The use of *2n* gametes for introgression breeding in Oriental × Asiatic lilies

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Chapter 1

General introduction

Lilies

Lilies have been cultured since ancient times, throughout the world and became one of the most important ornamental crops (Woodcock & Stern, 1950). They belong to the genus *Lilium,* a monocotyledonous bulbous crop of the Liliaceae family, with over 80 species (Comber, 1947). Lilies originated in the Himalayan region and they have extended over the mountain areas in the Northern hemisphere (De Jong, 1974). The genus has been classified into six sections (De Jong, 1974) from which the following contributed to the domestication of cut flowers:

a) Section Leucolirion, with Longiflorum hybrids. These hybrids are trumpet-shaped, with white flowers and a distinctive fragrance. They can be forced into flower year round (McRae, 1998).

b) Section Sinomartagon, with the Asiatic hybrids. These types represent the most widely cultivated group. They are preferred for their early flowering and their wide variation in colours, from bright to soft; they are white, pink, yellow, orange, and red (McRae, 1998). Probably the most important characteristics of some of these hybrids are their resistance to *Fusarium oxysporum* (Straathof & Van Tuyl, 1994; Straathof & Löffler, 1994a; 1994b), one of the most threatening pathogens to lilies, and their resistance to some viruses, for which no resistance is present in hybrids of other sections (McRae, 1998).

c) Section Archelirion, with Oriental hybrids. Hybrids form this section are used since the early 1950s in lily breeding (McRae, 1990). The number of commercial varieties, however, has increased within a span of a few years (Schenk, 1990). In general, Oriental hybrids are late-flowering, with big and showy flowers and possess a sweet fragrance (McRae, 1998). These hybrids show a wide variety of white, pink and yellow flowers. Most of them are resistant to *Botrytis elliptica,* a pathogenic fungus that affects most of the lilies from other sections.

Interspecific hybridization and breeding barriers

Lilies have a wide variety of agronomic characteristics of major importance, such as distinctive small or large flowers, simple or fancy shapes, up or down facing flowers, wide

variety of colours, different forcing times and foliage arrangements, variation in stem length and strength, but specially, resistance to certain pathogens that are restricted only to some hybrids within the different sections.
To combine agronomic traits in new hybrids, crosses within a section can be made with relative ease, but intersectional, interspecific hybridization is very difficult. This is, however, the most important tool to combine traits from different sections to generate completely new interspecific hybrids.

The production of intersectional hybrids was not possible due to i) pre-fertilization barriers caused by poor pollen tube growth due to stigmatic incompatibility (Asano & Myodo, 1977a; Asano, 1980c) and ii) post-fertilization barriers resulting in seeds having no endosperm and very small embryos that usually abort in early stage (Myodo, 1975; Asano & Myodo, 1977b).

From the sixties to the eighties major changes occurred in lily breeding with the introduction of special pollination techniques such as cut-style (Myodo, 1962) and intrastylar pollination (Asano & Myodo, 1977a), together with embryo culture (Myodo, 1975; Asano & Myodo, 1977b; 1980; Myodo & Asano, 1977; Asano, 1978; 1980a; 1980b) which were applied to overcome pre- and post-fertilization barriers, respectively. The application of these techniques resulted in a wide variety of new intersectional lily hybrids. At the end of the eighties and the beginning of the nineties even more inter-sectional hybrids were produced with the implementation of novel techniques to overcome pre- and post-fertilization barriers such as mentor pollen, *in vitro* pollination and ovary- and ovule culture (Van Tuyl, et al., 1982; 1988; 1991; Wolf & Van Tuyl, 1984). Examples of successful crosses performed are depicted in the crossing polygon shown in Figure 1.1. It shows the crossing compatibility within and between the sections (Van Tuyl et al., 2000).
Special interest exists today in breeding Oriental × Asiatic hybrids to combine the resistance to *Fusarium oxysporum* and viral diseases from Asiatic hybrids and the resistance to *Botrytis elliptica* from the Oriental hybrids into a new group of interspecific hybrids (Schenk, 1990; Lim et al., 2000a).

Hybrid sterility

Good examples of successful ornamental hybridization between distantly related species are present in the genera *Alstroemeria* (Ramanna, 1992; Lu & Bridgen, 1997; Buitendijk et al., 1997; Kamstra et al., 1999a; 2004), *Cyclamen* (Ishizaka & Uematsu, 1995), *Dendranthema* (Endo et al., 1997), *Gladiolus* (Ohri & Khoshoo, 1983a), *Impatiens* (Arisumi, 1973; 1974; Stephens, 1998), *Iris* (Yabuya, 1984; Eikelboom & Van Eijk, 1990), *Lupinus* (Gupta, 1996),

Narcissus (Brandham, 1986) *Nerine* × *Amaryllis* (Van Tuyl et al., 1992; Meijer et al., 1998), *Primula* (Kato et al., 2001), *Sandersonia* × *Littonia* (Morgan, 2001), *Tulipa* (Van Eijk et al., 1991) and *Zantedeschia* (Yao et al., 1995, Snijder, 2004). Often, these interspecific hybrids tend to be sterile. Interspecific hybrids within the genus *Lilium* are known for their sterility (Asano, 1982a; Van Tuyl et al., 2000; 2002b; Lim & Van Tuyl, 2002). In most cases, hybrid sterility is related to low chromosome pairing and irregular chromosome segregation during meiosis (Asano, 1982a; Ohri & Khoshoo, 1983b; Yabuya, 1991; Ishizaka, 1994), besides many other abnormalities.

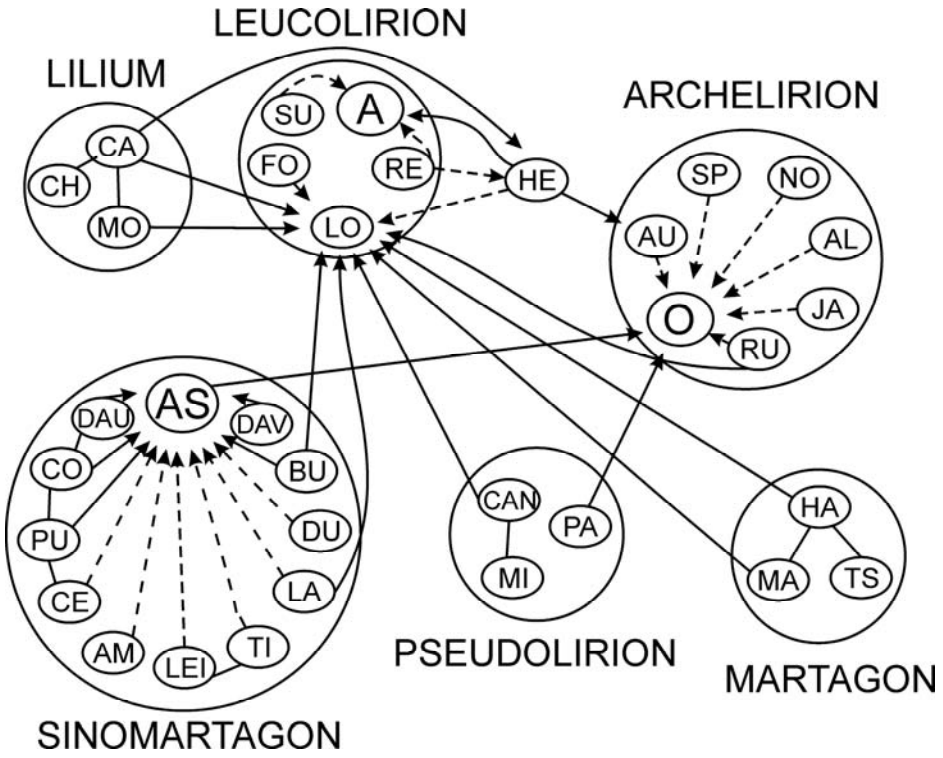

Figure 1.1. Crossing polygon of the genus *Lilium* including all the successful crosses of species between different sections of the genus *Lilium* developed at Plant Research International, Wageningen University and Research Centre, the Netherlands. In this figure, the connection between the Asiatic, Aurelian, and Oriental hybrid groups (large ellipses) are shown by dotted lines. In successful crosses between species (small circles) of different sections (large circles) the arrows point towards the female parent. Abbreviations: A: Aurelian hybrids; AL: *L. alexandrae*; AM: *L. amabile*; AS: Asiatic hybrids; AU: *L. auratum*; BU: *L. bulbiferum;* CA: *L. candidum*; CAN: *L. canadense*; CE: *L. cernuum*; CH: *L. chalcedonicum*; CO: *L. concolor*; DAU: *L. dauricum;* DAV: *L. davidii*; DU: *L. duchartrei*; FO: *L. formosanum*; HA: *L. hansonii*; HE: *L. henryi*; JA: *L. japonicum*; LA: *L. lankongense*; LEI: *L. leichtlinii*; LO: *L. longiflorum*; MA: *L. martagon*; MI: *L. michiganense*; MO: *L. monadelphum*; NO: *L. nobilissimum*; O: Oriental hybrids; PA: *L. pardalinum*; PU: *L. pumilum*; RE: *L. regale*; RU: *L. rubellum*; SP: *L. speciosum*; SU: *L. sulphureum*; TI: *L. tigrinum*; TS: *L. tsingtauense*.

The traditional method to restore fertility in interspecific hybrids is doubling the chromosome number by the use of spindle inhibitors such as colchicine (Arisumi, 1973; Asano, 1982b;

Yabuya, 1985; Eikelboom & Van Eijk, 1990; Ishizaka, 1994; Endo et al., 1997) and oryzalin (Van Tuyl et al., 1992; 2000; Lim et al., 2000b; 2003b; Takamura et al., 2002). Even though fertility is restored; the major drawback of this technique is that it promotes autosyndetic chromosome pairing, which hampers the recombination between the parental genomes (Lim et al., 2000b; Wendel, 2000; Van Tuyl et al., 2002b; Ramanna & Jacobsen, 2003). As a consequence, those allotetraploids, whose chromosomes from the parental genomes do not recombine, are properly called "permanent hybrids" and they offer little possibilities to create genetic variability.

2n gametes

Gametes with somatic chromosome numbers are known as "*2n*" or "unreduced" gametes. They occur in most of the angiosperm species and many authors attribute to unreduced gametes the origin of polyploid plant species (reviews by Harlan & De Wet, 1975; Veilleux, 1985; Ramanna & Jacobsen, 2003). Prior to the discovery of colchicine to double the chromosome number and restore fertility, *2n* gametes were used to produce polyploids. However, it was assumed that the production of such gametes was highly sporadic. As a consequence, their use was rapidly discarded by breeders and artificial polyploids (induced by chemicals) were preferred (Ramanna & Jacobsen, 2003). The artificially induced allopolyploids are found to have fixed heterozygosity (Soltis & Soltis, 2000) and differ in this respect to the naturally occurring sexual polyploids (originated through functioning *2n* gametes) (Bretagnolle & Thompson, 1995). In sexual polyploids heterozygosity is not fixed because recombination between the alien parental genomes is present and therefore they are more promising for breeding. Another advantage of *2n* gametes is that due to recombination introgression can be achieved (Karlov et al., 1999, Lim et al., 2001; Ramanna et al., 2003). Unreduced gametes have shown to be useful in breeding, some examples include: *Alstroemeria* (Kamstra et al., 1999a; Ramanna et al., 2003), *Lilium* (Lim & Van Tuyl, 2002; Van Tuyl et al., 2002a; Van Tuyl & Lim, 2003), *Medicago* (Bingham, 1980; Veronesi et al., 1986), *Primula* (Skiebe, 1958) and *Solanum* (Mendiburu & Peloquin, 1971; Mendiburu et al., 1974).

