 1975; Jackson 1976). The sexual polyploidization using 2*n* gametes is not only used to increase the numbers of genomes in the progeny but it also contributes towards the achievement of genetic variation in the progenies (Ramanna and Jacobsen 2003; Barba-Gonzalez et al. 2005a). Furthermore, it also represents the convergence of variability from divergent genotypes into a single individual resulting in heterosis (Ortiz 1997). These two characteristics make sexual polyploids superior to the somatic doubling of chromosomes, which only increase the number of respective chromosomes without creating any genetic variation in the progenies. Breeders of many vegetatively propagated crops have been taking advantage of the spontaneously occurrening 2*n* gametes for introgressing genes from wild diploid species to their cultivated polyploid crops through ploidy manipulations (Peloquin and Ortiz 1992). The usefulness of polyploids for crop improvement that originate through the functioning of 2*n* gametes has been demonstrated in some of the crops like potato (Mendiburu and Peloquin 1971; Mendiburu et al. 1974), alfalfa (Bingham 1980; Veronesi et al. 1986), red clover (Parrot and Smith 1984; Smith et al. 1985), blueberry (Lyrene et al. 2003), blackberry (Hall 1990), cassava (Ogburia et al. 2002), peanuts (Stalker and Moss 1987), strawberry (Bringhurst and Voth 1984), sugarcane (Bremer 1961), sweet potato (Lopez-Lavalle and Orjeda 2002) and banana (Ortiz and Vuylsteke 1995).

The species of the genus *Lilium* constitute the most important cut flower crops in The Netherlands. However, most of the cultivars have interspecific origin within three different

taxonomic sections. For example, the widely grown cultivars of *Lilium* belong to Longiflorum (L-genome), Asiatic (A-genome) and Oriental (O-genome) hybrids derived from sections Leucolirion, Sinomartagon and Archelirion respectively (Lim et al. 2000). The species *Lilium* possess a wide range of horticultural traits, such as flower size, shape, colours and their arrangement, different forcing times, variation in stem length and strength. However, the most important amongst these are the resistances to various pathogens which are restricted only to certain hybrids within the different sections.

It is most desirable to combine or introgress some important horticultural traits from different species into a single cultivar. In *Lilium*, however, it is difficult to obtain F1 interspecific hybrids between species or cultivars that belong to different taxonomic sections. Nearly all the F1 Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) hybrids are sterile. This sterility is due to irregular chromosome paring between the parental genomes during meiosis (Lim et al. 2000; Barba-Gonzalez et al. 2004). Somatic chromosome doubling of the F1 hybrids can produce allotetraploids in which homologous chromosomes can pair and restore fertility. This is one way to overcome the F1-sterility problem (Van Tuyl and De Jeu 1997). However, this method could not contribute much to introgression breeding due to the formation of the so-called "permanent hybrids" as their progenies never segregate for parental characters due to autosyndetic pairing (Ramanna and Jacobson 2003; Van Tuyl and Lim 2003). On the contrary, intergenomic recombination may occur in sexual polyploids induced through the use of 2*n* gametes (Ramanna et al. 2003; Ramanna and Jacobson 2003). Interestingly, some F1 LA and OA hybrids could produce functional 2*n* pollen to some extent. These 2*n* pollen producing Oriental × Asiatic (OA hybrids) and Longiflorum × Asiatic (LA hybrids) F1 hybrids and their triploid progenies were analysed cytologically (Barba-Gonzalez et al. 2005a; Zhou et al. 2008). It was found that homoeologous chromosomes in 2*n* pollen hybridize pair and recombine to certain extents. The subsequent backcross progenies obtained by back crossing these 2*n* pollen producing F1 LA and OA hybrids with Asiatic parents gave triploid progenies with certain amount of intergenomic recombination (Zhou et al. 2008; Barba-Gonzalez et al. 2005a). As 2*n* pollen were responsible for sexual polyploidization and genetic recombination in interspecific lily hybrids (Lim et al. 2001a; Barba-Gonzalez et al. 2004) then it is possible that 2*n* eggs could also be detected in F1 interspecific hybrids. Recently (Zhou et al. 2008) showed that some F1 LA hybrids also produced 2*n* eggs in considerable frequencies which transmitted the intergenomic recombination to certain extent to the subsequent progenies when backcrossed with Asiatic parents.

It was found that F1 interspecific lily hybrids producing 2*n* gametes results in homoeologous chromosomes recombination in the following generations which ultimately leads to considerable genetic variation (Barba-Gonzalez et al. 2004). This may result in the assembling of complementary characters from different species in to a single genotype in a back cross breeding program in *Lilium*. By keeping in view the nature of lily interspecific hybrids, attempts were made to back cross 2*n* gametes producing LA and OA parents with Asiatic cultivars. In this way an allotriploid BC1 interspecific lily hybrids could be achieved. Attempts were also made to cross F1 LA hybrids which produced both 2*n* pollen and 2*n* eggs (LA × LA). In this way allotetraploid lily interspecific hybrids could be achieved by the union of unreduced gametes from both parents. Until now there have been no cytogenetic reports for the occurrence of bilateral sexual polyploidization in interspecific lily hybrids.

The objective of this work was to evaluate the probable consequences of unilateral and bilateral sexual polyploidization cytologically and to discuss the prospects of uni- and bilateral sexual polyploidization in interspecific lily hybrids. Furthermore, the studies have been elaborated to illustrate how a backcross breeding program enables the transfer of L- and O-genome into A-genomes originated from functional 2*n* eggs and 2*n* pollen respectively. The GISH approach has been used here for the identification of parental genomes and to estimate the amount of intergenomic recombination in BC1 progenies of F1 LA and OA hybrids produced from functional 2*n* eggs and 2*n* pollen. The usefulness of 2*n* gametes in generating genetic variation by introgression breeding in *Lilium* has been discussed using allotriploid BC1 LA and OA hybrids as well as sib-mated allotetraploid F2 LA hybrids.

Materials and Methods

Plant material

Most of the diploid F1 Longiflorum × Asiatic hybrid (2n = 2x = 24) cultivars and some of the triploid interspecific hybrids (Table 4.1) were supplied by the Dutch lily breeding companies: De Jong Lelies BV, Royal Van Zanten BV, Testcentrum, Vletter and Den Haan BV and World Breeding BV. However, one F1 LA (024004-5) and Oriental × Asiatic (951502-1) were developed at Plant Breeding, Wageningen UR (Table 4.1 and 4.2). Because the three groups belong to three different taxonomic sections, the cultivars of different sections could be hybridized, or backcrossed, only through special techniques (Van Tuyl et al. 1991; Lim et al. 2001a; Barba-Gonzalez et al. 2004). 46 allotriploid (2n = 3x = 36) BC1 LA progeny plants were obtained by backcrossing three different F1 LA hybrids as female parents with nine different Asiatic cultivars to get progeny plants of LA × AA, indicated as LAA (Table 4.1) while in

reciprocal cross 15 plants were produced where three F1 LA hybrids were used as male parents and backcrossed with three different Asiatic cultivars. In case of OA BC1 progenies, two diploid Asiatic cultivars *viz.* 'Amarone' and 'Gironde' were used as female parents and crossed with one F1 OA hybrid to get 53 AA × OA genotypes (Table 4.2). In order to produce allotetraploid (2n = 4x = 48) F2 progenies, only one F1 LA hybrids (041502) was used as male parent and crossed with three different F1 LA hybrids (041546, 041548 and 041556) as female parents. So a total of 23 genotypes were obtained with a cross combination of LA × LA and 16 of them were analysed cytologically (Table 4.3). All the plant material is being maintained vegetatively at Wageningen UR Plant Breeding, Wageningen, The Netherlands.

Flow cytometry

Flow cytometry was done to evaluate the ploidy level of the BC1 and F2 progenies. The germinating embryos were transferred into the propagation medium and allowed to grow until leaves developed. One leaf or scale was collected from each seedling for testing ploidy level as described by Van Tuyl and Boon (1997).

Mitotic chromosome preparation, Genomic in situ hybridization (GISH) and identification and measurement of genome contribution

The mitotic metaphase chromosome preparation, Genomic *in situ* hybridization (GISH) and identification and measurement of various genome contributions were carried out according to Chapter 2 and 3.

Results

Unilateral sexual polyploidization

The number of genotypes of both LA and OA hybrids that produced notable frequencies of either 2*n* eggs or 2*n* pollen were generally few and in none of the cases any single genotype produced both types of gametes. Therefore, unilateral sexual polyploidization was carried out by using the 2*n* gamete producing genotypes either as male or female parents to backcross with Asiatic parents. For bilateral sexual polyploidization, however, it was necessary to cross (sib-mate) the genotypes that produced 2*n* eggs with those that produced 2*n* pollen. In the case of LA hybrids, there were genotypes that produced either 2*n* eggs or 2*n* pollen. This was not the case with OA hybrids in which only one genotype that produced 2*n* pollen was available. For this reason bilateral sexual polyploidization was possible in the case of LA hybrids but not in the case of OA hybrids.

Production of progenies from LA × AA and reciprocal

During present study, efforts were made to produce a fairly large number of backcross progenies from unilateral sexual polyploidization. In case of LA hybrids progenies were obtained by using both 2*n* eggs and 2*n* pollen. Three different F1 LA hybrids were used as female parents and backcrossed with nine different Asiatic cultivars to get LA × AA (LAA) progeny plants (Table 4.1). A total of 46 allotriploid (2n = 3x = 36) and two aneuploid (2n = 3x = 36 + 1) BC1 LA progeny plants were obtained. This indicated that it is possible to produce a large number of progenies by using 2*n* eggs of F1 LA hybrids. In the reciprocal cross 15 plants were produced where three F1 LA genotypes were used as male parents and backcrossed with three different Asiatic cultivars. Eleven of these plants were triploid (2n = 3x = 36) while four of them had aneuploid condition (2n = 3x = 36 +1 or -1). The occurrence of allotriploids in AA × LA (ALA) crosses indicated that F1 LA hybrids produced functional 2*n* pollen. Besides the progenies obtained from the use of 2*n* eggs and 2*n* pollen from LA hybrids in the present study, the genome constitution of some of the cultivars supplied by the lily breeders are also included in Table 4.1 (19 genotypes at the end of the Table 4.1). These had resulted from unilateral sexual polyploidization in the breeders nurseries (for proprietary reasons, the names of the cultivars and their parentage are not mentioned in the Table 4.1)

Production of progenies of AA × OA crosses

In order to generate OA progeny through unilateral sexual polyploidization from OA hybrids, only one F1 OA hybrid was used a male parent and backcrossed to two different Asiatic cultivars to get AA × OA (AOA) types of BC1 progenies. Although various parental combinations were tried (data not shown) to get allotriploid progenies through unilateral sexual polyploidization, there was no success. This was due to the frequency of 2*n* pollen produced by different F1 OA hybrids which was not sufficient for a successful backcross. A total of 53 plants were obtained from two different parental combinations (Table 4.2). The ploidy levels of all these progenies were determined through flow cytometry (data not presented) as well as by cytological counting of the chromosomes. Most of the resulting progenies were allotriploid (2n = 3x = 36) indicating the production of functional 2*n* pollen in F1 OA hybrids.

Table 4.1. Progeny plants obtained from unilateral sexual polyploidization of LA × AA and AA × LA crosses, their ploidy levels, genome composition (number of recombinant chromosomes) and the percentage of genome contribution of the parents.

Cross	Genotypes	Parents		Ploidy Level	Genome Composition		Genome %		No. of recombinant chromosomes
		Female	Male		$L^{L/A}$	$A^{A/L}$	L%	A%	
LA A	044525-1	041556	Mont Blanc	3x	12(1)	24(1)	34.8	65.2	2
LA A	044539-1	041558	Pollyanna	3x	12(1)	24(1)	33.3	66.7	2
LA A	044571-1	041557	Mont Blanc	3x	12(1)	24(2)	33.5	66.5	3
LA A	062035-1	041560	061092	3x	12(4)	24(2)	32.81	67.19	6
LA A	062035-2	041560	061092	3x	12(3)	24(3)	32.85	67.15	6
LA A	062071-1	041560	061091	3x	12(7)	24(5)	31.38	68.62	12
LA A	062071-2	041560	061091	3x	13(9)	23(5)	33.34	66.66	14
LA A	062074-1	041560	061085	3x	12(7)	24(7)	30.66	69.34	14
LA A	062074-3	041560	061085	3x	12(6)	24(6)	34.82	65.18	12
LA A	062074-4	041560	061085	3x	12(7)	24(7)	36.55	63.45	14
LA A	065051-2	024004-5	061095	3x	12(2)	24(4)	35.93	64.07	6
LA A	066828-2	041543	051072	3x	12	24(1)	36.14	63.86	1
LA A	066828-4	041543	051072	3x	12(1)	24(1)	33.33	66.67	2
LA A	066960-4	045143	031110	3x	12(4)	24(3)	32.37	67.63	7
LA A	066960-6	045143	031110	3x	11(4)	25(3)	32.20	67.8	7
LA A	066960-8	045143	031110	3x	12(4)	24	29.98	70.11	4
LA A	066960-9	045143	031110	3x	12(4)	24	30	70	4
LA A	066960-12	045143	031110	3x	12(4)	24(2)	33	67	6
LA A	066960-13	045143	031110	3x	12(3)	24(4)	34.17	65.83	7
LA A	066960-14	045143	031110	3x	12(1)	24(2)	37	63	3
LA A	066960-16	045143	031110	3x	12(4)	24(4)	30	70	8
LA A	066960-20	045143	031110	3x	11(4)	25(4)	33.53	66.47	8
LA A	066963-5	045143	031039	3x	12(6)	24(3)	31.37	68.63	9
LA A	066963-8	045143	031039	3x	12(3)	24(1)	34.08	65.92	4
LA A	066994-3	041560	051073	3x	13(11)	23(9)	33.91	66.09	20
LA A	066994-4	041560	051073	3x	12(6)	24(6)	35.60	64.4	12
LA A	066994-11	041560	051073	3x	12(8)	24(5)	32.63	67.37	13
LA A	066994-12	041560	051073	3x	12(5)	24(8)	35.96	64.04	13
L AA	066995-1	041560	031040	3x	12(5)	24(3)	37.01	62.99	8
A LA	044595-1	Pollyanna	041519	3x	10(2)	26(3)	29.65	70.5	5
A LA	044595-4	Pollyanna	041519	3x	12	24	33	67	0
A LA	044601-1	Mont Blanc	041502	3x	12(1)	24(2)	34.3	65.7	3
A LA	044601-2	Mont Blanc	041502	3x	10(2)	26(4)	31.04	68.96	6
A LA	044601-3	Mont Blanc	041502	3x-1	11	24(1)	32.0	68	1
A LA	044601-4	Mont Blanc	041502	3x	12(1)	24(1)	33.0	67.0	2
A LA	044601-5	Mont Blanc	041502	3x-1	11(1)	24	30.5	69.5	1
A LA	044601-6	Mont Blanc	041502	3x	12(2)	24(1)	31.6	68.4	3
A LA	044601-7	Mont Blanc	041502	3x	12	24(3)	33.7	66.3	3
A LA	044601-8	Mont Blanc	041502	3x+1	13(2)	24(1)	34.5	65.5	3
A LA	044638-1	Vivaldi	041502	3x-1	11(1)	24(1)	32.31	67.9	2
A LA	044638-2	Vivaldi	041502	3x	10	26(2)	30.25	69.75	2
A LA	044638-3	Vivaldi	041502	3x	10(1)	26(3)	31.4	68.9	4
A LA	044638-4	Vivaldi	041502	3x	12	24	33.3	66.7	0
A LA	044638-6	Vivaldi	041502	3x	12	24	33.3	66.7	0
LA A	041551*			3x	12(1)	24(1)	33.3	66.7	2
LA A	041552*			3x	12(2)	24(2)	32.7	67.3	4
LA A	041553*			3x	12(1)	24(2)	35.4	64.6	3
LA A	041554*			3x	12(1)	24	31.7	68.3	1
LA A	041555*			3x+1	12(1)	25	31.9	68.1	1
LA A	041568*			3x	12	24	33.3	66.7	0
LA A	041569*			3x	12(1)	24(1)	33.3	66.7	2
LA A	041571*			3x	12(2)	24(2)	32.3	67.7	4
LA A	041572*			3x+1	13(2)	24(1)	33.8	66.2	3
LA A	041573*			3x	12(1)	24(1)	33.3	66.7	2
LA A	041574*			3x	12(1)	24(1)	33.3	66.7	2

*Names of the cultivars and their parentage are not mentioned due to proprietary considerations

Table 4.1. Cont. Progeny plants obtained from unilateral sexual polyploidization of LA × AA and AA × LA crosses, their ploidy levels, genome composition (number of recombinant chromosomes) and the percentage of genome contribution of the parents.