2n gamete induction

Although most of the Angiosperms produce *2n* gametes in variable frequencies, it still requires an effort to detect plants producing them. Various approaches have been used to detect *2n* gametes. These are: pollen size examination (Den Nijs & Peloquin, 1977a,b; Ramanna, 1983; Veronesi et al., 1988; Van Tuyl et al., 1989), flowcytometry (Van Tuyl et al., 1989; Maceira et al., 1992) and progeny analysis (Bingham & McCoy, 1979; Iwanaga et al.,

1991; Veronesi et al., 1986; Werner & Peloquin, 1991). Detection of *2n* gamete producer plants does not assure that such plants can be readily used for crossing, because *2n* gamete production seems to be controlled genetically (Mok & Peloquin, 1975; Peloquin, 1982), but this genetic trait is elusive because these genes might be greatly influenced by the environment (Ramana & Jacobsen, 2003).

There are reports of different attempts to increase and even induce the production of *2n* gametes. These include: genetic selection (Jacobsen, 1976; Barcaccia et al., 2003), high solar level (Ortiz & Vuylsteke 1995; Negri & Lemmi, 1998), low temperature (Lutkov, 1937; Stein, 1970), heat (Lewis, 1943; Lokker, 2004) and caffeine treatments of immature flower buds (Levan, 1939; Rasmusson & Levan, 1939; Olden, 1954). Nevertheless, none of the previous attempts has shown to be completely efficient.

Origins of *2n* gametes and their genetic consequences

A vast amount of cytological as well as genetic research has been conducted in order to elucidate the different mechanisms of *2n* gametes formation. This showed that not a single mechanism is responsible for the formation of *2n* gametes in all cases. Research in potato is a good example showing evidence for several mechanisms (Mok & Peloquin, 1975; Den Nijs & Peloquin, 1977b; Ramanna, 1979; Veilleux, 1985). In the case of monocots, however, the mechanisms are not as well documented as in dicots. Nonetheless, research in the genus *Alstroemeria* (Kamstra, 1999a; Ramanna et al., 2003), *Lilium* (Lim et al., 2001, Ramanna & Jacobsen, 2003), *Triticum* (Xu & Joppa, 1995; 2000) and *Zea* (Roades & Dempsey, 1966) provided detailed descriptions of some of the cytological mechanisms responsible for the *2n* gamete formation. Although there are some minor differences between meiosis in monocots and dicots, the modes of *2n* gamete formation have some striking features in common. They share certain general meiotic events such as chromosome pairing, chiasma formation and cytokinesis. However, in most of the monocot plants, microsporogenesis is of the so-called "successive" type, where cytokinesis and the formation of a cell wall takes place after the first meiotic division (telophase I), following which, the second meiotic division takes place in different cells within a pollen mother cell. In the case of dicot plants, a majority possess the so-called "simultaneous" type of meiosis, where cytokinesis and cell wall formation takes place at the same time, after the second meiotic division (telophase II). Thus, when cytokinesis, chromosome disjunction and spindle abnormalities are involved in the formation of *2n* gametes, it is evident that different mechanisms are responsible for their formation (Ramanna & Jacobsen, 2003). Although there are several ways of nuclei restitution in the meiocytes, there are two broad mechanisms identified in plants. These are the first division restitution (FDR) and second division restitution (SDR). In the first case, the entire

chromosome complement divides equationally before telophase I and cytokinesis occurs, leading to the formation of a dyad, without further division (Figure 1.2). In the case of SDR, a normal first meiotic division occurs, i.e., the chromosomes divide reductionally and cytokinesis occurs,

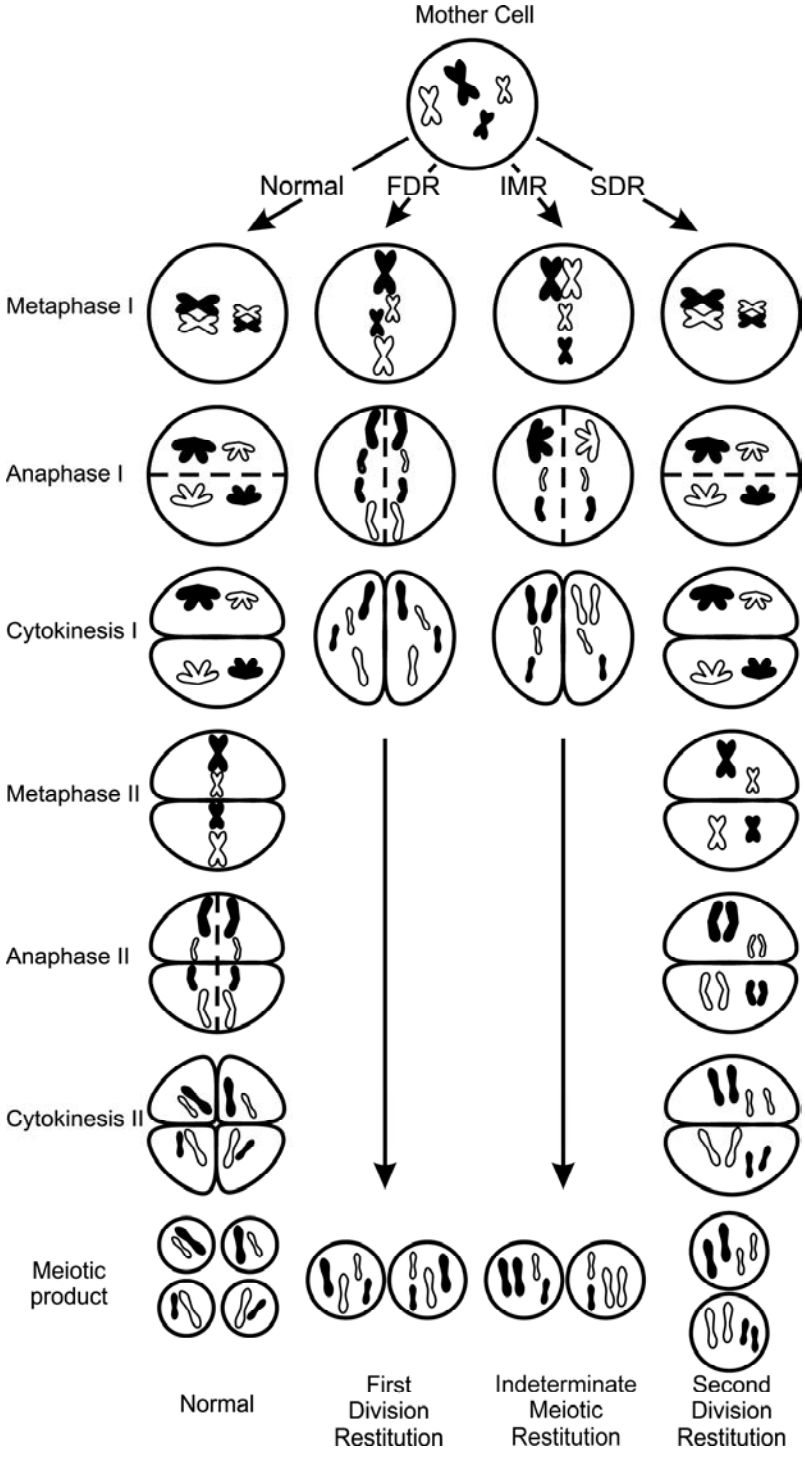

Figure 1.2. Schematic representation of the meiotic process and three restitution mechanisms in microsporogenesis in species having the monocotyledonous successive type of meiotic division (Lily type).

producing a dyad. Instead of the second division, the chromatids divide but the nuclei restitute in each of the two cells of a dyad (Figure 1.2). A third mechanism has been identified recently

in Longiflorum × Asiatic lily hybrids (Lim et al., 2001). In this case, during the first meiotic division some of the univalents divide equationally (as in FDR) and some bivalents disjoin reductionally (as in SDR) before telophase I, (Figure 1.2). This mechanism has been recognized as indeterminate meiotic restitution (IMR), because it cannot be characterized either as FDR or as SDR because it combines characteristics from both mechanisms. These mechanisms are illustrated in Figure 1.2. For the sake of simplicity, just the monocotyledonous successive type of meiosis known in lily is illustrated.

As can be inferred, the genetic consequences of the three mechanisms are different due to the dissimilar segregation of chromosomes in the three cases. In each mechanism, the presence or absence of recombination between the parental genomes must be considered to estimate the genetic consequences of each mechanism. FDR gametes will be identical to each other as well as the mother cell, when recombination is absent (Figure 1.2). With recombination, heterozygosity can be maintained for the part of the chromosome proximal to the crossover (in each recombinant chromosome). Gametes originated through SDR might be highly heterogeneous if the chromosome assortment is random and the gamete is constituted by chromosomes from both parents (Figure 1.2). If recombination is present, heterozygosity is maintained for the part of the chromosome distal to the crossover (in the recombinant chromosomes). The "intermediate" status of IMR gametes can offer the highest degree of genetic variation, due to the random assortment of some chromosomes as in SDR and equational segregation for the others (Figure 1.2). Both, distal and proximal heterozygosity can be maintained if recombination is present.

Introgression breeding and ploidy manipulation

Intergenomic recombination is essential for introgression. One of the most powerful and effective techniques to detect chromosomal recombination is genomic *in situ* hybridization (GISH). It allows the localization of DNA sequences determined by hybridization of a labeled DNA probe to the DNA of target chromosomes (Schwarzacher & 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. Some examples where this technique has been applied successfully include: potato (Jacobsen et al., 1995), *Allium cepa* × *A. fistulosum* (Khrustaleva & Kik, 2000), *Alstroemeria aurea* × *A. inodora* (Kamstra et al., 1999a), *A. pelegrina* × *A. inodora* (Ramanna et al., 2003), *Gasteria* × *Aloe* (Takahashi et al., 1997), *Musa* (Osuji et al., 1997; D'Hont et al., 2000) *Brassica* (Nagpal et al., 1996; Snowdon et al., 1997) among others. In lily this technique has been employed to study the recombinant chromosomes and the mechanisms of *2n* pollen formation in interspecific hybrids of *Lilium longiflorum* × Asiatic (LA-hybrids) (Karlov et al., 1999; Lim et al., 2001), *Lilium rubellum* ×

L. longiflorum (Lim et al., 2000b), the indeterminate meiotic restitution mechanism (Lim et al., 2001) and several analyses of BC_1 and BC_2 progenies (Lim et al., 2003a; 2004).

Selection of the parents in a crossing program allows the prediction of the ploidy levels of the offspring. In crosses involving *2n* gametes and diploid parents the progeny is expected to be triploid; in crosses involving *2n* gametes and tetraploid or other *2n* gamete producing parent, tetraploid progeny is expected. Analyses in the progeny of LA hybrids, obtained by crosses with diploid Asiatic (A) parents (by using functional *2n* gametes from the LA hybrid), showed that most of the progeny obtained was triploid as expected and considerable amounts of intergenomic recombination can occur (Lim et al., 2003a; Van Tuyl et al., 2003). A general belief is that triploid hybrids can not be used in breeding because of their low fertility. However, it has been demonstrated that triploid hybrids can produce aneuploid and euploid (*x*, 2*x* and 3*x*) gametes and have been used to produce progeny (reviews by Kuspira et al., 1986; Brandham, 1982; Ramsey & Schemske, 2002; Ramanna & Jacobsen, 2003). Furthermore, triploid hybrids might have contributed to the origin of a majority of new polyploids in nature (Husband, 2004). In the case of *Lilium*, triploid ALA (A × LA) hybrids derived from *2n* gametes, have been successfully used and the recombinant chromosomes have been transmitted to the progeny. Crosses of these triploids with diploids and tetraploids produced aneuploid near diploid and near pentaploid progenies, respectively (Lim et al., 2003a). Anueploid hybrids offer advantages over diploid hybrids; the case of *Hyacinthus* is a good example where aneuploid hybrids have been selected due to their superior ornamental traits when compared to diploid cultivars (Brandham & West, 1993).

Furthermore, the advantages of polyploids for breeding must be considered. It is well known that polyploids differ from their diploid progenitors in morphological, ecological, physiological and cytological characteristics (Lumaret, 1988; Soltis & 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 (review Ramsey & Schemske, 2002). Thus, the creation of polyploid interspecific hybrids with the use of *2n* gametes might be rewarded with a higher degree of genetic variation.

Scope of the thesis

The aim of this research was to evaluate the importance of *2n* gamete formation in F_1 hybrids from crosses of Oriental × Asiatic (OA) lilies and their relevance in relation to genetic variation and introgression. To accomplish this, the cytological modes of origin of *2n* gametes were investigated in **Chapter 2**. Through analysis of microsporogenic stages, it is shown that

unusual cytological events led to different types of restitution mechanisms; genomic *in situ* hybridization (GISH) analysis revealed the presence of intergenomic recombination between the parental genomes in the F_1 hybrids. The genetic implication of the formation of *2n* gametes for introgression is discussed. Additionally, pollen viability after anthesis was tested. In **Chapter 3**, a description is given of the way in which more than 700 F_1 OA hybrids were obtained. Pollen germination, the ability to produce embryos and in some cases bivalent formation in pollen mother cells were used to asses the fertility in a selected group of OA hybrids. Analyses in a BC_1 population, outcome of crosses of the selected F_1 OA hybrids with different diploid Asiatic and Oriental cultivars, as well as with tetraploid 4x-OA hybrids (obtained after chromosome doubling with oryzalin), shows the impact of *2n* gametes on the success of backcrossing. Furthermore, GISH analysis reveals the presence of chromosome recombination in the BC_1 hybrids. **Chapter 4** details the analysis of BC_1 plants and the identification of single and recombinant chromosomes by fluorescent *in situ* hybridization (FISH) and GISH techniques. These enabled the discovery of IMR restitution mechanisms in OA hybrids. The segregation of individual recombinant chromosomes is described and their possible genetic consequences are discussed. **Chapter 5** includes analyses in further crosses of the BC_1 allotriploids in different directions to assess the ploidy levels of the progeny. Estimated flowcytometric and observed chromosome numbers are compared and the possible chromosome contribution of the parental gametes is discussed. GISH and FISH analysis shows the transmission of recombinant chromosomes from the allotriploids into the next generations. **Chapter 6** reports the use of N_2O (laughing gas) treatments to successfully induce formation of viable *2n* gametes in sterile OA hybrids. Crosses with OA hybrids treated with N_2O resulted in a large number of progeny. In known *2n* gamete producers the production of *2n* pollen was increased considerably and in a single cross 28 embryos were produced. GISH analyses in the progeny demonstrate the occurrence of different restitution mechanisms due to the chromosome constitutions and the presence of recombinant chromosomes. Finally, in **Chapter 7** the importance of *2n* gametes, the transmission of recombinant chromosomes and its impact on genetic variation and the achievement of introgression are discussed.

Chapter 2

Occurrence of *2n* gametes in the F_1 hybrids of Oriental × Asiatic lilies (*Lilium*): relevance to intergenomic recombination and backcrossing

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Abstract

Cytological modes of the origin of *2n* gametes were investigated in six different genotypes of F_1 hybrids between Oriental and Asiatic (OA) lilies (*Lilium*, 2n=2x=24). Chromosome pairing between the parental genomes was very low, the average frequency range from 0.3 to 1.2 bivalents per cell among the genotypes. Within a genotype the frequency of bivalents varied from 0 to 6 in some cases. The normally occurring haploid pollen grains were totally sterile. In contrast, in different genotypes, variable percentages of *2n* pollen were found and shown to be fertile as estimated from pollen germination. A cytological analysis of metaphase I and subsequent stages of meiosis using genomic *in situ* hybridization (GISH) revealed that there was intergenomic recombination between the alien genomes. Following metaphase I stage, three different types of abnormal cytological events led to the formation of *2n* pollen: (i) Post Metaphase I division (PMI), (ii) Post Metaphase II division (PMII) and (iii) Asymmetric Cytokinesis of the pollen mother cell followed by chromosome division. All three cytological events led to First Division Restitution (FDR) gametes. Based on *in vitro* pollen germination it was proved for two genotypes that *2n* pollen was viable only during the first day of anthesis. It was possible to use *2n* pollen successfully for backcrossing. Implications of *2n* pollen for intergenomic recombination in BC_1 progenies are discussed.

Introduction

Gametes with somatic chromosome numbers, also known as *2n* gametes, occur in almost all plant species and they might have given rise to polyploid plants in nature (Harlan & de Wet, 1975). Despite this recognition, there have been relatively little efforts made to use *2n* gametes in crop breeding. Some progress has, however, been made in the case of autopolyploid crops such as potato, alfalfa and *Dactylis* among others (reviews, Veilleux, 1985; Mariani & Tavolleti, 1992; Bretagnolle & Thompson, 1995; Ramanna & Jacobsen, 2003). In breeding autopolyploids, *2n* gametes have been used for increasing plant vigor, yield, disease resistance and other agronomic characters.

In the case of allopolyploids, however, *2n* gametes have been used in recent years in two crops for inducing sexual polyploids. These are *Alstroemeria* (Ramanna, 1992; Buitendijk et al., 1997; Kamstra et al., 1999a, 2004; Ramanna et al., 2003) and *Lilium* (Karlov et al., 1999; Lim et al., 2001, 2003a; Van Tuyl et al., 2002a). In both crops, *2n* gametes were useful for overcoming sterility in the F_1 interspecific hybrids, for inducing intergenomic recombination as well as introgression of alien chromosome segments. In the case of lilies, extensive studies have been made on the hybrids between Longiflorum × Asiatic groups of hybrids (LA hybrids) and their backcross derivatives (Lim et al., 2001, 2003a; Van Tuyl et al., 2000, 2003).

Apart from LA hybrids, we have made a series of hybrids between Oriental × Asiatic groups of lilies (OA hybrids). These are potentially "useful" for combining desirable horticultural traits from the two parents through sexual polyploidization. For this purpose, we have selected a few genotypes of OA hybrids that produce considerable frequencies of *2n* pollen. In order to determine the modes of origin of *2n* pollen grains in OA hybrids, microsporogenesis was analyzed using genomic *in situ* hybridization (GISH) as well as traditional staining methods. The main objectives were to assess the extent of intergenomic recombination and to test the viability of *2n* pollen. The results are discussed in relation to the progenies that might be obtained in the BC_1 generation.

Material and Methods

Plant material

The two groups of cultivars, viz., Oriental and Asiatic hybrids, were all diploids (2n = 2x = 24) and were hybridized through cut-style pollination and cultured by either: embryo, embryo-sac or ovule culture methods (Van Tuyl et al., 1991; Van Creij et al., 2000). In all, 10 Oriental and six Asiatic hybrids were used for the production of F_1 OA hybrids, among which the *2n* gamete producing genotypes were selected (Table 2.1) (see later in Chapter 3). Because all the cultivars of lilies are intra sectional hybrids between different taxonomic species, it is not appropriate to mention the botanical names of the species and therefore avoided.

Table 2.1. Selection of OA hybrids

Genotype	Parents		Occurrence of *2n* Pollen
	Oriental	Asiatic	
951462-1	'Romero Star'	'Connecticut King'	+
951502-1	'Pesaro'	'Connecticut King'	+
951584-1	'Acapulco'	'Sancerre'	+
952400-1	'Mero Star'	'Gran Sasso'	+
969023-2	'Casa Blanca'	'Connecticut King'	-
952059-9	'Touch'	'Connecticut King'	-

Pollen germination

Pollen was collected at different stages of anthesis (on the day of opening and a day after), and cultured for 24 h at 25°C in artificial agar medium containing 100 g sucrose, 5 g bacteriological agar, 20 mg boric acid and 200 mg calcium nitrate per liter. The pollen was classified as large (2*n*) and small (*n*) depending on size and the large germinated pollen was counted to determine the germination range.

Cytological methods

Young anthers were collected and fixed in an ethanol acetic acid solution (3:1) for at least 12 h and stored at –20°C until use. The pollen mother cells (PMCs) were dissected from the anther and squashed in a drop of 1% aceto-orceine. For the genomic *in situ* hybridization, the PMCs were dissected from the anther in a drop of enzyme mixture containing 0.2% (w/v) pectolyase Y23, 0.2% (w/v) cellulase RS and 0.2% (w/v) cytohelicase in 10 m*M* citrate buffer (pH 4.5). The slides were incubated at 37°C for 10 minutes, squashed and frozen in liquid nitrogen. The cover slips were removed with a razor blade and subsequently the slides were dehydrated in absolute ethanol and air-dried.

For the determination of the different meiotic stages, we counted only the PMCs from anthers whose PMCs contained stages from metaphase I to anaphase II, where the orientation of chromosomes and/or chromatids were evident, and from telophase II to sporad stages and ignored those ones with earlier stages. In this way the different meiotic and sporad stages from normal meiosis and those resulting from restitution mechanisms could not be confused.

In situ *hybridization*

Sonicated genomic DNA (1–10 kb) from the Oriental cultivar 'Sorbonne' was used as a probe after labeling with Biotin-16-dUTP (Biotin-16–2'-deoxyuridine-5'-triphosphate) by nick translation according to the manufacturer's instructions (Roche, Germany). Autoclaved DNA (100–500 bp) from the Asiatic cultivar 'Connecticut King' was utilized for blocking the non-hybridized sequences.

The *in situ* hybridization protocol was carried out according to Lim et al. (2000b) with minor modifications. In brief, slides were pre-treated with RNase A (100 μg/ml) for 1 h and pepsin (5 μg/ml) for 10 min, both at 37°C, followed by paraformaldehyde (4%) for 10 min at room temperature, dehydrated with 70%, 90% and absolute ethanol for 3 min in each and air dried. Hybridization followed using a mixture consisting of 20x SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS, 25-50 ng/ml probe DNA and 5-10 µg/ml blocking DNA. The DNA was denatured by heating the hybridization mixture at 70°C for 10 min and then placed on ice for at least 10 min. For each slide, 40 μl hybridization mixture was used. The preparations were denatured at 80°C for 10 min. After overnight hybridization at 37°C in a humid chamber, slides were washed at room temperature in 2x SSC for 15 min and 0.1x SSC at 42°C for 30 min. Biotin-labelled DNA was detected with Cy3 labelled streptavidin (Amersham Biosciences, UK), and amplified with biotinylated goat-antistreptavidin (Vector laboratories, Burlingame, CA). Chromosomes were counterstained with 1 µg/ml DAPI (4,6-diamidino-2-phenylindole) and examined under a Zeiss Axiophot microscope equipped with a

triple filter. Images were photographed on 400 ISO colour negative film and scanned at 1200 dpi for digital processing in Photoshop (Adobe Inc.).