Cross	Genotypes	Parents		Ploidy Level	Genome Composition		Genome %		No. of recombinant chromosomes
		Female	Male		$L^{L/A}$	$A^{A/L}$	L%	A%	
LA A	041575*			3x	12(1)	24(1)	33.3	66.7	2
LA A	041576*			3x	12	24	33.3	66.7	0
LA A	041578*			3x	12(1)	24	31.4	68.6	1
LA A	041579*			3x	12	24	33.3	66.7	0
LA A	041580*			3x	12(3)	24(2)	31.2	68.8	5
LA A	041581*			3x	12	24(3)	38.2	61.8	3
LA A	041582*			3x	12	24	33.3	66.7	0
LA A	041583*			3x	12(1)	24(1)	33.2	66.8	2

*Names of the cultivars and their parentage are not mentioned due to proprietary considerations

Bilateral sexual polyploidization

This was possible only in LA hybrids because of the above stated reason. There was scarcity of genotypes in interspecific lily hybrids which produce both 2*n* eggs and 2*n* pollen simultaneously. So sib-matings were made between F1 LA hybrids which were found to produce either 2*n* eggs or 2*n* pollen in order to get a F2 population. Three F1 LA hybrids which produced 2*n* eggs were used as female parents and crossed with one F1 LA hybrid as male parent which produced 2*n* pollen. So the resultant progenies were expected to be allotetraploid. As a result of this combination (LA × LA) a total of 23 plants were obtained. The ploidy level of all these genotypes was determined through flow cytometry (data not presented) and 16 of these plants were analysed cytologically (Tables 4.3).

Genomic structure of BC progenies

Genome composition of LA × AA and AA × LA progenies

With GISH technique, it was possible to identify the chromosomes of parental genomes and recombinant segments in the progenies (Fig 4.1). Table 4.1 reports genome composition of BC1 genotypes obtained after unilateral sexual polyploidization. Here F1 LA hybrids backcrossed reciprocally with different Asiatic parents to get LA × AA and AA × LA type of progenies respectively. A total of 48 genotypes where F1 LA hybrid is used as female parent were analysed with GISH. Similarly, 15 genotypes were also investigated where F1 LA hybrid used as male parent. Most of the analysed genotypes were triploid (2n = 3x = 36). This observation of allotriploid BC1 progenies proved that the F1 LA hybrids had contributed balanced 2*n* chromosome complement. However, two genotypes had an aneuploid condition (3x + 1) in case of LA × AA type of cross while in reciprocal cross (AA × LA) four aneuploid (3x +1 and -1)

genotypes were obtained (Table 4.1). This condition could be explained on the basis of the 2*n* +1 and -1 egg produced in the F1 LA hybrids. In a majority of the triploid BC1 LA progeny plants, 12 chromosomes of the L genome and 24 of the A genome were clearly identified (in the case of recombinant chromosomes only the centromere was taken into consideration). However, there were four genotypes (062071-2, 066960-6, 066960-20, 066994-3) recovered in LA × AA progeny where there was deviation from the expected 12 L and 24 A chromosomes respectively (Table 4.1). On the other hand eight genotypes were found in case of AA × LA cross where there were 12 (12 + 1, + 2 or 12-1, -2) L and 24 (+1,-1 and -2) A chromosomes respectively. As far as recombination was concerned most of the BC1 LA hybrids exhibited recombination to certain extent. An illustration of these triploid genotypes is given (Fig. 4.1a).

Genome composition of the AA × OA progenies

A total of 53 BC1 progenies resulting from OA hybrids through unilateral sexual polyploidization were analysed during the present investigation. It was found that 48 (90.38%) genotypes were triploid (2n = 3x = 36), four (7.7%) were tetraploid (2n = 4x = 48) and one (1.92%) was aneuploid (3x + 1). As expected the triploid BC1 progenies of the OA hybrids indicated that the F1 OA hybrid donated 2*n* gametes to the progeny so that a complete set of OA chromosomes were transferred to their subsequent progenies. Most of the BC1 triploids had 24 A and 12 O chromosomes. However, like in LA hybrids, three genotypes in BC1 triploid progenies of OA hybrids were found to possess (12 + 1 or - 1) of O chromosomes from the expected 12 O- and 24 A-genome chromosomes (Table 4.2). The four tetraploids recovered had originated through the functioning of 2*n* eggs from the female Asiatic parents and hence contributed two sets of the A genome. Remarkably, one of these plants (022605-13) had 11 O and 37 A chromosomes instead of the expected 12 O + 36 A constitution. One aneuploid (3x+1) was also found in the BC1 progenies with one extra chromosome of A genome. This could have resulted from a univalent which might have lagged behind when the sister chromatids of the other univalents were segregating during FDR process of OA hybrid parents. This gave rise to the formation of 2*n* + 1 gametes (Table 4.2). Fig. 4.1 shows the triploid and tetraploid genotypes respectively with recombinant chromosomes.

Table 4.2. Progeny plants obtained from unilateral sexual polyploidization of AA × OA crosses, their ploidy levels, genome composition (number of recombinant chromosomes) and the percentage of genome contribution of the parents.

Cross	Genotypes	Parents		Ploidy Level	Genome Composition		Genome Percentage		No. of recombinant chromosomes
		Female	Male		$O^{O/A}$	$A^{A/O}$	O%	A%	
A OA	022538-1	Amarone	951502-1	3x	12(4)	24(3)	32.64	67.36	7
A OA	022538-3	Amarone	951502-1	3x	12(4)	24(2)	31.13	68.87	6
A OA	022538-5	Amarone	951502-1	3x	12(3)	24(2)	32.0	68.0	5
A OA	022538-7	Amarone	951502-1	3x	13(3)	23(3)	35.07	64.93	6
A OA	022538-8	Amarone	951502-1	3x	12(2)	24(2)	32.23	67.77	4
A OA	022538-9	Amarone	951502-1	3x	13(2)	23(2)	35.4	64.6	4
A OA	022538-14	Amarone	951502-1	3x	12(3)	24(2)	30.5	69.5	5
A OA	022538-15	Amarone	951502-1	3x	11(1)	25(3)	32.12	67.88	4
A OA	022538-16	Amarone	951502-1	3x	12(5)	24(3)	30.22	69.78	8
A OA	022538-17	Amarone	951502-1	3x	12(1)	24(1)	33.3	66.7	2
A OA	022604-6	Gironde	951502-1	3x	12	24	33.3	66.7	0
A OA	022604-9	Gironde	951502-1	3x	12(1)	24	27.0	73.0	1
A OA	022604-10	Gironde	951502-1	3x	12	24	33.3	66.7	0
A OA	022605-1	Amarone	951502-1	3x	12(1)	24(1)	32.00	68	2
A OA	022605-2	Amarone	951502-1	3x	12(1)	24(1)	32.02	67.98	2
A OA	022605-3	Amarone	951502-1	3x	12(4)	24(3)	20.0	80.0	7
A OA	022605-4	Amarone	951502-1	4x	12	36	25	75	0
A OA	022605-5	Amarone	951502-1	3x	12(1)	24(1)	32.5	67.5	2
A OA	022605-7	Amarone	951502-1	3x	12(3)	24(3)	29.0	71.0	6
A OA	022605-8	Amarone	951502-1	3x	12(2)	24(1)	32.82	67.18	3
A OA	022605-9	Amarone	951502-1	3x	12(4)	24(3)	34.35	65.65	7
A OA	022605-10	Amarone	951502-1	4x	12	36	25	75	2
A OA	022605-11	Amarone	951502-1	3x	12(1)	24(1)	33.3	66.7	2
A OA	022605-12	Amarone	951502-1	3x	12(1)	24(1)	33.22	66.8	2
A OA	022605-13	Amarone	951502-1	4x	11(1)	37(1)	20.25	79.75	2
A OA	022605-15	Amarone	951502-1	4x	12	36	25	75	0
A OA	022605-16	Amarone	951502-1	3x	12(2)	24(2)	33.3	66.7	4
A OA	022605-18	Amarone	951502-1	3x	12(2)	24(2)	28	72	4
A OA	022605-19	Amarone	951502-1	3x	12(4)	24(2)	30.5	69.5	6
A OA	022605-20	Amarone	951502-1	3x	12(4)	24(2)	31.23	67.57	6
A OA	022605-21	Amarone	951502-1	3x	12(4)	24(4)	32.43	67.57	8
A OA	022605-22	Amarone	951502-1	3x	12(1)	24(1)	33.3	66.7	2
A OA	022605-23	Amarone	951502-1	3x	12(3)	24(2)	31.7	68.3	5
A OA	022605-24	Amarone	951502-1	3x	12(3)	24(2)	30.0	70.0	5
A OA	022605-25	Amarone	951502-1	3x	12(3)	24(2)	31.3	68.7	5
A OA	022605-27	Amarone	951502-1	3x	12(1)	24(1)	33.33	66.7	2
A OA	022605-28	Amarone	951502-1	3x	12(1)	24	31.16	68.84	1
A OA	022605-30	Amarone	951502-1	3x+1	12(2)	25(2)	32.0	68	4
A OA	022605-31	Amarone	951502-1	3x	12(2)	24	29.55	70.45	2
A OA	022605-32	Amarone	951502-1	3x	12	24	33.3	66.7	0
A OA	022605-34	Amarone	951502-1	3x	12(2)	24(2)	33	67	4
A OA	022605-35	Amarone	951502-1	3x	12(6)	24(2)	30.83	69.17	8
A OA	022605-36	Amarone	951502-1	3x	12(2)	24	29.4	70.6	2
A OA	022605-37	Amarone	951502-1	3x	12(1)	24	31.27	68.8	1
A OA	022605-38	Amarone	951502-1	3x	12(1)	24(1)	33.3	66.7	2
A OA	022605-39	Amarone	951502-1	3x	12(2)	24(2)	33.3	66.7	4
A OA	022605-40	Amarone	951502-1	3x	12(2)	24(2)	33.3	66.7	4
A OA	022605-41	Amarone	951502-1	3x	12	24	35.96	64.04	0
A OA	022605-42	Amarone	951502-1	3x	12(1)	24(3)	33	67	4
A OA	022605-44	Amarone	951502-1	3x	12(1)	24(1)	33.3	66.7	2
A OA	022605-45	Amarone	951502-1	3x	12	24(3)	35.93	64.07	3
A OA	022605-46	Amarone	951502-1	3x	12(3)	24(3)	36.92	63.08	6
A OA	022611-4	Gironde	951502-1	3x	12(1)	24(3)	35.96	64.04	4

Genome composition of LA × LA hybrids

A total of 16 F2 plants derived from crossing LA hybrids that produced either 2*n* eggs or 2*n* pollen were analysed by GISH for their ploidy level, number of chromosomes from each genome and the number of recombinant chromosomes (Fig. 4.1 b). It was found that nine genotypes were tetraploids (2n = 4x = 48) indicated the contribution of 2*n* gametes from both parents. The other seven genotypes (064525-6, -7, -8, -14, -15,-18 and 064536-2) had an aneuploid condition with 4x -4, -5, +5, -1, -3, +1 -1 and -1 respectively (Table 4.3).

Table 4.3. Progenies obtained from bilateral sexual polyploidization using 2*n* gamete producing LA hybrids (LA × LA), their ploidy levels and genome composition (number of recombinant chromosomes).

Cross	Genotype	Parents		Ploidy level	Genome composition		Number of recombinant chromosomes
		Female	Male		$L^{(L/A)}$	$A^{(A/L)}$	
LA × LA	064525-1	041556	041502	4x	23	25	0
LA × LA	064525-6	041556	041502	4x-4	21(1)	23(2)	3
LA × LA	064525-7	041556	041502	4x-5	21(3)	22(2)	5
LA × LA	064525-8	041556	041502	4x+5	28(3)	25(2)	5
LA × LA	064525-9	041556	041502	4x	22(2)	26(3)	5
LA × LA	064525-10	041556	041502	4x	22(1)	26(1)	2
LA × LA	064525-13	041556	041502	4x	25(3)	23(1)	4
LA × LA	064525-14	041556	041502	4x-1	24(2)	23(1)	3
LA × LA	064525-15	041556	041502	4x-3	21(2)	24	2
LA × LA	064525-16	041556	041502	4x	25(4)	23(1)	5
LA × LA	064525-17	041556	041502	4x	22	26	0
LA × LA	064525-18	041556	041502	4x+1	22	27(1)	1
LA × LA	064525-19	041556	041502	4x	23(2)	25(1)	3
LA × LA	064525-20	041556	041502	4x	25(3)	23(4)	7
LA × LA	064534-1	041546	041502	4x	25(5)	23(5)	10
LA × LA	064536-2	041548	041502	4x-1	23(1)	24	1

Homoeologous recombination and frequency of introgression of L and O- genomes into A-genome

From the analysed data, it was found that in case of BC1 progenies of both LA and OA hybrids obtained from unilateral sexual polyploidization, 56 genotypes (90.30%) of LA × AA and its reciprocal cross (AA × LA) and 47 (88.5%) of the progenies of OA hybrids exhibited recombination at certain level (Table 4.1 and 4.2). There was however, a clear difference between the two groups of progenies with regard to number of plants with recombinant chromosomes. The numbers and frequency of recombinant chromosomes differed depending on the direction of the cross and the type of interspecific hybrids used for sexual polyploidization. It was found that the frequency of recombination recovered in BC1 progenies was higher in case of

LA hybrids as compared to OA hybrids. Similarly the frequencies of recombination events were also greater in LA hybrids than OA hybrids. Among the 48 BC1 LA genotypes which were derived from a cross where F1 LA hybrid was used as female parent, a total of 271 recombinant chromosomes were detected. Out of these 271 recombinant chromosomes, 148 were L/A types and 123 were of A/L type.

This contrasted with the 35 recombinant chromosomes recovered in 15 triploid BC1 progenies of LA hybrids where F1 LA hybrids were used as male parent. Here 13 chromosomes were of L/A and 22 were of A/L types (Table 4.1). In case of BC1 progenies of OA hybrids a total of 179 recombinant chromosomes were recovered where 98 were O/A and 81 were of A/O type of recombinant chromosomes (Table 4.2). Thus there was an obvious difference between the two types of interspecific hybrid with regard to the contribution of recombinant chromosomes to their resultant progenies. Furthermore the range of recombinant chromosomes varied from 0 – 20 in the crosses involved F1 LA hybrids as female parent where as in reciprocal cross (A LA) it ranged from 0 – 6 in various genotypes. On the other hand in case of OA hybrids, the number of recombinant chromosomes was far less than LA hybrids and it varied from 0 - 8 in different genotypes. As far as the crossover event were concerned there were evidences that single, double and multiple crossover events had occurred in both type of progenies resulting from unilateral sexual polyploidization.

In case of progenies derived from bilateral sexual polyploidization (LA × LA) 14 genotypes (87.5%) exhibited recombinant chromosomes. The total number of recombinant chromosomes recovered in F2 progeny derived from 2*n* - 2*n* cross was 56. Here 31 of these were L/A type while 25 were A/L type of recombinant chromosomes (Table 4.3). Taking the total length of three genomes i.e., A, L and O genomes in the BC1 triploid progenies, the percentages of each genome present in the BC1 progenies was estimated (Table 4.1 and 4.2). There is a little deviation from the expected values (L = 33.3% and A = 66.7%). The percentage of L genome varied from 29.98% (066960-8) to as high as 37.01% (066995-1). Similarly, in case of OA hybrids the amount of introgression of O genome in A also ranged from 20% (022605-3) to 36.92% (022605-46). However, in most of the cases almost an expected amount of Longiflorum and Oriental genomes were transmitted from LA and OA hybrids to respective BC1 progenies resulted from unilateral sexual polyploidization. In order to estimate the number and types of recombinant chromosomes and the lengths of recombinant segments, the karyotypes of the BC1 LA and OA progenies were constructed and some of them were shown in Fig. 4.2.

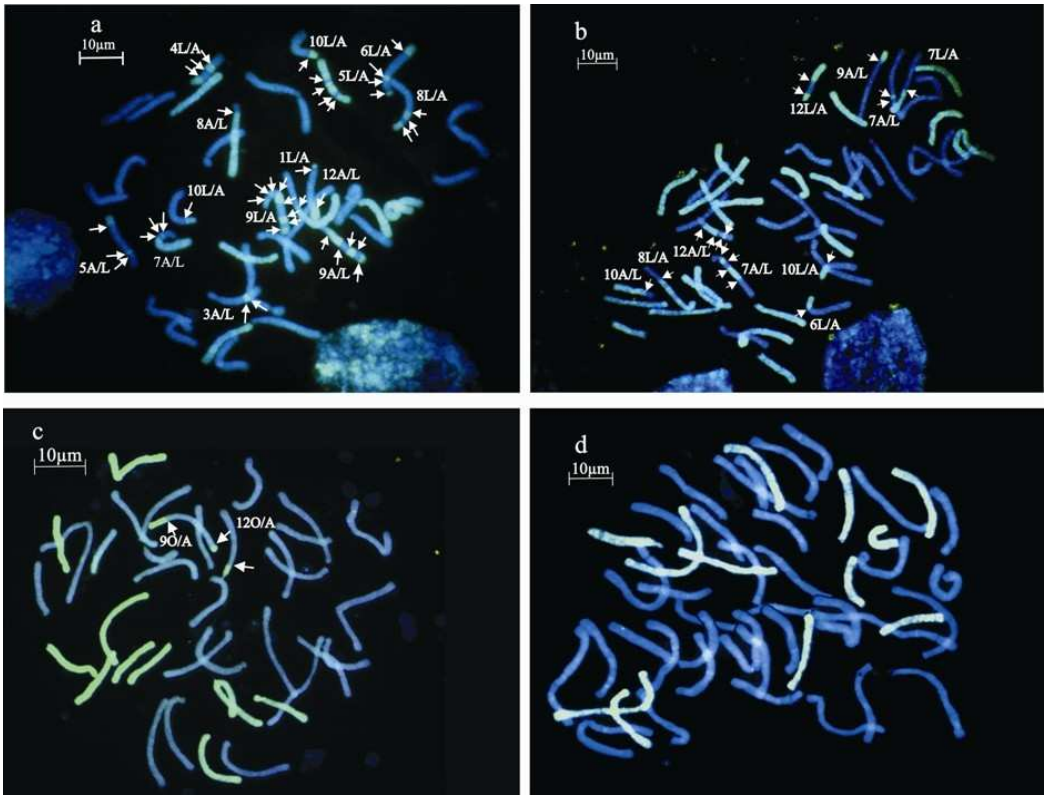

Fig. 4.1 (a-d). Triploid (2n = 3x = 36) and tetraploid (2n = 4x = 48) LA and OA hybrids obtained from uni- and bilateral sexual polyploidization. **a-** Chromosomes complement of triploid BC1 LA (062071-2) showing 12 L (green fluorescence) and 24 A (blue) chromosomes. **b-** Chromosomes complement of tetraploid F2 LA (064534-1) showing 25 L (green fluorescence) and 23 A (blue) chromosomes. **c-** Chromosomes complement of triploid BC1 OA (022605-36) showing 12 O (green fluorescence) and 24 A (blue) chromosomes. **d-** Chromosome complement of tetraploid BC1 OA (022605-4) showing 12 O (green fluorescence) and 36 A (blue) chromosomes. Arrows indicate the recombination break points.

Discussion

Present study revealed that uni- and bilateral sexual polyploidization through 2*n* gametes could successfully be carried out in interspecific lily (*Lilium*) hybrids to produce allotriploid and allotetraploid genotypes. Application of functional 2*n* gametes has become an important tool for the production of polyploid cultivars in different plant species like potato (Mendiburu and Peloquin 1977; Den Nijs and Peloquin 1977), wheat (Jauhar 2003), *Lolium* (Sala et al. 1989), *Asparagus* (Camadro 1994), *Medicago sativa* (Barcaccia et al. 1998) and *Lilium* (Lim et al. 2001a; Barba-Gonzalez et al. 2005a; Zhou et al. 2008). The traditional method to breed the triploid lilies is to obtain the tetraploid as a first step by mitotic polyploidization. These tetraploids are then crossed with normal diploids to achieve triploid progenies. This approach is less desirable because in the allotetraploid, which behaves like a ‘permanent hybrid’ due to autosyndetic chromosome pairing, no intergenomic recombination is expected to occur. As a result, there is very little scope for the selection of cultivars from mitotic polyploidization. On

the other hand the breeding schemes involving 2*n* gametes *via* unilateral sexual polyploidization in lily hybrids can overcome F1 hybrid sterility, facilitate intergenomic recombination leading to genetic variation and production of allotriploid progenies with some introgressed chromosomal segments. This has been clearly demonstrated in three different types of interspecific hybrids: Longiflorum × Asiatic (Lim et al. 2003; Zhou et al. 2008), Oriental × Asiatic (Barba-Gonzalez et al. 2004) and *Lilium auratum* × *L henryi* (Van Tuyl et al. 2002). During present study, allotriploid progenies were obtained in LA and OA hybrids through functional 2*n* eggs as well as 2*n* pollen. Previously, in most of the cases 2*n* pollen rather than 2*n* eggs have been used for unilateral sexual polyploidization.

One reason might be that it is easier to detect 2*n* pollen producing genotypes merely by staining the pollen and determine the size or growing them on artificial pollen germination medium. On the other hand, detection of 2*n* egg requires crossing and production of progeny that has to be tested for ploidy level. Although we have selected several interspecific F1 hybrids that produce either 2*n* pollen or 2*n* eggs, the frequencies of such genotypes are relatively low as compared to other liliaceous such as *Alstroemeria* and other plants (Kamstra et al. 1999a and 1999b; Ramanna et al. 2003; Ramsey and Schmske 1998).

The development of GISH technique facilitates discrimination of the parental genomes and predicts the level and amount of genome changes that occur in such crosses. Earlier reports on analysis of microsporogenesis during 2*n* pollen formation indicated the meiotic irregularities leading to the generation of gametes with differing genome compositions depending on the types of meiotic nuclear restitution (Lim et al 2001a; Ramanna and Jacobsen 2003; Barba-Gonzalez et al. 2004).

The first objective of our study was to produce interspecific triploid lilies (LA and OA) through 2*n* gametes (both male and female) *via* unilateral sexual polyploidization and to evaluate the nature of these BC1 triploid progenies by GISH. In this study, we have demonstrated the frequency of homoeologous recombination and amount of introgression from L- and O-genomes into A genome when BC1 progenies were obtained after unilateral sexual polyploidization. Flow cytometric analysis (data not presented) and chromosome counting after GISH confirmed the allotriploid natures of BC1 plants in both type of crosses, i.e. LL × AA and OO × AA. In case of BC1 progenies of LA hybrids (LA × AA type of cross), most of the genotypes were triploid (2n = 3x = 36) where 24 chromosomes were contributed by 2*n* gametes producing LA hybrid and *n* number of chromosomes by the Asiatic parent. As a result 24 chromosomes inherited from A and 12 chromosomes from L genome were present in BC1 progeny plants. However, it was

found that in some BC1 LA hybrid (LA × AA and AA × LA) there were deviation from the expected number of L and A chromosomes.

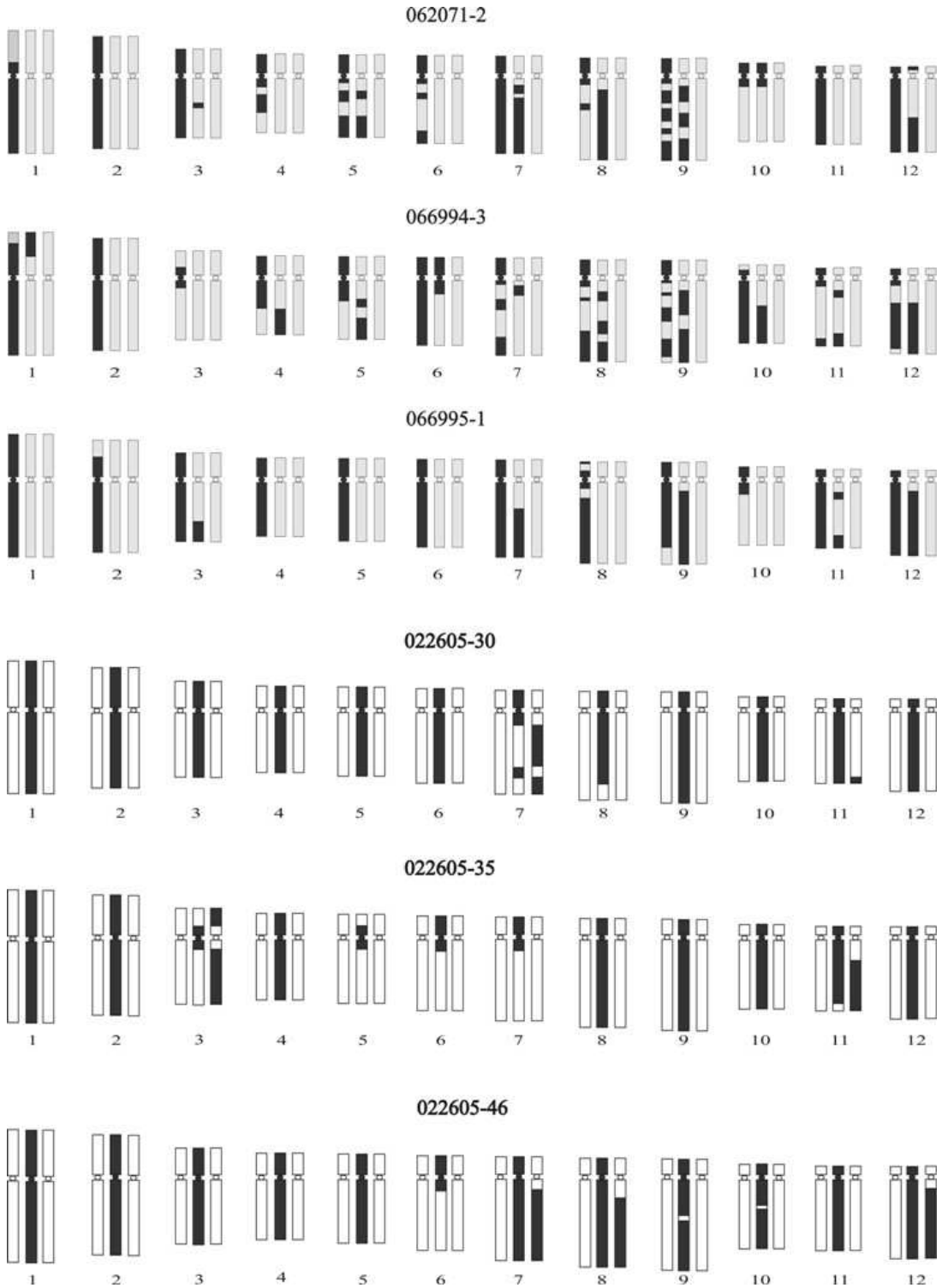

Fig. 4.2. A diagrammatic representation of recombinant chromosomes in BC1 triploid LA (062071-2, 066994-3 and 066995-1) and OA (022605-30, 022605-35 and 022605-46) hybrids. In this Fig. the black colour represents the Longiflorum and Oriental genomes while grey and white colour represents the Asiatic genome.

This deviation could be explained on the bases of IMR mechanism of 2*n* gametes formation in LA hybrids (Lim et al. 2001a). In case of BC1 progeny of OA hybrid, the progenies are mostly triploids (2n = 3x = 36) with few tetraploid genotypes. These tetraploid progenies could be the result of bilateral sexual polyploidization. Here, mostly we found the normal contribution of 24A (2*n* pollen contribute each set of 12 O and 12 A chromosomes) and 12 O genome composition in BC1 plants with few exception of 12 +1 or -1 O- chromosomes. This indicates that FDR was the mechanism of 2*n* gametes formation in most of OA hybrids (Ramanna et al. 2003; Barba-Gonzalez et al. 2004). Present study strengthens the findings of Lim et al. (2001a), Barba-Gonzalez et al. (2005a) and Zhou et al. (2008) who found that in case of both LA and OA hybrids more BC1 progenies recovered from FDR 2*n* gametes rather than IMR 2*n* gametes. This might be due to more stability and viability of FDR 2*n* gametes as compared to IMR 2*n* gametes in both types of F1 hybrids.

GISH, analysis of BC1 progenies have demonstrated an extensive genome recombination in the BC1 progenies of both type of crosses obtained after unilateral sexual polyploidization. Here it was shown that the choice of 2*n* gametes as female parents also has a significant effect as compared to 2*n* gametes producing male parent when backcrossed to a diploid Asiatic cultivars. This significant effect was in terms of the numbers of recombinant chromosomes recovered in allotriploid BC1 progenies of LA hybrids as compared to the numbers of intergenomic recombinants recovered from OA hybrid. It was found that 43 of the analysed plants (91.5%) of BC1 progeny of LA × AA had recombinant chromosomes in variable frequencies i.e., 1 - 20 recombinant chromosomes per genotype. While in reciprocal cross 13 genotypes (86.6%) ranged from 1 – 6 recombinant chromosomes per genotype. Moreover, on an average 31.3% recombinant chromosomes have been recovered in BC1 triploid progeny of LA × AA crosses while the percentage of recombinant chromosomes in reciprocal cross was 6.5%. On the other hand when OA F1 hybrid was used as male parent and backcrossed with two diploid Asiatic cultivars 47 out of 53 analysed BC1 plant (88.5%) exhibit recombinant chromosomes. Furthermore, the number of recombinant chromosomes is far less (1 - 8) than in the corresponding progeny of LA hybrids. Finally, the average number of recombinant chromosomes recovered in OA hybrids is also less (8.9%) as compared to LA hybrids (31.3%). The mean result of analysed genotypes in both types of crosses clearly reflected the low recombination events in OA hybrids as compared to LA hybrids. It could be explained by assuming that in case of LA hybrids different F1 LA hybrids have been used to cross with various Asiatic parents to produce interspecific BC1 progeny plants while in case of OA hybrids only two Asiatic cultivars were used to make a cross with one F1 OA hybrid.

In the current study, it was found that more or less the expected percentage (33.3%) of L and O genome is retained in BC1 progenies of LA and OA hybrids. However, irrespective of the direction and type of the cross, an imbalance was found between numbers of L- and O- genome introgressions on A chromosomes and *vice versa*. In both type of crosses the Asiatic genome introgression on L or O chromosomes is exceeded. Conversely, less introgression of L or O genome was found in Asiatic background. In LA × AA crosses, a total of 125 Asiatic introgressions were found on L genome while introgressions of L genome on Asiatic chromosomes was 85. Similarly in AA × OA hybrids a total of 57 A genome introgression were found on O chromosomes while in reciprocal case 42 introgressions were found in Asiatic background from O genome.

Another aim of the present study was to evaluate the relevance of bilateral sexual polyploidization in interspecific lily hybrids. However, it is worthy to have genotypes producing 2*n* gametes for the bilateral sexual polyploidization. In the absence of genotypes of lily hybrids that produce both 2*n* pollen and 2*n* eggs simultaneously, bilateral sexual polyploidization in interspecific lily hybrids has not been accomplished before. One idea was to use the F1 hybrid with good female fertility to be crossed with another F1 hybrid with good male fertility to produce tetraploids through bilateral sexual polyploidization. These ideas became evident only when the crosses were made between some F1 LA hybrids through sib-mating to generate F2 population. Such tetraploids have two important advantages: (i) Because of their fertility they can be used as parents repeatedly to produce triploid or tetraploid progenies. (ii) When the bilateral sexual polyploids possess recombinant chromosomes, they are likely to pair as multivalent and segregate randomly as if in an autopolyploid. This means, the loci present on recombinant chromosomes segregate whereas all other loci will not segregate as in the typical allotetraploid (Ramanna et al. 2003). Furthermore, using this method of sexual polyploidization, crops with maximum heterozygosity could replace those were obtained by artificial chromosome doubling (Den Nijs and Stephenson 1988; Hahn et al. 1990; McCoy 1992). Because of these attributes, such tetraploids might be potentially useful for introgression and for cytogenetic mapping purposes. The present study implies that crosses between 2*n* gametes producing parents produced allotetraploid progenies and in the following backcross 4x and 2x parents would predominantly produce triploid progenies in the 4x – 2x directions.