Results

Chromosome pairing

Meiosis was investigated in three F_1 hybrid genotypes that produced 5% or more pollen that would germinate. The main objectives were the assessment of chromosome pairing and the cytological mechanism(s) of nuclear restitution. Without exception there was reduced chromosome pairing at metaphase I in all cases (Figure 2.1a). Very low frequencies of bivalents, from 0.3 to 1.3 bivalents per cell, were observed between genotypes (Table 2.2). Within a plant, bivalent averaged from 0 to 6 per cell. As a result of high frequencies of univalent formation, meiotic division was chaotic as expected in a majority of PMCs. But in 5 to 45% of the cases, the stages following metaphase I the chromosomes did not separate into two groups (as in anaphase I). A notable feature was that the sister chromatids of each chromosome became clearly visible and it was the most ideal stage for detection of intergenomic recombination. For the sake of convenience, this stage will be mentioned hereafter as Post Metaphase I or PMI. In one of the PMCs four different recombination events were clearly visible (Figure 2.1b), they are single recombination events. However, there was one case where it could be explained as a four strand double crossover event (Figure 2.1b arrows).

Table 2.2. Chromosome pairing in three genotypes of OA hybrids.

Genotype	# of cells analysed	6_{II}, 12_I	5_{II}, 14_I	4_{II}, 16_I	3_{II}, 18_I	2_{II}, 20_I	1_{II}, 22_I	0_{II}, 24_I	Mean Frequency
951502-1	296	1	3	13	26	63	99	91	$1.3_{II} + 21.4_I$
952400-1	231	0	0	2	11	45	67	106	$0.8_{II} + 22.8_I$
962120-1	292	0	0	0	3	18	55	216	$0.3_{II} + 23.3_I$

II and I represent bivalent and univalent respectively

Nuclear restitution

We analyzed six F_1 hybrid genotypes in which meiosis was fairly asynchronous in each anther and as a result of that, it was possible to find meiotic stages ranging from metaphase I to anaphase II and from telophase I to sporad stages in one and the same preparation (Table 2.3). In some of the PMCs the first division (anaphase I) was generally followed by cytokinesis and a cell wall was formed at telophase I (Figure 2.2d). This was according to expectation in *Lilium*, which has the so-called successive type of cytokinesis. However, in a considerable

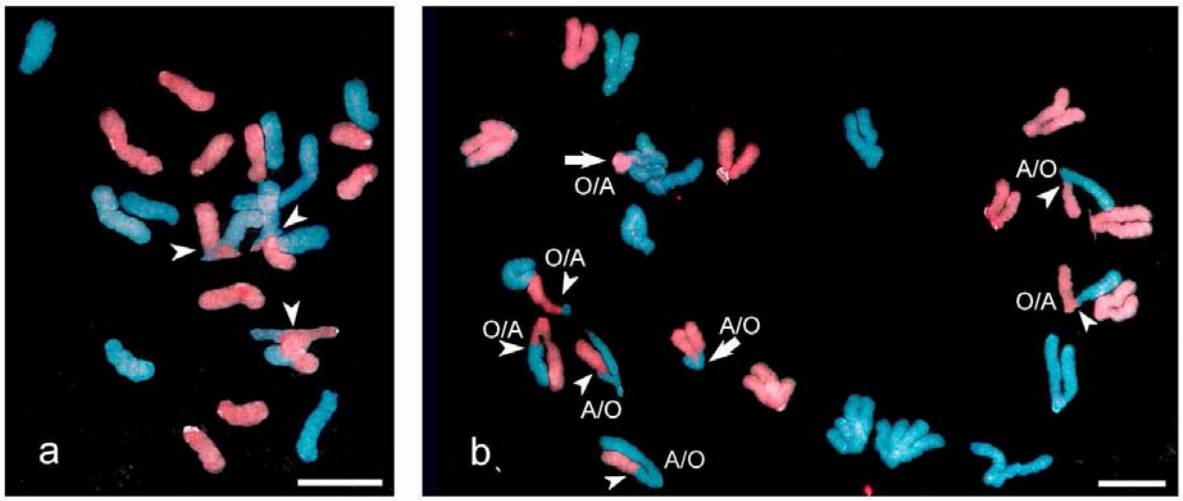

Figure 2.1. Chromosome pairing at metaphase I and post metaphase I stage in OA hybrid, 951502-1. The Oriental chromosomes were biotin-labeled and detected with Cy3-streptavidin system (pink fluorescence) and the Asiatic chromosomes were counterstained with DAPI (blue fluorescence). (**a**) Metaphase I showing 3 bivalents (arrowheads) and 18 univalents. (**b**) Post metaphase I stage in which sister chromatids of each chromosome are clearly visible, as are the recombinant chromatids in three pairs (arrowheads) and the four strand double strand crossover event (arrows). *Bar represents 10 μm.*

Table 2.3. Frequencies of deviating meiotic stages (PMI and PMII) in six different genotypes of OA hybrids and the frequencies (%) of sporads

Genotype	# of cells analysed	Anaphase separation				Sporads			
		PMI		PMII		Dyads		Others	
951502-1	293	37	(12.63)	8	(2.73)	117	(39.93)	131	(44.71)
952400-1	372	1	(0.27)	10	(2.69)	26	(6.99)	335	(90.05)
951462-1	317	6	(1.89)	21	(6.62)	26	(8.20)	264	(83.28)
951584-1	382	5	(1.31)	1	(0.26)	24	(6.28)	352	(92.15)
969023-2	553	10	(1.81)	20	(3.62)	88	(15.91)	435	(78.66)
952059-9	425	2	(0.47)	24	(5.65)	141	(33.18)	258	(60.71)

number of PMCs, the expected cytokinesis and cell wall formation were absent. In such PMCs, either the entire chromosome complement was aligned at the equatorial position (Figure 2.2e) and divided equationally or formed restitution nucleus (Figure 2.2a). The restitution nuclei gave rise to metaphase stage, (equivalent to metaphase II) and divided equationally (Figures. 2.2b and c). This type of division will be indicated as post metaphase II, or PMII division. These two different restitution mechanisms (PMI and PMII) occurred in different frequencies among the F_1 hybrids (Table 2.3) and obviously led to the formation of a dyad. In addition to PMI and PMII, there were also PMCs in which asymmetrical cytokinesis occurred so that the entire nucleus was included in a single cell (Figures 2.2f and g).

Regardless of the occurrence of PMI, PMII or the division of a nucleus in an asymmetrically divided PMC, they all led to an equational division of the whole complements. They obviously conformed to first division restitution (FDR) mechanism.

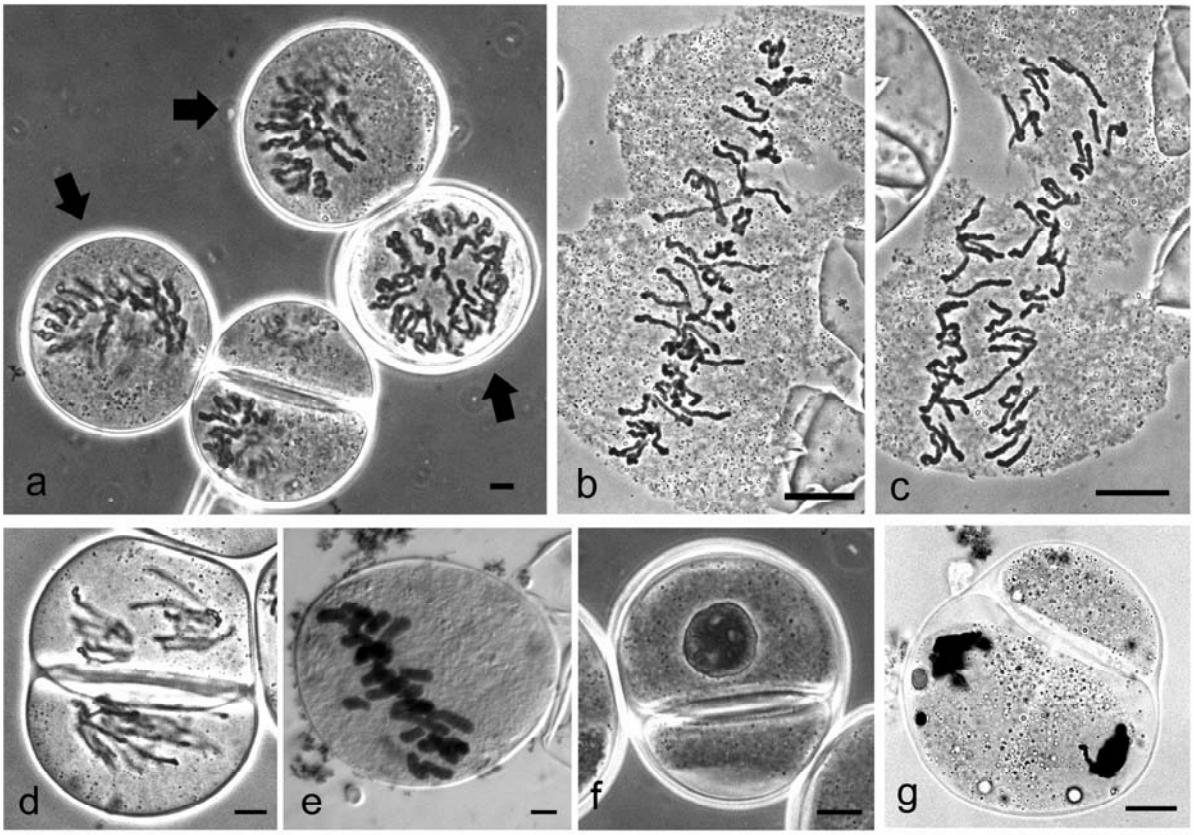

Figure 2.2. Stages of microsporogenesis in OA hybrid 951502-1, showing meiotic nuclear restitution. (**a**) Restitution nucleus formation in PMCs without cytokinesis (arrows). (**b**) Equivalent of metaphase II stage showing the orientation of all chromosomes in a single group. (**c**) Anaphase separation of chromatids in a stage subsequent to that shown in (b). (**d**) Cytokinesis and cell wall formation at the end of first meiotic division, so-called "successive type" of meiosis in lilies. (**e**) Post metaphase I orientation that can lead to the equational division of chromosomes (FDR). (**f**) Asymmetric cytokinesis in PMC in which the entire nucleus is included in one of the cells. (**g**) Nuclear division following asymmetric cytokinesis. *Bar represents 10 µm.*

Viability of 2n *pollen*

Two criteria were used for the assessment of the viability of *2n* pollen. a) *In vitro* germination of pollen and b) fruit set and embryo germination after using *2n* pollen in crossing. For *in vitro* germination, two diploid cultivars, one Oriental and one Asiatic, as well as two genotypes of OA hybrids were used (Table 2.4). Pollen from the two diploid cultivars (controls) showed 95 – 100% germination when fresh pollen from just opened flowers or flowers one day after opening were used. Also *2n* pollen from both genotypes of OA showed fairly good germination when fresh pollen was used (0 – 40% in 952400-1). But *2n* pollen almost completely failed to germinate from one day old flowers (with the exception of a few

flowers of 951502-1 which presented less than 5% pollen germination). This loss of viability of *2n* pollen in an OA hybrid was strikingly constant. Besides germination *in vitro*, fresh pollen grains could be successfully used in order to produce backcross progenies (results not included). A fairly good number of BC_1 plants were obtained when diploid Oriental and Asiatic hybrids were used as female parents in crosses with OA males. In two combinations, by using OA as female parent and a diploid Asiatic hybrid as male, BC_1 progeny was obtained. This was an indication for the occurrence of *2n* eggs in OA hybrids.