As mentioned earlier bilateral sexual polyploidization is rarer than its unilateral counterpart, the few known cases involved were *Trifolium pratense* (Parrott et al. 1985), *Solanum* (Jongedijk et al. 1991; Werner and Peloquin 1991), *Avena sativa* haploids (Rines and Dahleen 1990), *Dactylis glomerata* L. (Van Santen et al. 1991; Lumaret et al. 1992), *Triticum*-

Aegilops hybrids (Fukuda and Sakamoto 1992), *Madicago* spp. (Barcaccia et al. 1998), *Triticum turgidum* haploids (Jauhar et al. 2000), *Manihot esculenta* (Ogburia et al. 2002), and *Alstroemeria* (Ramanna et al. 2003).

Harlan and de Wet (1975) pointed out the three mechanism responsible for polyploid formation *viz.* i) by direct unilateral or bilateral sexual polyploidization by fusion of 2*n* gametes ii) by an intermediate step forming a hybrid with 2*n* gamete and iii) somatic doubling of diploid hybrid. It was argued that for direct bilateral sexual polyploidization, the mean frequency of 2*n* gamete production in a diploid population is so low that the chance of spontaneous tetraploidization, by fusion of two 2*n* gametes is negligible. However, some spontaneous bilateral sexual polyploids have been reported in *Manihot esculenta* (Hahn et al. 1990), *Dactylis glomerata* (Van Santen et al. 1991) and *Cyphomandra betacea* (Pringle and Murray 1992). It was found in the present study that interspecific F1 LA hybrids produced both 2*n* pollen and 2*n* eggs resulted in tetraploid or near tetraploid (aneuploid) progenies (Table 4.3). Presently we do not have good reasons about the production of these aneuploid genotypes.

Unilateral and bilateral sexual polyploidization resulted in the formation of triploid and allotetraploid progenies respectively. A general consideration is that those triploids are mostly sterile and have little interest in breeding because of sterility. However, a cursory look at the literature indicates that triploid have been used successfully in crossing programs and could produce diploid or circa diploid progenies in 3x - 2x or in reciprocal crosses (Lim et al. 2003; Barba-Gonzalez et al. 2006b; Proscevičius et al. 2007). These diploid or circa diploid progenies are potentially useful for breeding at the diploid level (Khan et al. 2009b). Unlike the triploids the allotetraploids obtained after bilateral sexual polyploidization could be fertile and can produce progenies by selfing or crossing with other genotypes. Different interspecific F1 hybrids with 2x - 2x crosses were made to estimate the 2*n* gametes formation. When no natural polyploids are available, certain treatments are done to induce the 2*n* gametes formation. Notably temperature and chemical agents were shown to affect the frequency of 2*n* gamete formation (Barba Gonzalez et al. 2006a; Wu et al. 2007). Beside these factors the production of 2*n* gametes is under strong influence of genetic control (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Although a lot of work has been done on the induction of 2*n* gametes but there is scarcity of research for molecular characterization of these 2*n* gametes formations. Recently, the first gene *AtPS1* responsible for 2*n* gametes formation has been isolated and characterized (d'Erfurth et al. 2008). This information can facilitate our understanding the origin of 2*n* gametes and speed up their use in crop breeding programs.

Chapter 5

Construction of a genetic linkage map in a F1 population of Longiflorum × Asiatic (LA) lily hybrids using Diversity Array Technology (DArT) markers

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Abstract

Genetic mapping in lilies (*Lilium* L.) is constrained by its large genome. Diversity Arrays Technology (DArT) can detect and type DNA variation at several hundreds of genomic loci in parallel without relying on sequence information. We developed a DArT platform for lilies to enable efficient and cost-effective genetic mapping with the production of a large number of markers in a single hybridization-based assay. Amongst several tested complexity reduction methods one was selected (*Pst*I + *Taq*I) that generated the largest frequency of polymorphic genomic representations for a genotyping array. Genomic DNA fragments from the Asiatic cultivar 'Connecticut King' were cloned and printed on the slides, thus assembling of a DArT genotyping microarray. Genomic representations of the 88 F1 Longiflorum × Asiatic (LA) hybrid plants were hybridized to these microarrays. This yielded a total of 687 DArT markers. Out of these 382 polymorphic DArT markers with an average call rate of 93.0% and a scoring reproducibility of 99.9% were used to construct a genetic linkage map in the F1 population. The genetic linkage map was comprised of 14 main linkage groups which are two more then the haploid chromosome number. The resulting linkage map with 382 DArT markers spanned 1329 cM (1 marker per 3.5cM on average). The DArT markers were very suitable to construct a genetic linkage map and to evaluate genetic relationships amongst a F1 population in the Longiflorum × Asiatic progeny. The results highlighted the potential of DArT as a genetic technique for genome profiling in the context of molecular breeding and genomics, especially in so-called molecular orphan crops, such as lily.

Introduction

Crop improvement mostly relies on the effective utilization of genetic diversity. Molecular marker technologies are promising tools for genetic and genomic studies, breeding and biodiversity research. Currently, several DNA-based molecular marker technologies are available for genetic diversity analysis and molecular assistant breeding in different crops e. g., Restriction Fragment Length Polymorphisms (RFLP) (Miller and Tanksley 1990; Foolad and Chen 1999), Random Amplified Polymorphic DNA (RAPD) (Williams et al. 1990; Gonzalez and Ferrer 1993), Simple Sequence Repeats (SSR) (Weber and May 1989; Wang et al. 2004), Amplified Fragment Length Polymorphisms (AFLP) (Vos et al. 1995; Mackill et al. 1996), and Single Nucleotide Polymorphisms (SNPs) (Wang et al. 1998).

Lilium is one of the most important ornamental crops, grown for a wide range of agronomical traits. Several molecular marker technologies have been developed and applied to study the patterns of genetic diversity in lily collections and in breeding programs, including RAPD markers for identification of *Lilium* species and interspecific hybrid (Yamagishi, 1995; Yamagishi et al. 2002) to study genetic variation (Persson et al. 2004) for tracing parentages (Haruki et al. 1998), identifying genetic diversity (Arzate-Fernández et al. 2005), and for finding linked RAPD-markers to *Fusarium oxysporum* resistance in Asiatic hybrids (Straathof et al. 1996). Van Heusden et al. (2002) used AFLP markers in order to construct a molecular map and to find out the level of polymorphism in the Asiatic lily hybrids. A total of 251 AFLP markers based on 100 descendents of a lily backcross population were used for the construction of a genetic map. Based on these approaches it was found that LMoV (TBV) resistance was clearly a monogenic trait and could reliably be mapped on linkage group 9. Despite the difficult screening for *Fusarium* resistance, four significant QTLs were mapped to linkage groups 1, 5, 13 and 16 respectively.

Although a lot of molecular marker techniques are used, most of these technologies suffer from a combination of impediments which affect the level of genome coverage, their discrimination ability, reproducibility and technical and time demand. Several marker technologies require DNA sequence information of the crop. However, *Lilium* spp. has very large genome among plants (Bennett and Smith 1976; 1991), and the whole genome for this genus is not sequenced yet.

Diversity array technology (DArT) does not require genome sequence information. It is a hybridization based approach that captures the value of the parallel nature of the microarray platform. With DArT, simultaneous screening of several hundreds of loci in a single assay can be performed without any sequence information. It generates genome wide fingerprints by scoring

the presence versus absence of DNA fragments in genomic representation generated from samples of genomic DNA. This technology was originally developed using rice (Jaccoud et al., 2001) and then extended to a range of other plants like *Arabidopsis* (Wittenberg et al. 2005), barley (Wenzl et al. 2004), cassava (Xia et al. 2005), Pigeonpea (Yang et al. 2006) and profiling of the hexaploid wheat genome (Akbari et al. 2006). DArT has been also applied to a number of animal species and microorganisms as well (http://www.diversityarrays.com).

Molecular markers are an important tool to speed up crop improvement (Langridge 2005; Varshney and Tuberosa 2007) and for the construction of genetic linkage maps, as the first step in the genetic dissection of the required traits. During the present study efforts were made to generate DArT markers and score them in the F1 population of a cross between Longiflorum and Asiatic (LA) lily hybrids. This allowed us to construct a genetic linkage map in the LA population by positioning polymorphic DArT markers on different linkage groups.

Materials and Methods

Construction of the microarray

Genomic DNA was isolated from young leaves of an Asiatic cultivar 'Connecticut King' according to Fulton et al. (1995) and was subjected to digestion with the methylation sensitive restriction endonuclease *Pst*I, and adapters (Table 5.1) were ligated to the ends of these fragments. The restriction fragments could be amplified by using adapter specific primers. In order to reduce the number of fragments that could be amplified, an additional restriction was performed that cut the majority of the fragments. Several restriction enzymes were evaluated (see Results). The best results were obtained for the frequent cutter *Taq*I without *Hin*III. The genomic representations were generated as described by Jaccoud et al. (2001) and Wenzel et al. (2004). Shortly, these were generated by cutting 250ng of DNA samples with 2U *Pst*I and the frequent cutter *Taq*I (New England Biolabs, NEB, Beverly, MA) with supplied buffer. The *Pst*I adapter (Table 5.1) was simultaneously ligated with T4 DNA ligase (NEB).

Amplification of the restriction ligation mixture from 'Connecticut King'

The amplification of the restriction ligation mixture was performed as described previously for rice and cassava (Jaccoud et al. 2001; Xia et al. 2005). The digestion-ligation reaction was diluted 10-fold, and a 10µl aliquot was used as a template in 50µl amplification reaction. The PCR amplification reaction was comprised of 10µl of RL mixture, 2.0µl of 5mM dNTPs, 10µl Promega buffer, 1.3U Taq polymerase (Promega, Madison, WI, USA), 0.25µl of 100µM primer and 27.35µl of H_2O. The PCR reaction was carried out as described by Wenzel et al. (2004).

Cloning and amplification of the fragments from 'Connecticut King'

The amplicons from the genomic representation of the Asiatic cultivar 'Connecticut King' were ligated into the PCR2.1-TOPO vector (Invitrogen) using the TOPO cloning kit and transformed into electro competent *Escherichia coli* strain TOP10F (Invitrogen) with electroporation. The transformated cells were plated on solid LB medium supplemented with Kanamycin (100µg/µl) and X-gal (40µg/ml) and incubated overnight at 37°C (Jaccoud et al. 2001). Individual white colonies (containing recombinant plasmids) were transferred and grown in 384-well microtiter plates at 37°C containing LB medium supplemented with 100mg/l Ampicillin and freezing mix (36.0 mM $K_2HPO_4.3H_2O$, 13.2 mM KH_2PO_4, 1.7 mM $Na_3C_6H_5O_7$, 0.4 mM $MgSO_4.7H_2O$, 4.4% glycerol). From each clone, a 0.5µl aliquot was transferred to 25 µl of 10× Possum Taq PCR buffer (a home made buffer). The 25µl reaction mixture contains 2.5µl 10× Possum Taq PCR buffer 0.5µl (100µM) each of Forward and Reverse M13 primers, 0.5µl of 10mM dNTPs, 1U of SuperTaq polymerase (Sphaero Q, Leiden, The Netherlands) and 20.75µl of H_2O. The reactions were then incubated in microtiter plates for 5 min at 95°C followed by 35 cycles of: 94°C for 30 s, 52°C for 30 s and 72°C for 1 min. The final extension was carried out at 72°C for 7 min. After amplification, a representation (25%) of the PCR products was checked on 1% agarose gel (Fig. 5.1). Then the amplification products were precipitated with an equal volume of isopropanol at room temperature and washed once with 100µl of 70% ethanol with immediate centrifugation at 3400 × g force for 30 min. The ethanol was removed and the products were air dried.

Spotting of DNA fragments and processing of microarray slides

Amplification products from the clones of genomic DNA of 'Connecticut King' were dissolved in spotting buffer i.e., DArT spotter2 by DArT P/L (http://www.diversityarrays.com/index.html). The reaction mixture was then spotted in triplicate on poly-L-lysine-coated slides (Erie Scientific Com) using a MicroGrid II micoarrayer (Biorobotics, UK). After spotting the slides were dried overnight at room temperature. The slides were then processed by washing the slides in water (95°C) for 2 minutes, followed by rinsing the slides in washing solution containing 0.1mM DTT and 0.1mM EDTA for 1 minute under constant agitation. The slides were dried quickly by centrifugation at 380 × g force. Each clone was given a name, i.e. LPT for *Lily PstI-TaqI*, followed by the plate location (plate number + well position).

Genotyping, using the microarrays

Preparation of the genomic representations of the F1 population

Longiflorum cultivar 'White Fox' and Asiatic cultivar 'Connecticut King' were crossed to get a F1 LA population. From this population 88 genotypes were obtained and used for the construction of a genetic linkage map. DNA from the F1 population and one of the parents i.e. 'Connecticut King' was isolated as described above. Several DArT arrays were built in the course of this study. For each of these arrays, a genomic representation was generated Asiatic cultivar using the *Pst*I-based complexity reduction method (see in the results). DNA from 88 individuals of the F1 LA population was digested by *Pst*I/*Taq*I and ligated to the *Pst*I adaptor, as described above. The digestion/ligation products were amplified using the primer (Table 5.1) and concentrated by precipitation by adding isopropanol (45µl) and centrifuging at 3400 × g force at room temperature for 30-45 min. The pellet was resuspended in 2µl of H_2O and stored at -20°C.

For each sample approximately 650ng of DNA from 88 individuals of F1 LA hybrids was labeled with Cy5-dUTP or Cy3-dUTP, the fluorescent dyes (Pharmacia) using random decamers and Klenow enzymes (NEB/Fermantas). The targets representations contained 2.0µl of genomic DNA, 0.5µl of 10x NEB buffer, 0.5µl of 500 µM Random decamers (Biolegio), 0.5µl of low dNTPs and 0.3µl H_2O. These target representations were denatured at 95ºC for 3 min, cool down and a mixture of 0.5µl of Klenow enzyme, 0.16µl of dUTP-Cy3 of Cy5 and 0.54µl of H_2O was added to target representation. The labeling reaction was the performed for 2 h at 37ºC.

Hybridization and washing

The Cy3-labelled representations (5µl each) were mixed with 2.0µl of 10 mg/ml herring sperm DNA (Sigma) dissolved in ExpressHyb hybridization solution (Clontech) and denatured at 96°C for 3 min. The denatured probes were mixed with 65µl of ExpressHyb hybridization solution, pipetted directly onto the microarray surface and covered with a glass cover slip (24 x 26 mm, Menzel Glaser). Slides were quickly placed into a humidification chamber in a 65°C water bath for overnight hybridization. After hybridization, the cover slips were removed, and slides were rinsed in 1× SSC with 0.1% SDS and 0.1 mM DTT for 5 min, 1× SSC and 0.1 mM DTT for 5 min, 0.2× SSC and 0.1 mM DTT for 2 min and 0.02× SSC and 0.1 mM DTT for 25 s. All these washings were done at room temperature. Slides were quickly dried by centrifugation at 380 × g force in a slide rack for 10 min at 25ºC.

Slide scanning and data extraction

Slides were scanned using a LASER micro array scanner (ScanArray Express HT Microarray Scanner) and images were generated for each of the fluorescent dyes using the appropriate laser/filter combination for Cy-3 and Cy-5. DArTsoft, a software package developed by DArT P/L (http://www.diversityarrays.com/index.html) was used to automatically analyze each batch of TIF image pairs generated in an experiment.

Table 5.1. The adapter and primer oligonucleotide sequences used for generation of genomic representation for cloning and genotyping.