Table 2.4. Time effect on the pollen germination of diploid Asiatic (AA) and Oriental (OO) cultivars and diploid OA hybrids *2n* gametes producers

Genotype (Genome)	Flower #	Pollen germination (Range %)	
		Fresh	1 day old
Pollyanna (AA)	12	95-100	95-100
Sorbonne (OO)	15	95-100	95-100
951502-1 (OA)	17	85-100	0-<5
952400-1 (OA)	20	0-40	0

Consequences of nuclear restitution

An important feature of meiotic nuclear restitution in OA hybrids with a high frequency of univalents (Table 2.2) is that it leads almost exclusively to FDR. In the absence of any recombination, FDR leads to *2n* gametes with identical genotypes (i.e., the same as parental sporophyte). The occurrence of chiasma formation and crossing-over in the OA hybrid (Figures 2.1a and b) gives rise to *2n* gametes with different genotypes. An example of the number of genotypes that can result from a random segregation of chromatids after a crossover between a pair of homoeologous chromosomes is illustrated in Figure 2.3. Thus, four different genotypes of *2n* gametes were expected to occur with: (a) no recombinant chromosomes, (b) two recombinant (O/A + A/O) (reciprocal products), (c) only O/A and (d) only A/O. From an analysis of BC_1 progenies of OA hybrids all the expected types of *2n* gametes have been found to be functional (see later in Chapter 4).

Discussion

In the past, we have critically investigated the occurrences of *2n* gametes in LA hybrids and used them for producing backcross progenies (Karlov et al., 1999; Lim et al., 2001, 2003a; Van Tuyl et al., 2000, 2002a). Besides these basic investigations, lily breeders have

extensively used *2n* gametes in breeding numerous cultivars (data not shown). In view of this successful story of LA hybrids, the OA hybrids of the present investigation are of practical interest as well as scientific importance. It is of scientific importance because, when using distantly related species in crop improvement, the traditional approach was to produce an allopolyploid from a F_1 hybrid through somatic chromosome doubling. Such allopolyploids were appropriately called "permanent hybrids" because the parental characters almost never segregated in their progenies. In the absence of any genetic variation in the progenies of allopolyploids they were not useful for the selection of cultivars. Therefore the interest of breeders for using somatically doubled allopolyploids diminished, if not completely vanished. However, as is evident from the present investigation, considerable genetic variation can be generated if *2n* gametes from F_1 hybrids can be used for backcrossing.

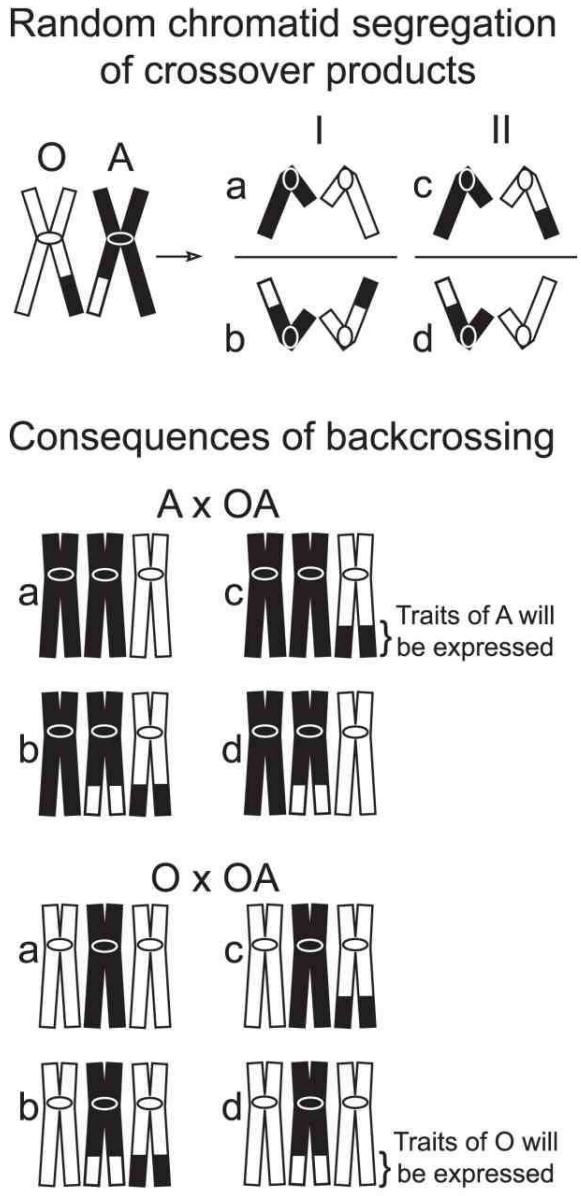

Figure 2.3. Random chromatid segregation scheme and its implication for the expression of either Asiatic (A) or Oriental (O) traits in subsequent backcrosses. Note: homozygosity can occur for the distal recombinant segment in the BC_1 progeny.

Most importantly, parental traits may be expressed in the BC_1 progenies depending on the recurrent parent used. Thus, considering the use of *2n* pollen where only the O/A recombinant chromatid of a single chromosome is included and if an Asiatic cultivar is used as recurrent parent, the Asiatic traits for those homoeologous chromosomes will be expressed. The opposite case when Oriental traits may be expressed will be when the *2n* pollen includes only the A/O recombinant chromatid and an Oriental cultivar is used as a recurrent parent (Figure 2.3).

There are three types of cytological events in OA hybrids that lead to *2n* pollen formation, viz., PMI, PMII divisions and asymmetric cytokinesis followed by nuclear division. In the six genotypes that were studied, all the three types appear to occur in variable frequencies (Table 3). In certain other monocotyledonous taxa such as wheat × *Aegilops squarrosa* (Fukuda & Sakamoto, 1992); *Triticum turgidum* × *Secale cereale, T. turgidum* × *Ae. squarrosa* (Xu & Joppa., 1995); *Alstroemeria* interspecific hybrids (Ramanna, 1992; Ramanna et al., 2003) and *S. cereale* × *Ae. squarrosa* (Xu & Joppa, 2000) restitution mechanisms similar to PMI have been described. Despite all these investigations there appears to be no clear-cut cytological mechanism solely responsible for FDR gamete formation. Nevertheless, there have been claims that the trait of *2n* gamete formation is genetically controlled and can be localized to certain chromosomes in wheat and oat (Xu & Joppa, 2000; Kynast et al., 2001). It would be useful to investigate whether genetically controlled restitution mechanisms are present in lily hybrids.

A clear difference was observed with regard to the viability of *2n* pollen grains based on *in vitro* germination (Table 2.4). It is not clear whether the difference is physiological. Nevertheless, from the point of view of making crosses, it is important to note that the use of fresh *2n* pollen grains can ensure success. This is illustrated from the successful crosses that were obtained in both O × OA as well as A × OA combinations (results not included). Viability of *2n* pollen is one of the considerations that one has to take into account while making crosses. Besides, factors such as the occurrence of very low frequencies of *2n* gametes and environmental influence can also be limitations in some cases. Nevertheless, from the success we have achieved in producing a large number of backcrosses through sexual polyploidization proves this method is quite practical in breeding lilies.

Chapter 3

Use of *2n* gametes for the production of sexual polyploids from sterile Oriental × Asiatic hybrids of lilies (*Lilium*)

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Abstract

Sixteen Oriental and 12 Asiatic cultivars were crossed in 158 different combinations. 708 F_1 hybrids were obtained from only 86 different combinations of 15 Oriental and 11 Asiatic cultivars. Because the *Lilium* cultivars (2n=2x=24) used for the production of these OA-hybrids belong to two different taxonomic sections, viz., Archelirion (O) and Sinomartagon (A) respectively, the F_1 hybrids (OA) could be obtained only through embryo, embryo sac rescue, ovary slice or ovule culture. Most of the F_1 hybrids were highly sterile (did not produce viable *n* gametes) due to the failure of chromosome pairing. However, in a few cases F_1 plants were found that produced viable *2n* pollen in variable frequencies. These *2n* pollen grains were successfully used for the production of backcross progenies. Using GISH, it was found that intergenomic recombinant chromosomes were present in the sexual polyploid progenies. The results of the present study indicate the prospects for combining important horticultural traits from the two main groups of cultivars of lilies through sexual polyploidization more effectively.

Introduction

Lilium L. is a genus of the monocotyledonous family Liliaceae, which comprises over 80 species (Comber. 1947; De Jong, 1974). All species are distributed over the mountainous area in the Northern Hemisphere, mainly in Asia, North America and Europe (Lim et al., 2000a). Comber (1947) classified the genus into six sections: Lilium (Liriotypus), Pseudolirium, Martagon, Sinomartagon (Asiatic hybrids), Archelirion (Oriental hybrids) and Leucolirion.

The most important hybrid groups cultivated for cut flower production are the Longiflorum, Asiatic and Oriental hybrids. Crosses within a section can be made with relative ease. To develop complete new hybrids, interspecific hybridization is the most important tool. However, there exist pre- and post-fertilization barriers which have to be overcome. There is special interest in breeding Oriental ×Asiatic hybrids because there is a need to transfer characteristics such as the resistance to *Fusarium* and viral diseases from Asiatic hybrids to Oriental hybrids. On the other hand, some Orientals are resistant to *Botrytis*. This trait would be valuable in breeding Asiatic lilies (Schenk, 1990; Lim et al., 2000a).

As in other plant taxa, the F_1 hybrids between distantly related *Lilium* species are mostly sterile and as such they will be useless in breeding. The traditional method of restoring fertility in such cases is to double the chromosome numbers of the F_1 hybrid and produce allopolyploids that might be fertile (Darlington, 1967; Grant, 1981, Van Tuyl et al., 1992). Such allopolyploids are appropriately called “permanent hybrids” because their progenies

never segregate for parental characters. This is because of the strictly autosyndetic pairing of different genomes in an allopolyploid. Therefore, from the point of view of creating genetic variation for breeding purposes, allopolyploids produced through somatic chromosome doubling have limited possibilities. In an earlier attempt to combine desirable characteristics of two diploid species of *Lilium* (2n=2x=24), *L. longiflorum* Thumb. and *L. rubellum* Baker, were hybridized and the chromosome number of the F_1 (LR) hybrid was doubled through oryzalin treatment (Lim et al., 2000b). In the BC_1 and BC_2 progenies derived from LLRR allotetraploid, however, not even a single cross-over between the L and R genomes was ever found (Lim et al., 2000b).

On the contrary, intergenomic recombination can occur readily in sexual polyploids induced through *2n* gametes originating from the F_1 hybrids of distant species. This has been clearly demonstrated in the case of distant hybrids of *Gasteria* × *Aloe* (Takahashi et al., 1997), *Alstroemeria aurea* × *A. inodora* (Kamstra et al., 1999b), *A. pelegrina* × *A. inodora* (Ramanna et al., 2003) and *L. longiflorum* × Asiatic lily hybrids (Karlov et al., 1999; Lim et al., 2001; 2003a). A cardinal feature of intergenomic recombination in allopolyploids is that they can lead to genetic segregation of parental characters in the progenies so that such polyploids do not behave as permanent hybrids (Ramanna & Jacobsen, 2003). Thus, sexual polyploidization offers attractive possibilities for breeding allopolyploids of lilies.

In the present investigation, with an aim to identify F_1 hybrids that produce *2n* gametes, a large number of hybrids were produced by crossing between cultivars of Oriental and Asiatic lilies. The F_1 hybrids were screened for the production of *2n* gametes, and those genotypes that had *2n* gametes were used for producing sexual polyploid progenies. The results on the frequencies of *2n* gametes in the progenies of 12 different F_1 hybrids as well as their use in producing BC_1 progenies are reported. Furthermore, intergenomic recombination that was detected through GISH in the progenies is reported and discussed.

Material and methods

Plant material

Cultivars from two groups of lilies were used for hybridization. They are the so-called Oriental and Asiatic hybrids belonging to two different taxonomic sections, Archelirion and Sinomartagon, respectively. Because all the cultivars in both sections are derived from hybridization of closely related, intra-sectional diploid (2n=2x=24) *Lilium* species (Van Tuyl et al., 2000), the accessions used in the crossing program are mentioned by their cultivar names, but not as botanical species. The Oriental and Asiatic cultivar (Table 3.1) genomes

will be mentioned as O and A respectively. Using the symbols, the F_1 hybrid is indicated as OA, and BC_1 plants as OOA and AOA depending on the parent used in the backcross. The oryzalin induced tetraploid OA hybrids will be indicated as 4x-OA (Van Tuyl et al., 1992).

Pollen germination

Pollen was cultured during 24-h at 25°C in artificial agar medium containing 100 g sucrose, 5 g bacteriological agar, 20 mg boric acid and 200 mg calcium nitrate per litre. The pollen was classified as large (*2n*) and small (*n*) and the germination percentage was scored counting only the large germinated pollen grains.

Embryo, embryo sac ovary slice and ovule culture

Embryo, embryo sac, ovary slice and ovule culture methods were used in order to overcome post-fertilisation barriers (Asano, 1980a; Okazaki et al., 1994; Van Tuyl et al., 1991; Van Creij et al., 1993).

Flow cytometry

Leaves from BC_1 plants were collected in order to determine the ploidy level as described by Van Tuyl & Boon (1997).

Chromosome preparation

For the study of somatic metaphase chromosomes, root tips were collected early in the morning from *in vitro* plantlets, pre-treated in a 0.7 mM cyclohexamide solution at room temperature for 4 h. For the analysis of meiotic chromosomes, young anthers were collected. Both, anthers and pre-treated root tips were fixed in the ethanol acetic acid solution (3:1) for at least 12 h and stored at –20°C until use. The root tips were incubated in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase Y23, 0.2% (w/v) cellulase RS and 0.2% (w/v) cytohelicase in 10 m*M* citrate buffer (pH 4.5) at 37°C for about 1–1.5 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. Slides were dehydrated in absolute ethanol and air-dried. The pollen mother cells (PMC) were dissected from the anther and squashed in a drop of 1% aceto-orceine.

DNA probe preparation

Sonicated genomic DNA (1-10 kb) from Oriental cultivar ‘Sorbonne’ was used as a probe after labelling with Biotin-16-dUTP (Biotin-16–2′-deoxyuridine-5′-triphosphate) by nick translation according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Autoclaved DNA (100-500 bp) from the Asiatic cultivar 'Connecticut King' was utilized for blocking the non-hybridized sequences. The genomic *in situ* hybridization protocol was carried out according to Chapter 2.

Results

Production of F_1 hybrids

Because hybridization between cultivars of two different sections could not be achieved through normal crossing and seed production, pollination was carried out by the cut style method and either embryo, embryo sac rescue, ovary slice or ovule culture was required in all cases (Van Tuyl & De Jeu, 1997). In order to rescue embryos, the ovules from capsules that had developed anytime between 20 to 70 days after pollination were used. This means, the embryo and endosperm were allowed to develop *in vivo* as if in the normal course of seed development for a considerable amount of time. There were instances in which the embryo, endosperm, or both had failed to develop in the ovules *in vivo*. Only those ovules that had developed embryos were dissected and used for *in vitro* culture. In spite of the embryo rescue being laborious, 708 F_1 plants were obtained from the 86 crossing combinations. Besides confirming the F_1 hybrids on the basis of plant morphology, chromosome pairing (Figure 3.1a-b) as well as a high degree of male sterility were used as criteria.

Pollen of F_1 hybrids and their viability

With the exception of a small fraction of the F_1 hybrids, all of them possessed aborted small pollen grains and were completely sterile (Figure 3.1c). However, in a small number of OA hybrids, well-filled large pollen grains (Fig. 3.1d) were present in some genotypes and these were considered as *2n* pollen grain producers as will be explained. Out of 708 OA hybrids that were produced, only 12 hybrids (1.7%) were found to produce *2n* pollen in notable frequencies and these were investigated further for pollen viability through germination and their ability to produce embryos after using as male and female parents (Tables 3.1 and 3.2). The frequencies of *2n* pollen grains in all the genotypes were highly variable, probably due to environment (data not included). In order to establish whether these *2n* pollen grains were viable and functional, they were germinated *in vitro* as well as used as male parents in crossing with 2x parents, 2x-OA and 4x-OA hybrids. The results on the average percentage and range of pollen germination in different genotypes, and the outcome of crossing, as evidenced from viable embryo formation *in vitro*, of 12 OA hybrids are presented in Table 3.1. For calculating the average germination percentages only the germinated ones among the large pollen grains were taken into account. Although *2n* pollen germinated in all the 12 hybrids, nearly a 40- fold variation was observed for germination percentages

Table 3.1. Twelve selected OA hybrids that produced well filled *2n* pollen: their germination percentages and ability to produce viable embryos after using them as pollen parents

Genotype	Parents		Pollen germination (%) (Range %)	Occurrence of embryos
	Oriental	Asiatic		
951462-1	'Romero Star'	'Connecticut King'	31.4 (0 – 75)	+[a]
951447-1	'Bel Paso'	'Gran Sasso'	2.0 (0 – 2)	-
951502-1	'Pesaro'	'Connecticut King'	16.6 (0 – 100)	+
951584-1	'Acapulco'	'Sancerre'	23.3 (5 – 60)	+
952088-1	'Expression'	'Au Revoir'	25.0 (n.a)	+
952381-5	'Mero Star'	'Connecticut King'	2.6 (0 – 10)	-
952400-1	'Mero Star'	'Gran Sasso'	0.5 (0 – 20)	+
952462-1	'San Marco'	'Connecticut King'	37.5 (20 – 50)	+
962119-1	'Acapulco'	'Connecticut King'	6.0 (0 – 40)	+
962120-1	'Bernini'	'Connecticut King'	1.9 (0 – 25)	+
962254-2	'Tenerife'	'Lanzarote'	2.1 (0 – 30)	-
962433-1	'Sissi'	'Mirella'	24.4 (0 – 75)	+

a + = Embryos were produced; – = no embryos were produced; n.a. = not available

among different genotypes of the same hybrid as well as among the 12 different hybrids that were investigated (Table 3.1). There was, however, no strict relationship between germination percentage and the formation of viable embryos *in vitro*. For example, out of the nine cases where embryo formation was observed, seven had fairly higher percentages of germination whereas two (952400-1, 962120-1) had low percentages (2% or less). Embryos were formed when higher pollen germination was found (Table 3.1). This absence of a strict relationship between pollen germination and viable embryo formation can be explained from the fact that the environment might greatly influence the formation and the viability of *2n* pollen. This implies that those genotypes that showed a lower percentage of germination had formed a higher percentage of viable pollen at the time of making the crosses. In an estimate of germination, percentages of *2n* pollen in eight clones of one genotype (962433-1), showed nearly 30- to 40-fold variation between the different stems as well as among the individual flowers of the same plant (results not included). This means, a repeated testing of *2n* pollen may be required before concluding whether a pollen parent could be used successfully.

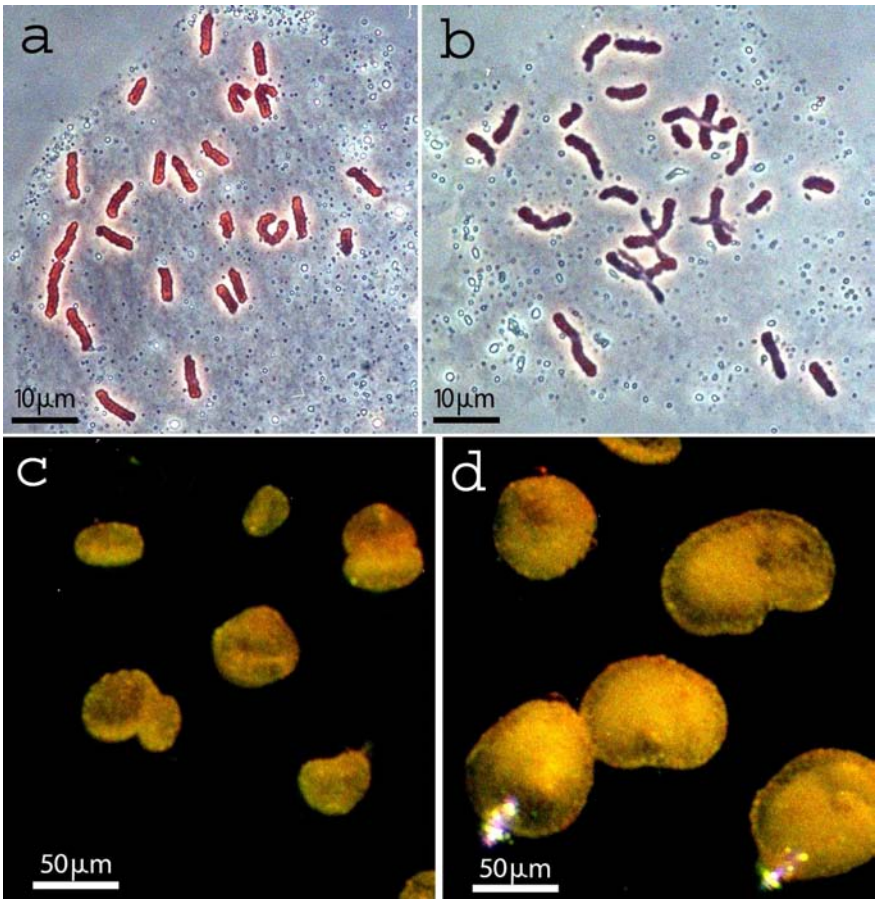

Figure 3.1. Metaphase I stage during microsporogenesis in a OA hybrid (951502-1) (**a**) showing 24 univalents and (**b**) 4 bivalents + 16 univalents in two pollen mother cells. Pollen grains in the same OA hybrid showing (**c**) sterile and (**d**) "fertile" *2n* pollen grains.

Use of 2n *pollen for backcrossing*

In order to investigate whether the selected *2n* pollen producers were suitable for sexual polyploidization, all the selected genotypes of the 12 OA hybrids were used as male and some as female parents for crossing with the diploid parental cultivars as well as with the 2x-OA and 4x-OA hybrids. The successful results obtained in four different sets of hybridizations are mentioned in Table 3.2. Of all the *2n* pollen producing genotypes tested as male parents, nine of them gave rise to germinating embryos. In the backcrosses, a relatively larger number of germinating embryos was obtained in the case of Asiatic × OA hybrids (2.9 embryos/pollination) as compared to Oriental × OA hybrids (1 embryo/pollination) (Table 3.2).

Besides the male fertility of *2n* gamete producing genotypes, one genotype (952400-1) showed positive results for female fertility (Table 3.2). In this case, four Asiatic cultivars as well as two 4x-OA hybrids were used as male parents. The occurrence of female fertility in the OA hybrids indicated the potential for using *2n* eggs for (bilateral) sexual polyploidization. Finally, the crosses with 4x-OA hybrids with four *2n* pollen-producing genotypes were successful. From these crossing data it was evident that most of the selected genotypes (Table 3.1) were potentially useful for producing a large number of backcross and other progenies through unilateral as well as bilateral sexual polyploidization.