Purpose	Adapter sequence	Primer sequence
Making the array, using DNA from 'Connecticut King'	5´GTT CAG TCA TAG ATG GTG CA 3´ 5´CCA TCT AAC TTG ACT G 3´	5´CAG TCA AGT TAG ATG GTG CAG 3´

Results

Evaluation of complexity reduction method and array development

The initial tests of DArT performance in *Lilium* were performed on a few F1 LA hybrids. Based on positive experience with *Pst*I-based genomic representations (Jaccoud et al. 2001) several combinations of *Pst*I with different frequently cutting restriction enzymes (RE) were evaluated as a complexity reduction approach for *Lilium.* The genomic DNA was subjected to digestion with the methylation sensitive restriction endonuclease *Pst*I, and an adapter (Table 5.1) was ligated to the ends of these fragments. In order to reduce the number of fragments that could be amplified, an additional restriction was performed that cut the majority of the fragments. The restriction fragments could be amplified by using primers that could anneal to the adapters. Cut fragments contained only one adapter at one end, and therefore could not be amplified anymore efficiently. Only non-cut fragment with adapters at both sides could still be amplified. If the number of restriction fragments is very low, the number of polymorphic markers will be low too. If the number of restriction fragments is very high, the number of markers might be high, but the hybridization mixture is diluted, leading to low signals and unreliable results. An optimal number of fragments are 10,000 to 15,000 (Kilian, personal communication). Several restriction enzymes were evaluated (*Pst*I+*Mse*I, *Pst*I+*Taq*I, *Pst*I+*Bst*N, *Pst*I+*Msp*I, *Pst*I+*Hind*III, *Pst*I+*Mse*I+ *Hind*III, *Pst*I+*Taq*I+ Hind III, *Pst*I+*Bst*N+ HindIII, *Pst*I+*Msp*I+*Hind*III, *Pst*I+*Hind*III+*Hind*III). The best results were obtained for the frequent cutter *Taq*I and *Mse*I without *Hin*III. However, after first test it was found that *Pst*I/*Taq*I gave the most polymorphic clones. Sometimes the other RE combinations gave a smear with one or more dominant bands representing the highly repetitive fragments. These fragments correspond to abundant repetitive

sequences in the representation, which should be avoided (Kilian et al. 2005). The average DArT marker size was 800-1200bp.

Genetic relationships between F1 LA hybrids revealed by DArT

The selected DArT clones were tested for their ability to resolve genetic relationships among a set of 88 genotypes. The technical reproducibility of the DArT genotyping array was successfully validated by independent assays from the same DNA. DArTsoft analysis (see Materials and Methods) identified 382 markers polymorphic among 88 genotypes printed on the array. The PIC (Polymorphism Information Content) values of these 382 markers were high with over 67% of the markers having a PIC value between 0.4 and 0.5 (Table 5.2). The average PIC was 0.39, almost equal to the previous DArT studies in barley which was 0.38 (Wenzel et al. 2006) and less than cassava which was 0.42 (Xia et al. 2005). The relationship between the quality of the DArT markers (measured as the % of total variance which existed between the two clusters: present and absent) and the performance of the DArT markers as determined through call rate and PIC was analyzed (Table 5.3). As expected, in most of the cases the average call rate decreased with average Q value. The markers with the highest Q values had high average call rates (98%), while the markers in the lower quality marker classes had lower average call rates (Table 5.3).

Table 5.2. Polymorphism Information Content (PIC) values for 382 DArT markers.

PIC Value	No of DArT markers	% DArT Markers
0.5 - 0.4	259	68
0.4 - 0.3	60	15
0.3 - 0.2	23	6
0.2 - 0.1	40	11

Image analysis and scoring for polymorphism

The software package DArTsoft from DArT P/L (http://www.diversityarrays.com/index.html) was used to analyze each batch of scanned microarray images automatically. DArTsoft localized the spots, rejected those with weak reference signals, computed and normalized the relative hybridization intensities, and identified clones that revealed genetic differences according to dominant scoring. These polymorphic clones were detected by means of a combination of ANOVA and fuzzy C-means clustering (Wenzl et al. 2004). A clone was considered to be polymorphic if the normalized hybridization signals from the different plants could be grouped into two distinct clusters, i.e. a cluster of low hybridization signals and a cluster of high signals, for which the between-cluster variance was at least 80% of the total variance. A polymorphic

clone was incorporated into a 0/1 dominant scoring table if the probability of belonging to one of the two classes was above 0.95 averaged across all slides of the batch of images, and if it was scored with $P > 0.95$ in at least 90% of all these slides. So as a whole a total of 687 DArT markers were scored. Markers that showed conflicting scores between the replicates or could not be scored in either of the replicates were scored as unknown.

Construction of a genetic linkage map using DArT markers

We constructed a linkage map for a cross between a Longiflorum cultivar 'White Fox' and an Asiatic cultivar 'Connecticut King'. Only the markers present in Asiatic parent 'Connecticut King' were mapped on linkage groups segregating in 1:1 fashion from the parent. A total of 382 DArT markers were mapped on different linkage group in the F1 LA hybrids (Table 5.4; Fig. 5.2). The automatically generated scoring table from DArTsoft was converted into a format suitable for the software package JoinMap Version 3.0 (http://www.kyazma.nl) for construction of a genetic linkage map. Markers that showed an identical scoring pattern were removed from the scoring table, leaving one marker per unique segregation pattern. For the markers that showed an identical scoring pattern, we did not know whether the underlying clones had identical sequences or co-segregated because of close linkage.

Map length and coverage

A total of 382 DArT markers, including the markers with identical segregation pattern were mapped on 14 linkage groups in F1 LA hybrids (Table 5.4; Fig. 5.2). The genetic linkage map spanned 1329 cM. The genetic linkage map has an average DArT marker density of 1/3.5 cM. Besides these markers there were 91 more markers which did not map to these linkage groups but ended up in 11 smaller clusters. The total length of these smaller clusters is 352 cM. Table 5.4 represents the distribution of DArT markers on various linkage groups.

Discussion

This study applies DArT in lily by creating new genomic libraries of clones and arrays. It adds markers to the existing ones in Asiatic population based on AFLP (Van Heusden et al. 2002) and NBS-profiling (unpublished data). Up to now, we have selected 382 polymorphic DArT markers that can be typed in a single assay. DArT profiling has proven to be useful to construct a linkage map and to elucidate the pattern of marker distribution amongst various linkage groups in the F1 progenies of LA population. The number of available DArT markers, their cost-effectiveness and

relatively high polymorphism content are ideal characteristics for extensive genome-wide screening for QTL discovery.

Table 5.3. The relationship between the quality and performance of DArT markers. Markers were selected for P > 0.95.

Q-Values	Call rate	No. of markers	PIC values
68.91	100	61	0.294
70.72	94.4	72	0.370
68.97	88.88	90	0.408
66.06	88.33	79	0.489
65.55	77.77	40	0.475
63.09	72.27	27	0.470
62.17	61.1	12	0.462
47.46	55.55	1	0.18

The high-throughput fingerprinting technique DArT can detect DNA polymorphisms by scoring the presence *v/s* absence of specific DNA sequences in a defined genomic representation through hybridization to microarrays (Jaccoud et al. 2001; Wenzl et al. 2004). Initially DArT technique has been applied in rice to detect polymorphisms (Jaccoud et al. 2001). Its usefulness for constructing genetic linkage maps has been demonstrated in barley (Wenzl et al. 2004), cassava (Xia et al. 2005), Arabidopsis (Wittenberg et al. 2005), pigeonpea (Yang et al. 2006), wheat (Akbari et al. 2006; Mantovani et al. 2008), and *Sorghum bicolor* (Mace et al. 2008). We used DArT technique to develop a genetic linkage map in F1 population of Longiflorum × Asiatic (LA) hybrids in *Lilium* to determine the position of different polymorphic DArT clones located on various linkage groups. This is the first report of the use of DArT technology in *Lilium* and our results demonstrate that *Lilium* DArT markers are of high quality, as assessed by different sets of parameters like their call rate, scoring reproducibility and PIC values (Table 5.2; 5.3). The DArT marker quality parameters measured for the *Lilium* array are comparable to those obtained for pigeonpea (Yang et al. 2006), barley (Wenzl et al. 2004), cassava (Xia et al. 2005), wheat (Akbari et al. 2006; Mantovani et al. 2008) and *Sorghum bicolor* (Mace et al. 2008). Reproducibility of DArT marker was found to be up to 99.9% and similar values have been reported for DArT in barley (Wenzl et al. 2004) and Arabidopsis (Wittenberg et al. 2005).

Distribution of DArT markers in Lilium genome

The genetic linkage map comprising DArT markers clearly demonstrates that the DArT markers behave in a Mendelian manner. In total, 382 DArT markers were distributed on 14 linkage groups. The final genetic linkage map spanned a total length of 1329 cM, with an average DArT marker density of 1 marker per 3.5cM. Linkage group I was the longest (219 cM) while the

shortest LG was number 14 (47 cM). The average linkage group length was 95 cM. Although the DArT markers are distributed across the genome in a similar pattern there are genomic regions containing significantly more markers, e.g. linkage group 2 (Fig. 5.2) has a higher than average marker density of 1/1.6 cM and linkage group 12 is marker-poor with an average of 1/6.3 cM. The map distance between adjacent markers varied from 0.25 cM (linkage group 6) to 37 cM (Linkage group 3).

Table 5.4. Summary of the genetic linkage map of LA population with total length of each linkage group and the number of markers per linkage group are detailed.

Linkage groups	No. of Markers	Length (cM)	Average marker distance (cM)
1	65	219	3.4
2	52	87	1.7
3	43	122	2.8
4	35	70	2.0
5	34	107	3.1
6	24	86	3.6
7	23	72	3.1
8	20	99	4.9
9	16	69	4.3
10	16	81	5.0
11	16	87	5.5
12	16	101	6.3
13	13	82	6.3
14	09	47	5.2
Total	**382**	**1329**	**3.5**

These regions of low marker density may be therefore associated with genomic regions that were identical by descent or that had very limited genetic variability in the initial diversity representation, or have a high recombination frequency. Another explanation is that these areas are methylated: as *Pst*I is methylation sensitive, DArT markers reside mainly in non-methylated areas. The marker-dense regions probably correspond to the centromeric regions with low recombination frequencies, a feature that has been observed previously in *Sorghum* (Bowers et al. 2003). It should however be noted that clustering around the centromeres is observed for both DArT and all kind of other markers in almost all plant species (Mace et al. 2008). This might be due to the centromeric suppression of recombination. However, interesting observations were found in case of barley genomes where DArT markers were significantly less clustered at most centromeric regions (Wenzel et al. 2006), possibly because of methylation in these genomic areas in barley. The high number of DArT markers generated in a single assay not only provides a precise estimate of genetic relationships among genotypes, but also their even distribution over the genome offers real advantages for a range of molecular breeding and genomics applications.

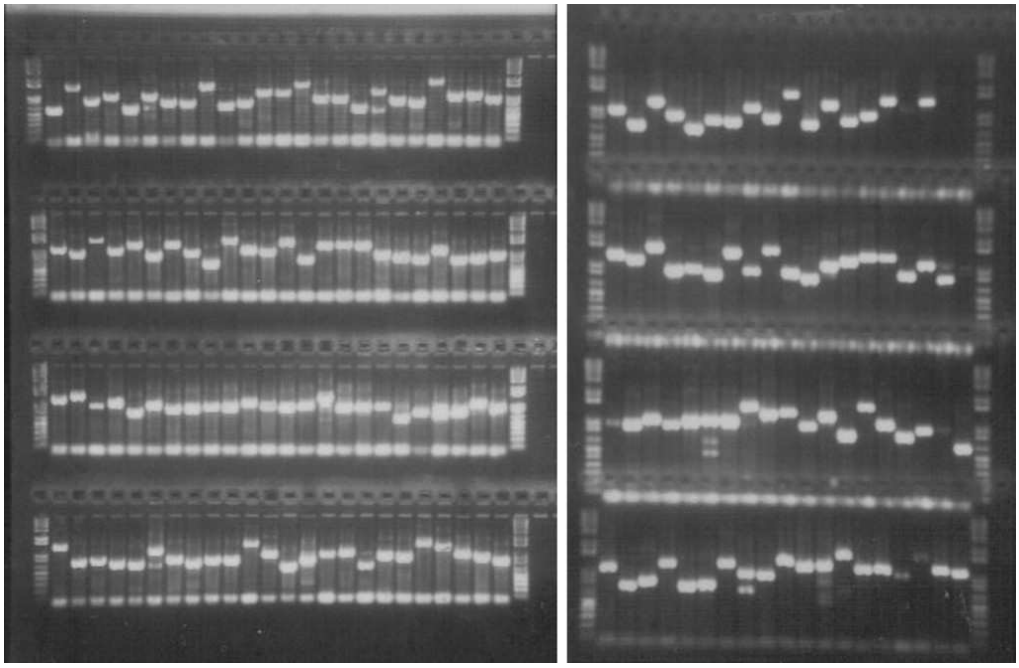

Fig. 5.1. Gel images of PCR product of DArT markers, representing the genomic representation of the DArT clones to be spotted for micro array analysis.

Beside 14 main linkage groups there are 11 smaller clusters spanning a size of 352 cM which carry 91 additional DArT markers. Considering the extreme large genome size of lily it can be emphasized that although the marker density is high some genomic regions could not be linked together due to large blocks of repetitive DNA sequences. The other reason as mentioned above is that these areas of genome are methylated and *Pst*I is methylation sensitive. As a result the DArT markers mainly reside in non-methylated areas. These reasons may lead to higher number of linkage groups compared to basic chromosome number of lily (n = 12). It is expected that when in course of time and more markers become available, that these smaller clusters will join with other linkage groups.

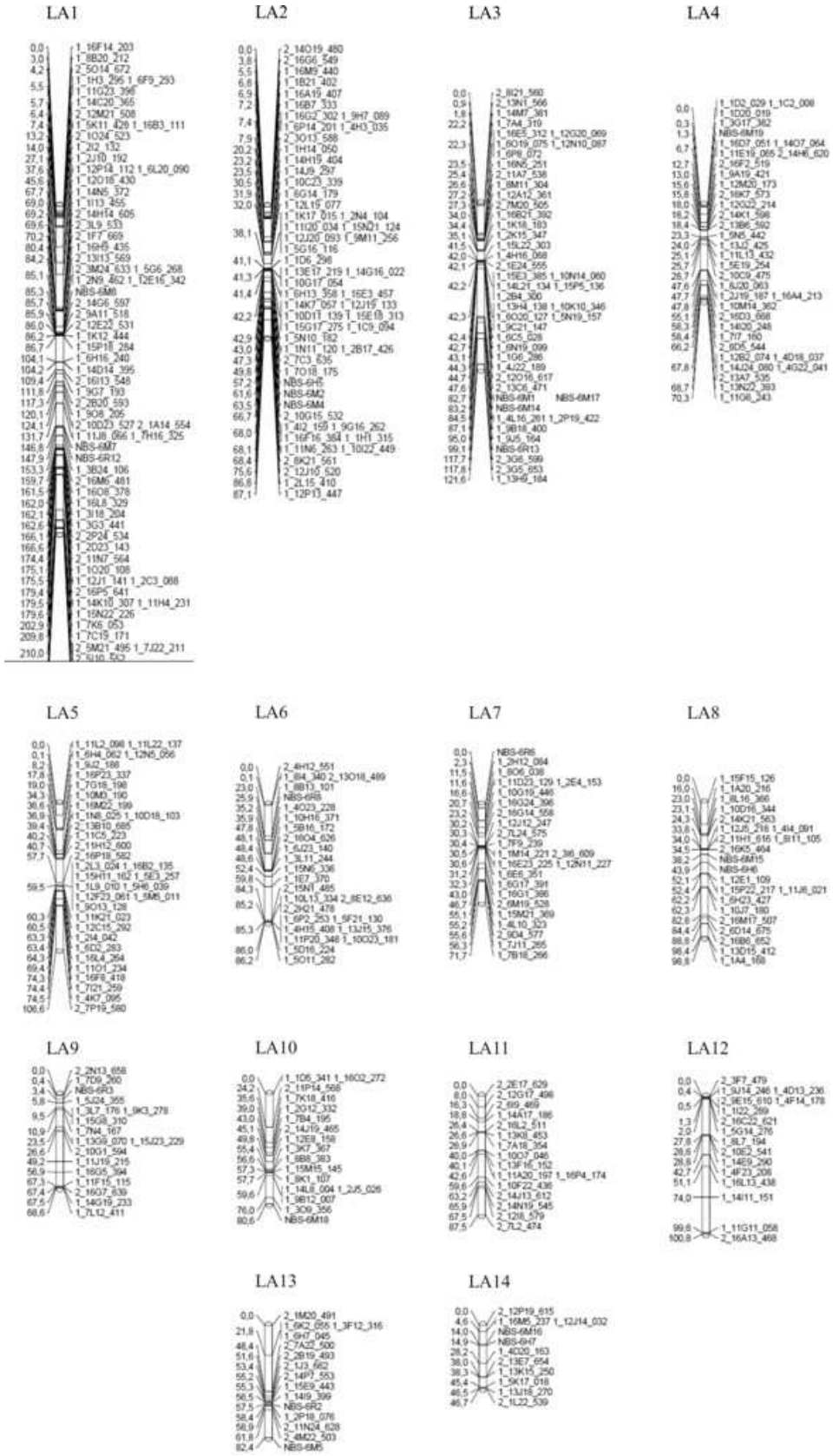

Fig. 5.2. Genetic linkage map for Longiflorum × Asiatic population. Map distance (cM) and name of markers are shown on the left and right side of the linkage group respectively.