Table 3.2. Results of crossing of *2n* pollen producing OA hybrids with parental diploid, 2x OA and 4x – OA genotypes

Parents		Number of flowers pollinated	Number of germinations	Based on theoretical estimate of 100 crosses
Female	**Male**			
Asiatic				
'Amarone'	951462-1	9	5	56
'Gironde'	951502-1	24	54	225
'Amarone'	951502-1	23	160	696
'Mont Blanc'	951502-1	16	44	275
'Amarone'	951584-1	2	10	500
'Gironde'	952400-1	16	27	169
'Amarone'	952400-1	5	25	500
'Mont Blanc'	952400-1	5	15	300
'Gran Sasso'	952400-1	5	2	40
'Lanzarote'	952400-1	18	36	200
'Gironde'	952462-1	6	1	17
'Amarone'	952462-1	6	26	433
'Mont Blanc'	952462-1	3	20	667
'Mont Blanc'	962119-1	2	3	150
'Amarone'	962120-1	2	1	50
'Gironde'	962433-1	12	10	83
'Amarone'	962433-1	13	26	200
'Mont Blanc'	962433-1	14	59	421
		Total 181	524	2.9 e/p[a]
Oriental				
'Sorbonne'	951502-1	28	45	161
'Lombardia'	951502-1	31	27	87
'Lombardia'	951584-1	11	14	127
'Sorbonne'	952462-1	5	8	160
'Lombardia'	952462-1	4	6	150
'Tiber'	952462-1	6	3	50
'Lombardia'	962119-1	2	4	200
'Lombardia'	962120-1	2	2	100
'Sorbonne'	952400-1	8	2	25
'Time out'	952400-1	5	2	40
'Bramante'	952400-1	4	1	25
		Total 106	114	1 e/p
OA Hybrid				
952400-1	'Gironde'	7	12	171
952400-1	'Connecticut King'	2	4	200
952400-1	'Amarone'	11	6	55
952400-1	'Mont Blanc'	4	11	275
952400-1	'Mero Star' x 'Connecticut King'	12	5	42
952400-1	'Expression' x 'Lady Rosa'	14	2	14
		Total 50	40	0.8 e/p

a = embryos / pollination.

Table 3.2 cont. Results of crossing of *2n* pollen producing OA hybrids with parental 4x – OA genotypes

Parents		Number of flowers pollinated	Number of germinations	Based on theoretical estimate of 100 crosses
Female	**Male**			
4x – OA				
'Romero Star' x 'Lady Rosa'	951502-1	10	1	10
'Expression' x 'Lady Rosa'	951502-1	19	8	42
'Expression' x 'Lady Rosa'	952088-2	2	1	50
'Romero Star' x 'Connecticut King'	952400-1	12	2	17
'Expression' x 'Lady Rosa'	952400-1	11	3	27
'Expression' x 'Lady Rosa'	952462-1	2	7	350
		Total 56	22	0.4 e/p

a = embryos / pollination.

Analysis of the sexual polyploid progenies (BC_1 + 4x-OA × OA crosses)

In 263 BC_1 seedlings derived from three different combinations, viz., AOA, OOA and OAA and 6 4x-OA × OA, seedlings were analysed for their ploidy levels by determining DNA values through flow cytometry according to Van Tuyl & Boon (1997). Assuming that *2n* gametes from OA hybrids have contributed 24 chromosomes, it was expected that the BC_1 progenies would possess triploid (2n=3x=36) chromosome numbers and the 4x-OA × OA progenies to have tetraploid (2n=4x=48) chromosome numbers. Out of the 263 BC_1 progenies that were analysed, 246 (93.5%) were triploid and 14 (5.3%) were tetraploid (Table 3.3). In the 6 4x-OA × OA progenies, one was a triploid, four were tetraploid and one was a hexaploid (not shown). The observation that the BC_1 progenies were mostly triploids proved that the F_1 OA hybrids had contributed balanced diploid chromosome complements. And, as expected, the 4x-OA × OA progeny was mostly tetraploid. The few tetraploids that occurred in the BC_1 progenies had obviously originated through the functioning of *2n* gametes from both, the diploid Asiatic and the OA backcross parent. A notable result was the presence of one hexaploid in 4x-OA × OA which was probably the result of the production of a *2n* egg in the mitotically doubled OA hybrid. The results of flow cytometric analysis were confirmed through cytological analysis of the somatic chromosomes in several triploids as well as tetraploids (Figure 3.2 a-b).

Of particular interest of the BC_1 progenies was that through GISH analysis the presence of intergenomic recombinant segments could be demonstrated (Figure 3.2a). Such recombination was anticipated because the cytological analysis of metaphase I stages of the F_1 OA hybrids revealed bivalent formation between O and A genomes (Figure 3.1b).

Discussion

Failure to obtain intergenomic recombination in the inter-sectional species hybrids of *L. rubellum* × *L. longiflorum* (Lim et al., 2000b) was a clear illustration of the limitation of using somatically doubled allotetraploids in breeding lilies. In contrast, the results of the present investigation clearly demonstrate the value of sexual polyploidization in obtaining crossovers between O and A genomes. There are indeed BC_1 progenies that are predominantly triploid, as well as 4x progenies from BC_1 and 4x-OA × OA crosses that possess more than one recombinant chromosome (results not included). Allotriploid BC_1 plants, although difficult to hybridize, our previous results from *L. longiflorum* × Asiatic crosses have shown that triploids can be crossed either with diploid or tetraploid parents (Lim et al., 2003a). In these cases the progenies that posses near diploid or near pentaploid chromosome numbers have to be selected for the presence of recombinant chromosomes.

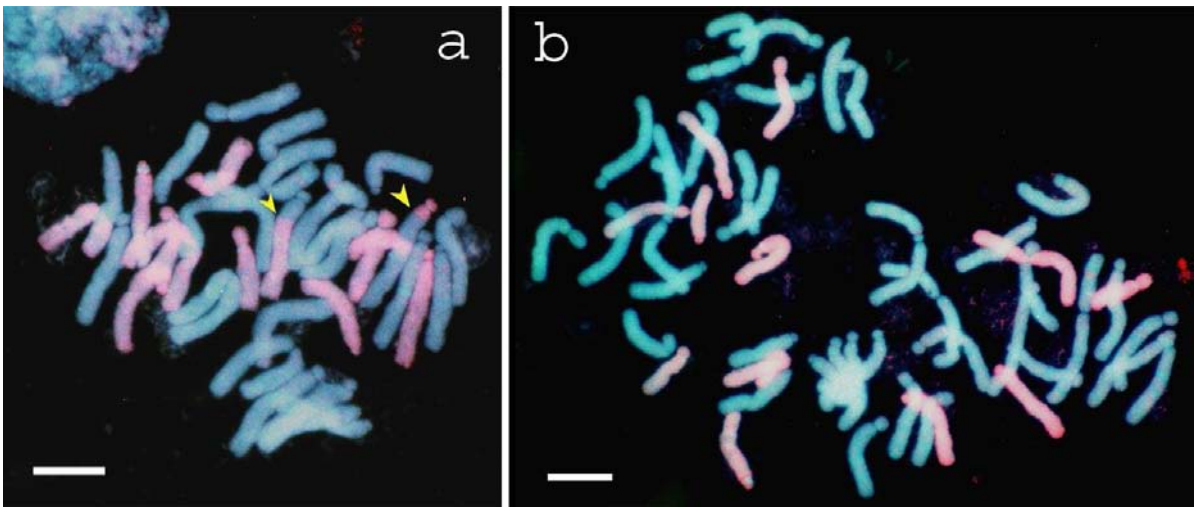

Figure 3.2. Chromosome complements of the BC_1 progenies showing a triploid and a tetraploid chromosome number. (**a**) 36 chromosomes of the BC_1, 022217-3, plant with two recombinant chromosomes (arrows). The biotin-labeled Oriental DNA of 12 chromosomes was detected with the Cy3-streptavidin system (pink fluorescence) and 24 Asiatic chromosomes were counter stained with DAPI (blue fluorescence) (**b**) A tetraploid chromosome complement of the BC_1 plant, 022218-6, showing 12 Oriental and 36 Asiatic chromosomes. The probe DNA used and detection were the same as for (a). *Bar represents 10 μm*.

In the case of 4x-OA × OA crosses most of the progeny plants are allotetraploids. Some of these possess recombinant chromosomes. The allotetraploids are expected to behave almost like permanent hybrids but for those chromosomes that have recombinant segments that can form multivalents and assort independently. Thus these tetraploids are much more directly accessible for breeding than the triploid BC_1 progenies.

Table 3.3. Number and types of progenies obtained from different types of backcrosses

Genotype	Parents		# of progenies analyzed	Ploidy levels of the progenies	
	Female	Male		3x	4x
A OA					
002433	'Gran Sasso'	952400-1	2	2	0
002526	'Lanzarote'	952400-1	20	20	0
002529	'Lanzarote'	952400-1	16	16	0
002531	'Gironde'	952400-1	21	21	0
022217	'Gironde'	952400-1	6	6	0
022218	'Gironde'	952400-1	9	6	3
022219	'Mont Blanc'	952400-1	4	3	1
022538	'Amarone'	951502-1	14	12	0
022542	'Amarone'	962433-1	3	3	0
022604	'Gironde'	951502-1	16	15	1
022605	'Amarone'	951502-1	75	71	4
022610	'Mont Blanc'	951502-1	18	17	1
022611	'Gironde'	951502-1	12	11	1
022612	'Amarone'	951502-1	25	23	2
022643	'Amarone'	951502-1	3	2	1
O OA					
992682	'Sorbonne'	952400-1	2	2	0
992738	'Time Out'	952400-1	2	2	0
022552	'Lombardia'	951584-1	1	1	0
022572	'Lombardia'	962119-1	1	1	0
022574	'Lombardia'	952462-1	1	0	0
022582	'Sorbonne'	952462-1	3	3	0
022609	'Sorbonne'	951502-1	2	2	0
022624	'Lombardia'	951502-1	1	1	0
022636	'Lombardia'	951502-1	1	1	0
OA A					
022215	952400-1	'Mont Blanc'	1	1	0
022204	952400-1	'Connecticut King'	4	4	0
Total			263	246	14

The occurrence of *2n* gametes observed in the present study is similar to that reported in distant F_1 hybrids in various other plant taxa (Ramanna & Jacobsen, 2003). Most of these cases share certain common features: first, they all have disturbed chromosome pairing; second, they are highly sterile in the sense that they do not produce functional *n* gametes; third, they all produce exclusively first division restitution (FDR) gametes. Some of the other comparable instances are: wheat × *Aegilops squarrosa* (Fukuda & Sakamoto, 1992), *Aegilops squarrosa* × *Triticum durum* (Sasakuma & Kihara, 1981), rye × *A. squarrosa* (Xu & Dong, 1992; Xu & Joppa, 2000), *Alstroemeria* interspecific hybrids (Ramanna et al. 2003) and *Lilium longiflorum* × *Asiatic* hybrids (Lim et al., 2001). An important feature in many of these cases is that the frequencies of *2n* gametes are greatly influenced by the environment. This appears to be also in the present *Lilium* hybrids (unpublished data).
There is a general view that *2n* gametes may not be widely applicable in breeding because their occurrence is not always predictable. Although there are some instances in which *2n* gamete formation has been claimed to be genetically controlled, this is not always the case (Ramanna & Jacobsen, 2003).

At least in the present study the cultivars of both Oriental and Asiatic hybrids are not known to produce FDR gametes in any noticeable frequencies. Yet there are a few genotypes among the F_1 hybrids (Table 3.1) that produce considerable frequencies of *2n* pollen through FDR. The success in the present study has probably been achieved for the following two reasons: a) a fairly large number of F_1 hybrids using several genotypes have been produced and b) all the F_1 hybrids were carefully screened for the occurrence of *2n* gametes. Because of the relative ease with which the pollen could be screened, we have found several *2n* pollen producers and there is evidence that there are also *2n* eggs producing genotypes (952400-1). However it will be important to identify more *2n* egg producers, because it can facilitate bilateral sexual polyploidization.