Integration of genetic linkage groups with physical maps in Lilium based on FISH technique

An important goal is to integrate the genetic maps constructed based on molecular markers to the corresponding cytogenetic maps through *in situ* hybridization techniques. This would give a real image in the investigation of crop genomic structure. Several attempts have been made to correlate molecular maps with cytogenetic maps using *in situ* hybridization techniques in various crops. Examples are *Brassica oleracea* (Howell et al. 2002), *Lolium- Festuca* (King et al. 2002a), *Allium* spp. (Khrustaleva et al. 2005) and *Lycopersicon esculentum* (Koo et al. 2008). Based on FISH technique it would be possible to detect the position of different DArT markers distributed amongst various linkage groups on the physical structure of the chromosomes. This may help us to end up with 12 linkage groups equal to the number of haploid (*n*) chromosomes in *Lilium*. By doing so we may also be able to locate the markers linked to some specific traits on various chromosomes and the transfer of these genes could be monitored in the subsequent progenies. The availability of the cytological maps based on recombination sites in three genomes of *Lilium* (Khan et al. 2009a) and the DArT markers which are linked to some specific traits to be used as probes in FISH technique may offer very exciting opportunities for assessing these markers located on different genetic linkage groups with the markers (recombination sites) on the cytological map on various chromosomes.

Chapter 6

General Discussion

The results presented in the four experimental chapters of this thesis mainly describe a molecular cytogenetic approach to study the intergenomic recombination and introgression in interspecific lily hybrids and their progenies. The reported results in different chapters (chapters 2-5) on different interspecific lily hybrids can pave the path for more rational approaches for cytogenetic mapping and introgression of certain traits into lily cultivars. In this context, the following topics will be discussed in more detail in subsequent paragraphs to draw the attention to the practical and theoretical aspects of introgression of alien segments at different ploidy levels.

- Production of different types of gametes in interspecific lily hybrids and their use in breeding schemes
- Recombination events and the development of cytological maps and their significance
- Prospects for integrating molecular linkage groups and cytogenetic maps

Different types of gamete production and their use in lily breeding

Chromosome pairing, crossing over and the distribution of chromosomes are the most important event during meiosis (Sybenga 1975). The outcomes of these events lead to the formation of different types of gametes in interspecific lily hybrids. The products of meiosis are interrelated with chromosome pairing at metaphase I and chromosome distribution at anaphase I. If all of the homoeologous chromosomes pair and normal meiosis occurs, it produces four haploid gametes. When homoeologous chromosomes fail to pair, first division restitution (FDR) can occur and produce two 2*n* FDR gametes; if bivalents disjoin and univalents divide simultaneously at anaphase I, such meiosis leads to indeterminate meiotic restitution (IMR) and produce two 2*n* IMR gametes. The mechanism of 2*n* gamete formation with a single pair of chromosome assortment is illustrated in the Fig. 6.1. It was found that most of the LA and OA hybrids have possibilities to produce a high amount of aneuploid gametes and a small amount of 2*n* gametes. However few LA hybrids were shown to produce *n* gametes as well (Chapter 2). The aneuploid gametes are responsible for the sterility of F1 hybrids. Based on the homoeologous distribution at anaphase I, the F1 LA hybrids have more potential to produce IMR 2*n* gametes than FDR 2*n* gametes (Zhou 2007). However, it was found that most of the BC1 progenies result from FDR 2*n* gametes and less from IMR 2*n* gametes (Lim et al. 2001a; Barba-Gonzalez et al. 2005b; Zhou et al. 2008). The same results were also found in the present research (Chapters 3 and 4). It might be argued that FDR 2*n* gametes have better viability than

IMR 2*n* gametes due to the balanced chromosomal composition of FDR 2*n* gametes as compared to IMR 2*n* gametes.

Ploidy manipulation and its significances in lily breeding

One of the most important drivers of genetic variation is the occurrence of intergenomic recombination – whether in restitutional meiosis or somatically doubled allotetraploid. Thus in the absence of intergenomic recombination, an allotetraploid gives rise to identical 2x gametes without any potential for genetic variation. On the contrary, intergenomic recombination is most likely to occur in the diploid hybrid during restitutional meiosis and has the potential to produce considerable genetic variation. F1 distant hybrids usually have abnormal meiosis and are highly sterile. The traditional approach of chromosome doubling or the so-called somatic doubling, of the sterile hybrids is less fruitful because the resulting allopolyploids are not amenable to intergenomic recombination due to autosyndetic pairing. In view of this, the use of 2*n* gametes from LA and OA hybrids would be the most logical approach, because in addition to overcoming F1 sterility, intergenomic recombination can also be accomplished. However, 2*n* gametes occur in very low frequencies in OA-hybrids, while in other interspecific lily hybrids such as LR, LO or OP hybrids, there was no evidence for 2*n* gamete formation exists (Van Tuyl personal comm.). Detailed studies were made on the production of functional 2*n* gametes in lily distant hybrids (Van Tuyl et al. 1989; Asano 1982; Lim et al. 2001a; Barba-Gonzalez et al. 2005b). In this thesis, genotypes which produce functional 2*n* gametes as well as *n* gametes were found (Chapters 2, 3 and 4). The advantages of 2*n* gametes and production of BC progenies *via* unilateral sexual polyploidization have been documented (Lim et al. 2001a; Barba-Gonzalez et al. 2005b; Zhou et al. 2008). In this thesis, bilateral sexual polyploidization in LA hybrids and its potential advantages over mitotic doubling of chromosomes in lily breeding has been elucidated (Chapter 4).

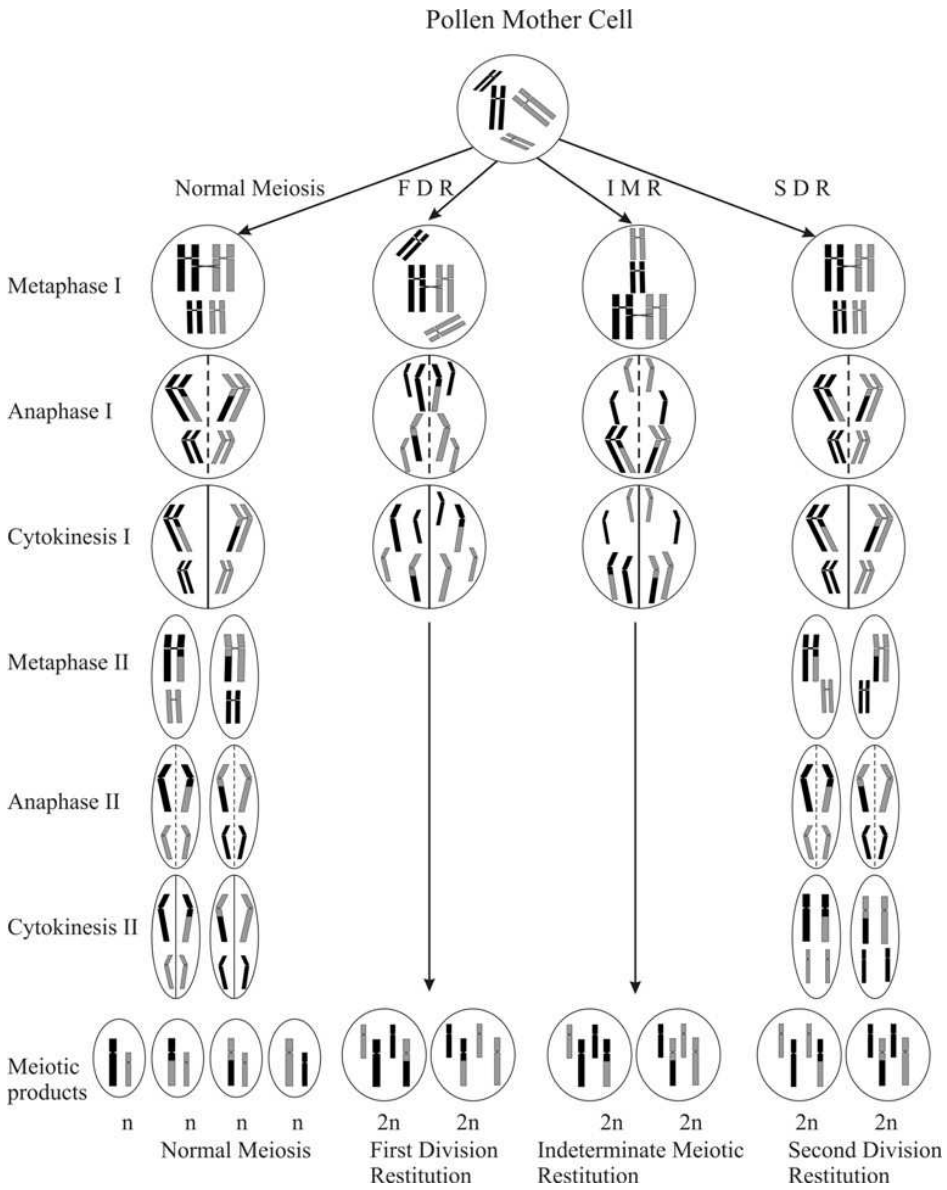

Fig. 6.1. An illustration of meiotic process and three types of restitution mechanisms of 2*n* gametes formation in interspecific lily hybrids

In some crops introgression can be accomplished at the diploid level even though the species involved belong to different genera. A well known example is *Festuca – Lolium* hybrids in which the desirable characters are introgressed at the diploid level – even though the genomes are quite differentiated (Zwierzykowski et al. 1998; Thomas et al. 2003). The same approach can be used in the case of LA hybrids (Chapter 2). This also implies that, like the *Festuca – Lolium* hybrids, introgression in LA hybrids can be achieved at the diploid level. The diploid (distant) interspecific hybrids that can produce both *n* as well as 2*n* gametes provide an important opportunity for the introgression of different traits which can be more readily selected for characters at diploid level. Once the selection has been made at the diploid level, then the

unilateral sexual polyploids can be achieved by using 2*n* gametes to reach the optimum ploidy level, e.g. triploid in lilies (Van Tuyl and Lim 2003; Zhou et al. 2008). The occurrence of *n* gametes in the F1 LA hybrids and the production of diploid BC1 progenies have been reported recently. It was found that diploid BC1 progenies have more interstitial recombinant chromosomes (Chapter 2), and it is expected that they may have relatively good fertility. This finding might open a new way of introgression breeding, the 'analytic breeding' in interspecific LA hybrids. This approach involves the reduction of polyploids to diploid level followed by intensive selection of superior genotypes through breeding at this level and in the final step optimum ploidy level is achieved (through sexual polyploidization) from the selected diploid parents (Jacobsen et al. 1991). Besides their relevance to analytic breeding, the diploid BC progenies can be useful for molecular mapping as well as for molecular markers assisted selection (MAS) for useful traits. A hypothetical analytic breeding scheme for lily LA hybrids is presented in Fig. 6.2.

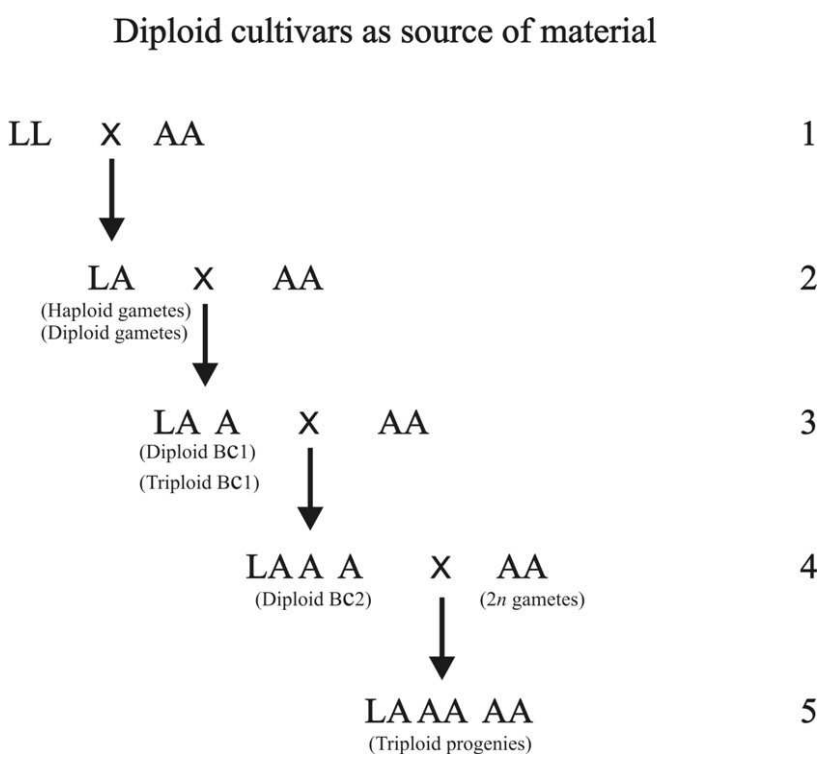

Fig. 6.2. Hypothetical analytic breeding scheme for LA hybrids, L (Longiflorum genome); A (Asiatic genome)

The variation caused by 2*n* gametes considerably increases the chance of selection at BC1 level. The occurrence of bilateral sexual polyploidization by sib-mating of F1 LA hybrids and the production of tetraploid F2 progenies have been reported for the first time in lily (Chapter 4). The tetraploid F2 progenies contained recombinant chromosomes and it is expected

that they may have a relatively good fertility. One advantage of bilateral sexual polyploidization is that fertile 4x progenies with intergenomic recombination can be used for generating considerable genetic variation either by selfing or crossing with other suitable genotypes as has been illustrated in *Alstroemeria* (Ramanna et al. 2003). This finding might open a new way for lily introgression breeding with considerable genetic variation in the subsequent progenies. This method can be used to overcome the disadvantages of mitotic chromosome doubling which is less suitable for creating any genetic variation.

Introgression breeding in interspecific lily hybrids between Longiflorum × Asiatic or Oriental × Asiatic plants mostly depends on the production of 2*n* gametes. These 2*n* gametes producing hybrids are backcrosses with one of the parental plant for introgression and selection for the trait of interest. However, hybridization of such allopolyploid with elite cultivars adds the entire genomes of the alien species rather than only the chromosome segments that possess the desirable traits. The addition of entire genomes, obviously, adds many undesirable traits along with the desirable ones into the cultivars enhancing so called, linkage drag (Hospital 2001). The amount of linkage drag is dependent on the frequency of crossover events around the target gene and the number of backcrosses. One effective way of preventing linkage drag is the addition of trait specific recombinant segments (Servin et al. 2004). To achieve this, homoeologous recombination must be accomplished in the hybrids of the putative parental species followed by their recovery in the progenies of the F1 hybrids. These two requirements are most ideally achieved in the case of LA and OA hybrids (Chapter 2 and 4) with a wide range of recombinant chromosomes with varying numbers and lengths of recombinant segments have been recovered in the BC1 progenies. Furthermore, the variation caused by the use of 2*n* gametes considerably increases the chance of selection at BC1 level. The progenies obtained after unilateral sexual polyploidization could express the recessive loci by attaining the nulliplex condition (Fig. 6.3). Various genotypes in LA and OA hybrids (Chapter 4) exhibited nulliplex condition. This could facilitate the selection of genotypes at BC1 level. Similarly, the tetraploid F2 progenies resulting from bilateral sexual polyploidization contained recombinant chromosomes when backcrossed with one of the putative parents and then the expression of recessive loci could be enhanced further as shown in Fig (6.3). These triploid BC plants obtained after unilateral sexual polyploidization and those derived from bilateral sexual polyploidization could further be used in introgression breeding for the further dissection of genome with minimum linkage drag.

In spite of all the attractive features of using 2*n* gametes in breeding, there is a limiting factor in some cases in terms of the genotypes which produce 2*n* gametes. Only few genotypes can produce 2*n* gametes on a regular basis. Besides this, their production is highly subjected to

environmental conditions. Moreover, most of the genotypes produce either only 2*n* pollen or 2*n* eggs but not both simultaneously. In view of these difficulties, the induction of 2*n* gametes through N_2O treatment (Barba-Gonzalez et al. 2006b; Wu et al. 2007) is a positive development for using 2*n* gametes in lily breeding. The production of 2*n* gametes in interspecific lily hybrids might respond in a positive way to the N_2O treatments, broadening the possibilities to combine important traits present in the sterile hybrids and generate even more interspecific hybrids. It was found that N_2O treated F1 OA hybrids gave progenies with recombinant chromosomes (Barba-Gonzalez et al. 2006b) which shows the advantages of this technique to restore fertility while genetic variation is generated.

In view of this, introgression of desirable segments can be accomplished in lilies, through hybridization of distant species and backcrossing. This is possible because both *n* and 2*n* gametes can be used in breeding and techniques of DNA *in situ* hybridization facilitate the monitoring of alien chromosomes and recombinant segments in every step.

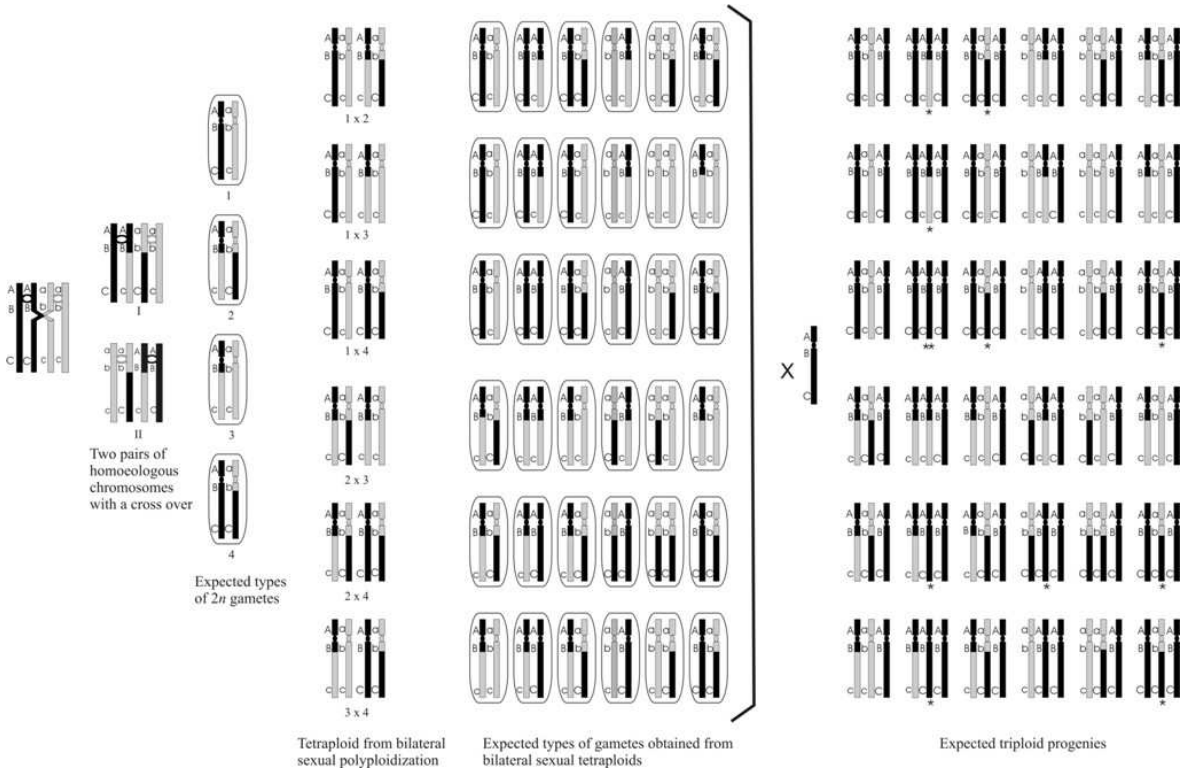

Fig. 6.3. A schematic representation of gametes resulted from bilateral sexual polyploidization and their subsequent progenies for selection of traits. (* Nulliplex condition; ** Complete substitution of chromosome)

Molecular genetic basis of 2*n gametes production*

The reliance of 2*n* gametes in plant polyploidization has been realized since a long time and they are known to occur in diploid populations as a result of meiotic failure, led to change in the genetic consequences of the subsequent progenies (Harlan and de Wet 1975). It is believed that

2*n* gametes are the major route for polyploidy formation in interspecific lily hybrids, particularly leading to the formation of triploids with recombinant chromosomes segments (Lim et al. 2001a; Barba-Gonzalez et al. 2005b; Zhou et al. 2008). 2*n* gametes are also important for the genetic improvement of several polyploid crops, where useful genes from diploid relatives are incorporated into cultivated genotypes (Ramanna and Jacobsen 2003). Given their importance in lily breeding, 2*n* gametes have got a focal point of research and a considerable amount of research work has been done (Lim et al. 2001a; Barba-Gonzalez et al. 2005b and 2006b). The production of 2*n* gametes is sporadic and strongly genetically controlled. However, limited information is available about the genetic control of 2*n* gamete formation. There is a need for the identification and characterization of genes responsible for 2*n* gamete formation in lilies. Recently, (d'Erfurth et al. 2008) isolated and characterized a gene from *Arabidopsis thaliana (AtPS1)* which causes the formation of high frequency of 2*n* gamete in the model plant. In the near future an Expressed Sequence Tag (EST) library of *Lilium* cDNA will be constructed and sequence analysis will be compared with *Arabidopsis* and other sequenced crops having genes with known functions. It could be possible that some of these ESTs from generative cells show sequence similarity to the *Arabidopsis thaliana* gene (*AtPS1*) responsible for 2*n* gamete formation. The identification and incorporation of such genes involved in 2*n* gamete formation in interspecific lily hybrids can open the door to speed up the process of introgression breeding in lilies.

Molecular and cytogenetic mapping

Molecular cytogenetics has not only revolutionized the genetic analysis of plant genomes, but has also provided plant breeders and geneticists with a tool to identify genes involved in determining various traits. The development of the genomic *in situ* hybridization (GISH) technique for cytogenetic analyses in interspecific lily hybrids has represented a major advance in the genome analysis of lily hybrids (Karlov et al. 1999; Lim et al. 2001; Barba-Gonzalez et al. 2004). This technique has been employed to demonstrate the intergenomic recombination in the intergeneric and interspecific hybrids of different species in crop complexes like, *Gasteria* × *Aloe* (Takahashi et al. 1997), *Festuca* × *Lolium* intergeneric hybrids (King et al. 2002a and b), *Alstroemeria* spp. (Kamstra et al. 1999a and b; Ramanna et al. 2003) and *Allium* spp. (Khrustaleva and Kik 1998). In the present investigation, GISH is employed to construct the chromosomal recombination maps, the so-called ‘cytomolecular maps’ in three genomes of *Lilium*. For this purpose, BC progenies from distant lily hybrids were used for mapping without which the constituent genomes and the recombination sites on individual chromosome could not

be distinguished through GISH. An attractive feature of the cytomolecular maps is that large numbers of recombination sites become available as physical land marks on individual chromosomes. Using such sites or land marks, molecular markers, like AFLPs, and RFLPs can be assigned to specific positions on individual chromosomes as has been done in the case of *Festuca-Lolium* substitution line (King et al. 2002) and *Allium cepa* × (*A. roylei* × *A. fistulosum*) interspecific crosses (Khrustaleva et al. 2005). Mapping of individual chromosomes in these two cases show that proper integration of molecular maps with respective chromosomes can only be accomplished when reliable cytological markers are available. We described here for the first time the cytomolecular mapping of three genomes of *Lilium* obtained after using *n* and 2*n* gamete producing LA and 2*n* gamete producing OA hybrids. These BC1 progenies of LA and OA hybrids have been used as the starting point for the estimation of recombination sites on each chromosome and potentially it can be used successfully to transfer and locate the genes or markers linked to genes of interest in interspecific hybrids in *Lilium* during a backcross breeding programme. Cytomolecular maps of genomes such as the ones reported here can pave the way for the construction of more meaningful maps where there is integration of molecular data on the morphological features of the chromosomes. It was found that chromosome 9 in different interspecific lily hybrids had the maximum recombinant segments leading to maximum number of recombination sites after GISH analysis (Chapter 3). The recovery of these recombination sites at chromosome 9 in different interspecific hybrids indicated that the L- genome is much more closely related to the A- genome as compared to the O- genome. The high frequency of homoeologous recombination makes this chromosome unique among lily genome from evolutionary point of view. These results are likely to pave the way for molecular approaches for breeding new cultivars, such as marker assisted breeding, where with the combination of molecular markers and FISH techniques it becomes possible to localize markers for specific genes in the chromosomes and trace them in the progeny of subsequent crosses.

Mapping of plants with large genome

Fluorescent in-situ hybridization (FISH), a molecular cytogenetic technique which can be used for the integration of molecular genetics maps with cytogenetic maps. It has opened the possibilities for localizing large numbers of cloned DNA sequences directly on chromosomes for mapping purposes. It has become a standard technique to localize, orient, and map genes in the genomes of a wide range of species. These cytogenetic maps are constructed for several organisms and are considered essential to relate genetic loci and molecular sequences to morphological features of chromosomes (Fransz et al. 2000; Cheng et al. 2001). Several

successful attempts were made in the past to relate and integrate genetic linkage groups on individual chromosomes cytogenetically through FISH. For example maize (Ananiev et al. 1998), tomato (Zong et al. 1996), rice (Cheng et al. 2001) and potato (Song et al. 2000). Furthermore, the application of GISH/FISH techniques in the physical mapping of different genes in different introgression lines derived from festuca-lolium intergeneric hybrids has also been reported (Humphreys et al. 2005).

In several crops BAC libraries are constructed and individual BAC clones containing the markers linked to various traits were physically localized on different chromosomes using FISH. High resolution cytogenetic maps provide important biological information on genome organization and function, as they correlate genetic distance with chromosome structures. *Lilium* has one of the largest genomes in plant kingdom, almost 250 fold larger than the *Arabidopsis* genome (Bennet et al. 2003; Leitch et al. 2007) which is mostly comprised of highly repetitive DNA sequences. To establish a feasible mapping system for *Lilium*, DArT technique was used to construct a genetic linkage map in interspecific LA hybrids. This genetic map ended up with 14 linkage groups instead of the expected 12, the basic number of the lily genome. This finding could be argued on the basis of the extremely large and highly repetitive genome size of lily which hinders some of the genomic regions to be linked together. The occurrence of a higher number of linkage groups compared to the basic chromosome number creates an ambiguity as it is not possible yet to link or assign any linkage group to specific chromosomes of lily. One possibility is that the DArT markers genetically mapped on different linkage groups could be probed and their physical location can be determined on chromosomes by FISH. Integration of existing information from genetic maps with the cytological structure of chromosomes will provide a comprehensive data in cytogenetic map. However, it is noted that the average size of these DArT markers is around 800-1200 bp and they are single or low copy sequences. Detection of single copy genes on plant chromosomes is difficult. So it is hard to monitor the location of these individual markers used as probes on mitotic chromosomes which are highly condensed and possess highly repetitive DNA sequences.

The sequencing and mapping of plants with large and complex genomes are disadvantageous as their sequencing is expensive, time consuming and is sometimes hampered by a significant content of repetitive DNA repeats. Because the material used for mapping is of interspecific origin, the allopolyploid nature of lily and the presence of homoeologous genomes also cause a serious problem in the mapping. One possibility is the dissection of the genome into its component chromosomes and chromosome arms by flow cytometeric sorting (Doležel et al. 2007). This could provide a probable solution to these problems. However, the identification of

individual chromosomes and its discrimination from the rest of the genome might be complicated. Once the individual chromosomes have been sorted out, it is advantageous to construct BAC libraries specific for a defined chromosome or genome segments, and this represents an area in which chromosome sorting can play a crucial role. These BAC clones can be probed on chromosomes through FISH. This could be an elegant approach in sequencing and mapping complex and large genomes (Paux et al. 2008).

Furthermore, technical refinements are needed in FISH for successful detection of single copy genes or markers on *Lilium* mitotic metaphase chromosomes. This technique has a great potential to complement genetic mapping on orphan and large genomes and could be used to integrate the genetic linkage group with physical features of the individual chromosomes. We are trying to construct a cytogenetic map using DArT markers anchored to the genetic map. The probing of a set of DArT clones on one of the linkage group could be localized on specific chromosomes by FISH. This will be useful for the integration of existing information from genetic maps with the cytological structure of chromosomes and will provide a comprehensive genetically anchored cytogenetic map.

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Summary

Lilies (*Lilium* L.) are one of the most important ornamental bulbous crops for cut flower industry grown extensively in The Netherlands for last few decades. The genus *Lilium* consists of seven different sections with about 80 species. The species within genus *Lilium* ($2n = 2x = 24$) comprise a range of desirable and complementary characters. Besides being an important horticultural crop, lily (*Lilium*) also serves as an interesting model plant for molecular cytogenetic research and introgression breeding for several reasons like, i). Lily is a model crop for interspecific hybridization and it includes plants of different taxonomic species each of which possess valuable horticultural traits that need to be combined in the new cultivars. ii) Through careful selection *n* and 2*n* gametes can be obtained in interspecific hybrids. iii) The genomes of different species are so well differentiated genetically that the parental chromosomes can be clearly identified in the F1 hybrids as well as in the progenies through DNA *in situ* hybridization techniques. iv) The chromosomes are large enough and the number and position of homoeologous recombination sites can be clearly detected. v) The large and easily identified chromosomes in different lily species could be a potential source for the cytological mapping of the *Lilium* genomes. Taking advantage of these favourable attributes of lily, a molecular cytogenetic investigation was conducted to evaluate the amount of recombination and introgression of characters between Longiflorum - Asiatic (LA) and Oriental - Asiatic (OA) hybrids through the use of *n* and 2*n* gametes.

For this purpose different F1 Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) hybrids were backcrossed with different Asiatic cultivars. Ovule and embryo rescue techniques were employed to get backcross (BC) progenies. Most of the F1 LA appeared to be sterile but some hybrids were able to produce only 2*n* gametes in considerable frequencies. However, in rare occasions it was also found that normal meiosis took place in few of the LA hybrids which resulted into the formation of normal *n* gametes. Ploidy level and intergenomic recombination was studied in LA interspecific hybrids in order to assess the possibility of functional *n* gametes and their potential use in introgression at diploid level in lily. A total of 104 BC1 LA interspecific lily hybrids were obtained, 27 diploids ($2n = 2x = 24$), 73 triploids ($2n = 2x = 36$) and 4 aneuploids ($2x - 1$, $2x + 2$ or $2x + 3$). Similarly, triploid BC1 (LAA) plants were backcrossed to diploid Asiatic parents. As a result 14 diploid BC2 progenies were produced. The intergenomic recombination and amount of introgression of respective genome (L and A) was assessed in these diploid genotypes through GISH (Genomic *in situ* Hybridization). Extensive intergenomic recombination was found among the chromosomes in LA hybrids. A large of amount of L- genome was transmitted from F1 LA hybrids to their subsequent BC1 progenies.

However, very few segments of L- genome were introgressed from the BC1 diploid and triploid (LAA) plants to the BC2 progenies (Chapter 2). GISH identifies a considerable amount of recombination events amongst different interspecific lily hybrids (LA and OA) obtained from functional 2*n* gametes. Based on recombination sites on different chromosomes identified by GISH, cytological maps of three genomes of *Lilium* were constructed. For this purpose, BC progenies of two diploid interspecific hybrids of lily, *viz*., Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) were used. The BC progenies of LA hybrids consisted of both triploid (2n = 3x = 36) and diploid (2n = 2x = 24) with some aneuploid genotypes and those of OA hybrids mostly consisted of triploid (2n = 3x = 36) and some aneuploid genotypes. In LA hybrids 248 recombination sites were cytologically localized on 12 different chromosomes of each genomes (i.e., L and A). Similarly, 116 recombinant sites were marked on 12 chromosomes each from the BC progenies of OA hybrids (O and A genomes). The distances of the recombination sites from the centromeres are measured (in micrometres). Based on these recombination sites four cytological maps were constructed. Since an Asiatic parent was involved in both hybrids, *viz*., LA and OA, two maps were constructed for A genome which were indicated as Asiatic (L) and Asiatic (O) and one each for Longiflorum (A) and Oriental (A) genomes (Chapter 3).

With a view to generate genetic variation *via* homoeologous recombination in BC progenies of LA and OA hybrids the most logical approach was the use of 2*n* gametes. 63 BC1 LA (LA × AA or AA × LA) and 53 OA (AA × OA) progeny plants were obtained after unilateral sexual polyploidization. 16 genotypes from F2 LA populations were obtained after bilateral sexual polyploidization through sib-mating of F1 LA hybrids. GISH was employed for the identification of the parental genomes, mode of origin of these progenies and measurement of the introgression in different interspecific lily hybrids. Most of the BC1 progeny plants (LA and OA) had originated through 2*n* gametes by First Division Restitution (FDR) mechanism. However, there were 12 genotypes in LA hybrids and four genotypes in OA hybrids that originated through 2*n* gametes formation as the result of Indeterminate Meiotic Restitution (IMR). A higher amount of recombination was found in LA hybrids as compared to OA hybrids. Intergenomic recombination was also determined in the sib-mated F2 LA population. In this case both parents had contributed gametes with the somatic number of chromosomes (i.e., 2*n*-2*n*) thus confirming the event of bilateral sexual polyploidization in interspecific LA hybrids. Based on these results, the relevance of interspecific lily hybrids obtained from uni- and bilateral sexual polyploidization leading to allotriploid and allotetraploid formation in interspecific lily hybrids is discussed in the context of introgression and mapping (Chapter 4). Molecular markers are an

important tool for the construction of genetic linkage maps, as the first step in the genetic dissection of the required traits leading to crop improvement followed by the marker assisted breeding in different plants. *Lilium* has one of the largest genome in plant kingdom and genetic mapping in lilies is constrained by its large genome. DArT (Diversity Array Technology), a molecular marker technique can detect and type DNA variation at several hundred genomic loci in parallel without relying on genome sequence information. The DArT technique was developed for Longiflorum × Asiatic (LA) lily hybrids to enable an efficient and effective genetic mapping with the production of a large numbers of markers in microarrays-based assay. The restriction enzyme *Pst*I + *Taq*I combination generated the largest frequency of polymorphic genomic representations for a genotyping array. Genomic representations from 88 F1 LA plants were used to assemble a DArT genotyping microarray. A total of 687 DArT markers were developed and 382 polymorphic markers were mapped on 14 main linkage groups which is two more then the haploid chromosome number (i.e. n = 12). The resulting linkage map with 382 DArT markers spanned 1329 cM (3.5 cM/marker on average). The results highlighted the potential of DArT as a genetic technique for genome profiling in the context of molecular breeding and genomics, especially in crops with large genome sizes where other techniques proved to be less useful (Chapter 5).

The results of the present investigation are of practical implication. These results show the advantages of the *n* gametes and their subsequent progenies which opened a new approach of lily breeding 'the analytic breeding' in the allopolyploids. It also shows the possibility of using certain triploid hybrids for further breeding. A comparison has been made between different types of interspecific crosses, the amount of intergenomic recombination and introgressions of chromosomal segments obtained after unilateral sexual polyploidization. Furthermore, bilateral sexually polyploidization *via* sib-mated F1 hybrids producing 2*n* gametes has been proven. The use of allotetraploids obtained from bilateral sexual polyploidization could be a novel approach in the breeding of LA-hybrids. These allotetraploid with recombinant chromosomal segment may be a potential source to generate genetic variation in subsequent progenies. The molecular cytogenetic GISH and FISH techniques proved to be a powerful tool useful for the construction of cytogenetic maps in interspecific crosses in crops with large genomes sizes like lily. These techniques are also used for the identification and integration of genetic maps with chromosome maps. FISH also helps to monitor the introgressed chromosome segment or marker of interest in the subsequent progenies. Application of the DArT technique proved to be an effective method to construct genetic linkage maps especially crops (like *Lilium*) with large genome sizes where other techniques might be less useful.

Samenvatting

Lelies *(Lilium* L.*)* behoren tot de belangrijkste bolgewassen voor de snijbloemteelt en worden de laatste decennia op grote schaal in Nederland geteeld. Het geslacht *Lilium* (2n = 2x = 24) wordt ingedeeld in zeven taxonomische secties met in totaal circa 80 species die een reeks van gewenste en elkaar aanvullende eigenschappen bezitten. Naast een belangrijk tuinbouwgewas, is lelie (*Lilium*) ook een interessant modelgewas voor moleculair cytogenetisch introgressie onderzoek en wel om de volgende redenen: i) Het gewas is een modelgewas in soortkruisingsonderzoek en omvat genotypen uit verschillende taxonomische soorten die elk waardevolle, tuinbouwkundige eigenschappen bevatten die gecombineerd moeten worden in nieuwe cultivars ii) Door een juiste selectie kunnen bij interspecifieke hybriden zowel *n* als 2*n* gameten verkregen worden iii) De genomen van de verschillende species zijn genetisch zo goed te onderscheiden dat de ouderchromosomen zowel in de F1 hybriden als in hun nakomelingen duidelijk geïdentificeerd kunnen worden met behulp van genomische *in situ* hybridisatie (GISH) technieken iv) De chromosomen zijn groot en het aantal en de locatie van homoeologe recombinatie punten kan duidelijk gedetecteerd worden v) De grote en gemakkelijk te identificeren chromosomen van verschillende lelie species zijn een potentiële bron voor het cytologisch in kaart brengen van de *Lilium* genomen. Gebruikmakend van deze gunstige attributen van lelie, is moleculair cytogenetisch onderzoek uitgevoerd naar de mate van recombinatie en introgressie in Longiflorum – Aziaat (LA) en Oriental – Aziaat (OA) hybriden uitgaande van *n* en 2*n* gameten.

Voor dit onderzoek werden verschillende LA en OA F1 hybriden teruggekruist met verschillende Aziatische cultivars. Zaadknop en embryo reddingstechnieken werden toegepast om terugkruisingspopulaties (BC) te verkrijgen. Veel F1 LA-hybriden bleken steriel te zijn, maar een aantal uitsluitend 2*n*-gameten producerende hybriden kon worden geïdentificeerd waarbij voldoende gameten werden geproduceerd voor vervolgonderzoek. In uitzonderlijke gevallen bleek bij LA hybriden deels een normale meiose plaats te vinden resulterend in de vorming van *n*-gameten. Om de potentie van *n*-gameten en de toepassing hiervan ten behoeve van analytische veredeling op diploïd niveau in lelie te onderzoeken werd het ploïdie niveau en de intergenomische recombinatie in nakomelingen van deze LA-hybriden onderzocht. In totaal werden 104 BC1 LA-hybriden bestudeerd; hiervan waren 23 diploïd (2n = 2x = 24), 73 triploïd (2n = 2x = 36) en 4 aneuploid (2x – 1, 2x + 2 of 2x + 3). Op soortgelijke wijze werden triploïde BC (LAA) planten teruggekruist op diploïde Aziatische ouders. Dit resulteerde o.a. ook in 14 diploïde BC2 nakomelingen. De intergenomische recombinatie en de mate van introgressie van de beide genomen (L en A) in deze diploïde genotypen werd door middel van GISH

(Genomische *in situ* Hybridisatie) vastgesteld. Tussen de chromosomen van deze LA-hybriden werd een omvangrijke intergenomische recombinatie waargenomen. Een groot deel van het L-genoom werd doorgegeven van de F1 LA-hybriden naar de BC nakomelingen. Er werden echter slechts enkele segmenten van het L-genoom van de diploïde en triploïde BC1 (LAA) planten overgedragen naar de BC2 nakomelingen (Hoofdstuk 2). Met GISH is een groot aantal recombinatie gebeurtenissen geïdentificeerd die in verschillende LA en OA-hybriden werden verkregen met behulp van functionele 2*n*-gameten. Met behulp hiervan zijn recombinatie kaarten van de 3 *Lilium* genomen geconstrueerd. Hiertoe zijn BC nakomelingen van twee diploïde interspecifieke leliehybriden gebruikt, te weten LA en OA-hybriden. De BC nakomelingen van de LA-hybriden bestonden uit triploïde (2n = 3x = 36) diploïde (2n = 2x = 24) en enkele aneuploïde genotypen en die van de OA-hybriden bestonden hoofdzakelijk uit triploïde (2n = 3x = 36) en enkele aneuploïde genotypen. In de LA-hybriden werden 248 recombinatie plaatsen gelokaliseerd op de 12 verschillende chromosomen van elk genoom (L en A). Evenzo werden bij de OA-hybriden 116 recombinatie plaatsen gelokaliseerd op de 12 chromosomen van het O- en A-genoom. De afstand (in micrometer) van de recombinatie plaatsen tot de centromeren werd bepaald. Op basis van deze recombinatieplaatsen werden vier cytologische kaarten geconstrueerd. Omdat de Aziatische ouder in beide hybriden (LA en OA) aanwezig is, werden twee kaarten geconstrueerd voor het A-genoom aangeduid als Aziaat (L) en Aziaat (O) en één voor de Longiflorum (A) en Oriental genomen (Hoofdstuk 3).

De meest logische benadering bij het creëren van genetische variatie via homoeologe recombinatie in BC nakomelingen van LA en OA hybriden is de toepassing van *n*- en 2*n*-gameten. In totaal, werden 63 BC1 LA (LA × AA en AA × LA) en 53 OA (AA × OA) nakomelingen verkregen via unilaterale polyploïdisatie. Daarnaast werden 16 F2 LA nakomelingen verkregen via bilaterale sexuele polyploïdisatie door onderlinge kruising van F1 LA-hybriden. GISH werd toegepast voor identificatie van de ouder genomen, de ontstaanswijze en de bepaling van de mate van introgressie bij de verschillende lelie hybriden. De meeste BC1 nakomelingen (LA en OA) ontstonden uit 2*n*-gameten via het zogenaamde First Division Restitution (FDR) mechanisme. Er werden echter negen LA- en vier OA-genotypen geïdentificeerd waaraan het zogenaamde Indeterminate Meiotic Restitution (IMR) mechanisme van 2*n* gameetvorming aan ten grondslag lag. In de LA-hybriden werd in vergelijking met de OA-hybriden meer recombinatie gevonden. Intergenomische recombinatie werd ook vastgesteld in de F2 LA-populaties. In dit geval hebben beide ouders met 2*n*-gameten bijgedragen waarmee de bilaterale sexuele polyploïdisatie gebeurtenis in de LA-hybriden wordt bevestigd. De relevantie van de interspecifieke lelie hybriden verkregen na uni- en bilaterale polyploïdisatie

resulterend in allotriploïden en allotetraploïden wordt bediscussieerd in relatie tot introgressie en de genetische kartering (Hoofdstuk 4).

Moleculaire merkers zijn een belangrijk hulpmiddel voor de constructie van genetische koppelingskaarten, als eerste stap naar de genetische ontrafeling van eigenschappen die leiden tot gewasverbetering. Hiermee kan vervolgens door moleculaire merkers ondersteunde veredeling worden toegepast. Het genus *Lilium* heeft één van de grootste genomen in het plantenrijk en genetische kartering bij lelies wordt bemoeilijkt door het grote genoom. DArT (Diversity Array Technology) is een op microarrays gebaseerde moleculaire merker techniek die variatie in DNA op enkele honderden loci simultaan kan detecteren zonder specifieke informatie van genoomsequenties. De DArT techniek werd aangepast voor LA-hybriden om efficiënt genetische kartering mogelijk te maken. De combinatie van de restrictie enzymen *Pst*I + *Taq*I genereerde de hoogste frequentie van polymorfe genomische representaties voor een genotypen array. Genomische representaties van 88 F1 LA planten werden gebruikt om een DArT microarray samen te stellen. In totaal werden 687 DArT merkers ontwikkeld en 382 polymorfe merkers konden worden geplaatst op 14 koppelingsgroepen. Dit is twee meer dan het haploïde chromosoomaantal (n = 12). De verkregen koppelingskaart met de 382 DArT merkers is 1329 cM (3.5cM/merker) lang. De resultaten onderstrepen de potentie van DArT als genetische techniek voor genoom karakterisering, speciaal voor zogenaamde moleculaire probleemgewassen en gewassen met grote genomen waarvoor andere technieken minder bruikbaar zijn (Hoofdstuk 5).

De resultaten van dit onderzoek hebben praktische implicaties. De resultaten tonen de voordelen van *n*- gameten waarmee een nieuwe aanpak van de lelieveredeling, de zogenaamde “analytische veredeling”, bij de veredeling van de allopolyploïde LA’s kan worden toegepast. Het toont aan dat het mogelijk is om bepaalde triploïde hybriden te gebruiken voor verdere veredeling. Een vergelijking is gemaakt tussen verschillende typen interspecifieke kruisingen, de mate van intergenomische recombinatie en introgressie van chromosoom segmenten verkregen na unilaterale sexuele polyploïdisatie. Verder is de mogelijkheid van bilaterale sexuele polyploïdisatie via onderlinge combinatie van 2*n*-producerende F1 hybriden aangetoond. Het gebruik van allotetraploïden verkregen via bilaterale polyploïdisatie zou een vernieuwende aanpak van de LA-veredeling kunnen betekenen. Deze allotetraploïden met recombinante chromosoomsegmenten is een potentiële bron van genetische variatie in de nakomelingschappen. De moleculair genetische technieken GISH/FISH hebben getoond krachtige instrumenten te zijn voor de constructie van cytogenetische kaarten bij interspecifieke kruisingen in gewassen met grote genomen zoals lelie. Deze technieken zijn ook gebruikt voor de identificatie en integratie

van genetische kaarten met chromosoomkaarten. FISH maakt het mogelijk om overgedragen chromosoomsegmenten of merkers te volgen in de nakomelingschappen. Toepassing van de DArT techniek is een effectieve methode om genetische koppelingskaarten te construeren, speciaal in gewassen met grote genomen (zoals *Lilium*) waarvoor andere technieken minder bruikbaar zijn gebleken.

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Nadeem Khan

Wageningen UR

Curriculum Vitae

Muhammad Nadeem Khan was born in Rawalakot, District Poonch of Kashmir, Pakistan on 1st January, 1976. He studied his BSc in Biochemistry, at the University of the Punjab. He obtained his MSc degree in Botany from University of Peshawar in 2001. After that he completed his MPhil in Plant Physiology from Quaid-i-Azam University Islamabad in 2004. He spent one year 2005-06 at Kookmin University, Republic of Korea to work in a project entitled extraction, purification and characterization of extracellular enzymes from a wood rot fungus. He started his PhD at Wageningen University and Research Centre. His research was mostly focused on intergenomic recombination and introgression of chromosomal segments in interspecific lily hybrids. This thesis is the result of the work carried out from March 2006 up to June 2009 to obtain his PhD degree.

List of publication

Nadeem Khan, Shujun Zhou, M. S. Ramanna, Paul Arens, Jeronimo Herrara, Richard G. F. Visser and Jaap M. Van Tuyl (2009). Potential of analytic breeding in allopolyploids: an illustration using Longiflorum × Asiatic hybrid lilies (*Lilium*). Euphytica 166: 399-409

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Nadeem Khan, Rodrigo Barba-Gonzalez, M. S. Ramanna, Paul Arens, Richard G. F. Visser and Jaap M. Van Tuyl. Relevance of unilateral and bilateral sexual polyploidization in relation to intergenomic recombination and introgression in *Lilium* species hybrids (submitted)