There appear to be certain trends in different plant species hybrids that can help to identify the genotypes that might produce *2n* gametes. For example, in wheat × *Aegilops* hybrids the later emerging spikes and secondary culms produced a higher frequency of *2n* gametes (Fukuda & Sakamoto, 1992). In *Alstroemeria,* hybrids between Brazilian and Chilean species produced much higher frequencies of *2n* gametes than those between the Chilean species did (Ramanna et al., 2003). In the genus *Saccharum*, interspecific hybrids between *S. officinarum* × *S. spontaneum* produced *2n* eggs on a more regular basis (Bremer, 1961). In the present investigation, the hybrids involving the Asiatic cultivar, 'Connecticut King' as male parent predominantly produced *2n* pollen. But it is important to note that we have screened a

relatively larger number of hybrids from this combination for *2n* pollen production. The genotype dependence of *2n* gamete production has been observed in some of the crop plants (Ramanna & Jacobsen, 2003), but it remains to be established whether such genotype dependence is also present in lilies. In view of the variable tendencies in different plant species hybrids, it might be helpful to identify them, if any, in each instance.

In *Lilium,* embryo rescue is required for producing the F_1 hybrids, as well as the BC_1 and partly for BC_2 and subsequent progenies. A comparable situation is observed in the case of interspecific hybrids of *A. aurea* × *A. inodora* where embryo rescue is required for backcrossing (Kamstra et al., 1999b). In other interspecific hybrids of *Alstroemeria,* involving Brazilian and Chilean species, however, seed set occurs *in vivo* after selfing the F_1 hybrids as well as after backcrossing (Buitendijk et al., 1997; Ramanna et al., 2003). In many of the cereal interspecific, intergeneric hybrids and polyhaploids, both *2n* pollen and *2n* eggs occur so frequently that selfing and backcrossing can be practised regularly (Ramanna & Jacobsen, 2003). Despite embryo rescue being time consuming and laborious in *Lilium* hybrids, as this investigation shows, success can be achieved in using *2n* gametes for sexual polyploidization.

Chapter 4

Intergenomic recombination in F_1 lily hybrids (*Lilium*) and its significance for genetic variation in the BC_1 progenies as revealed by GISH and FISH

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Abstract

Intergenomic recombination was assessed in a BC_1 population of Oriental × Asiatic lilies (*Lilium*) backcrossed to Asiatic parents. This population consisted of 38 plants and originated through the functioning of *2n* gametes from two genotypes (951502-1 and 952400-1) of diploid F_1, Oriental × Asiatic lilies (2n=2x=24) as parents. In the majority of BC_1 plants, there was evidence that First Division Restitution (FDR), with and without crossovers, resulted in functional gametes. However, there were five BC_1 plants in which *2n* gametes had originated from Indeterminate Meiotic Restitution (IMR). Based on the presence of the number of recombinant chromosomes for a particular homoeologous pair, three types of plants were identified: (i) with both reciprocal product of a crossover ($^{O}/_{A}$, $^{A}/_{O}$, where O represents the centromere of the O genome and A the recombinant segment of Asiatic chromosome and vice versa); (ii) with one normal chromosome of O genome and a recombinant chromosome (O, $^{A}/_{O}$) and (iii) with one normal chromosome of A genome and a recombinant chromosome (A, $^{O}/_{A}$). An important feature of A × OA backcross progeny was the occurrence of substitutions for the segment distal to the crossover wherever the recombinant chromosome $^{O}/_{A}$ was present. In the case of IMR, the substitution occurred for both proximal as well as distal recombinant segments. The significance of these substitutions is that they offer the potential for the phenotypic expression of recessive genes in polyploids (i.e., nulliplex genotype).

Introduction

There are three main groups of lily hybrids (*Lilium*) that are important in commercial horticulture, viz., Longiflorum, Asiatic and Oriental hybrids. These groups belong to different taxonomic sections, Leucolirion, Sinomartagon and Archelirion, respectively. The cultivars in all these groups are mostly diploid (2n=2x=24) and they are easy to hybridize within the same section. However, it is difficult to hybridize the cultivars or species that belong to different sections and the hybrids can be produced only through special techniques such as cut-style method (Asano & Myodo, 1977*a*; 1977*b*), grafted style followed by *in vitro* pollination (Van Tuyl et al., 1991), *in vitro* pollination and rescue methods such as embryo, ovary slice and ovule culture, among others (Van Creij et al., 2000).

With a view to combine desirable horticultural traits that are available in the species of different sections, we have made many inter-sectional, hybrids. These include Longiflorum × Asiatic (LA hybrids) and Oriental × Asiatic (OA) hybrids (Lim et al., 2003a; Van Tuyl et al., 2002a). Because of the high degree of sterility of the F_1 hybrids, they cannot be directly utilized for backcrossing. Therefore, the chromosome numbers of the F_1 hybrids are doubled by colchicine or oryzalin treatment to restore fertility (Van Tuyl et al., 1992; Lim et al.,

2000b) and the allotetraploid is used for backcrossing. A great drawback of this approach was that, because of autosyndetic pairing in the allotetraploid, there was no intergenomic recombination in the BC_1 and subsequent progenies (Lim et al., 2000b). An alternative approach was to use numerically unreduced (*2n*) gametes from the F_1 hybrids for backcrossing. Although it is generally difficult to find the genotypes of F_1 hybrids that produce high frequencies of *2n* gametes, it was possible to select such genotypes in both LA and OA hybrids and backcross them extensively in both cases (Lim et al. 2000a; Chapter 3).

An important feature of the BC_1 progenies of the LA hybrids is that it was possible to obtain intergenomic recombination in the BC_1 progenies (Lim et al., 2003a; Van Tuyl et al., 2003). This recombination included not only crossing over between homoeologous chromosomes but also chromosome assortment. The crucial factors for the occurrence of recombination in LA hybrids were elucidated as due to two types of nuclear restitution mechanisms: First Division Restitution (FDR) with crossing over, and Indeterminate Meiotic Restitution (IMR) (Lim et al., 2001). As a consequence of intergenomic recombination, it was also evident that substitutions for recombinant segments as well as for whole chromosomes could occur in the BC_1 progenies of LA hybrids (Lim et al., 2001). The occurrence of FDR has also been elucidated in OA hybrids (Chapter 2).

The aims of the present investigation on BC_1 progenies are to: a) estimate the extent of intergenomic recombination; b) determine the chromosome composition through genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) and c) determine whether substitutions occur for recombinant segments in the BC_1 progenies of OA hybrids.

The implications of the results for the occurrence of genetic variation are discussed.

Material and Methods

Plant material

A BC_1 population consisting of 38 plants originated through sexual polyploidization was used for estimating the extent of intergenomic recombination and for determining the chromosome constitution. Three diploid Asiatic cultivars (A genome) viz., 'Gironde', 'Mont Blanc' and 'Amarone' (2n=2x=24), two tetraploid OA hybrids (4x-OA) from PRI collection (991108 and 991110) and a diploid OA genotype (952400-1) were used as female parents. With three exceptions, all these BC_1 progeny plants were derived from crossing a diploid cultivar with either one of the *2n* pollen producing OA hybrids, viz, 952400-1 (13 plants) and 951502-1 (23 plants). Only one BC_1 plant originated through OA × A cross in which *2n* eggs from OA must have been functional (Table 4.1). Bulbs from all hybrids and cultivars were planted in a greenhouse and grown in standard condition for lily growth and development (Van Tuyl & Van Holsteijn, 1996).

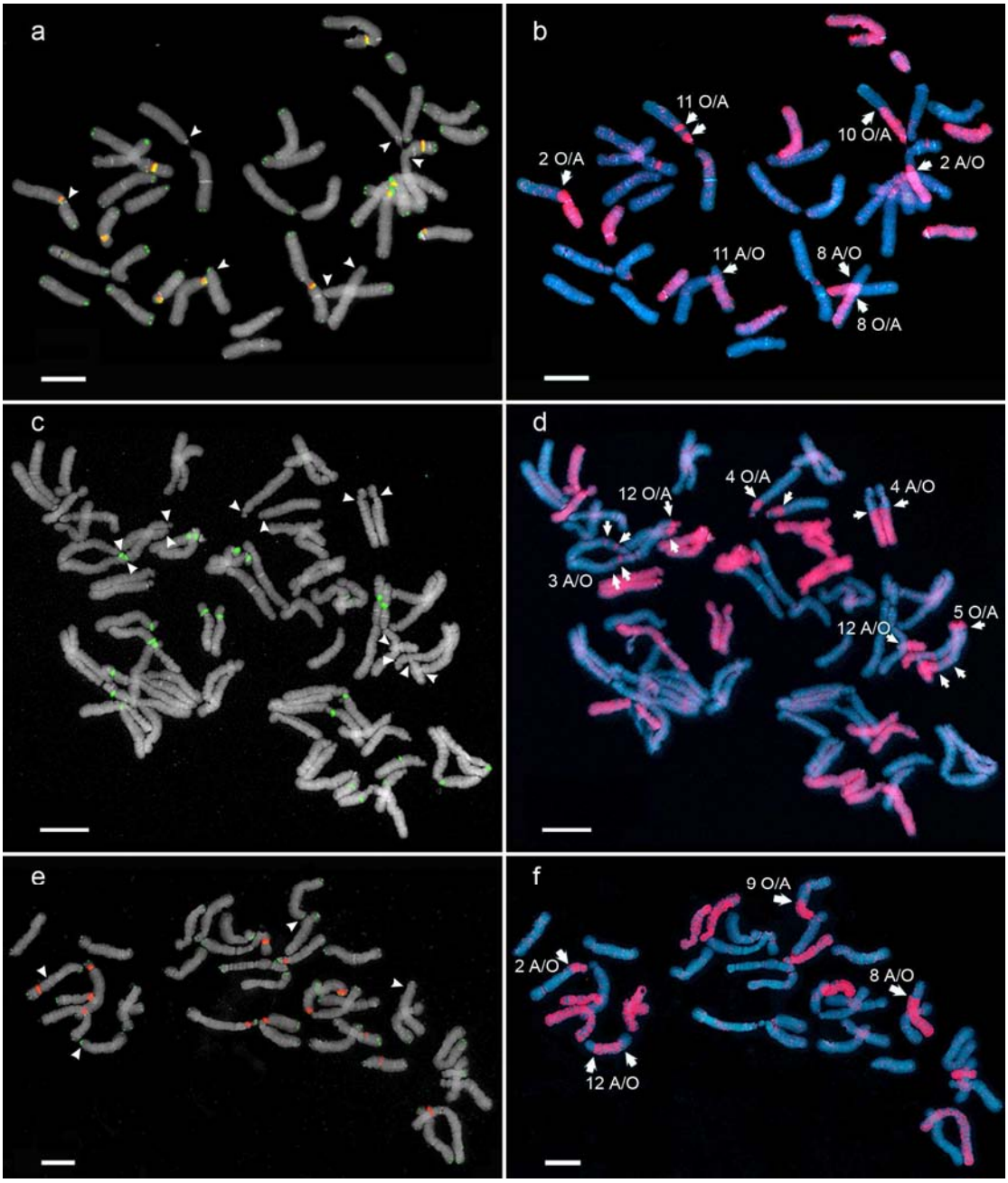

Figure 4.1. Chromosome identification and detection of intergenomic recombination in three BC_1 progenies. In all cases, FISH images (left), show 45S rDNA sites, probed with either biotin or digoxigenin, and telomeric sites, probed with digoxigenin. Biotin probes were detected with the Cy3-streptavidin system (orange-red fluorescence) and digoxigenin probes were detected with the anti-digoxigenin detection system (green fluorescence). The centromeres are marked with arrow heads for the relevant chromosomes only. In all GISH images (right) the respective recombinant chromosomes are mentioned appropriately (e.g., O/A or A/O) and the arrows indicate the recombinant segments. The biotin-labeled Oriental DNA was detected with the Cy3-streptavidin system (pink fluorescence) and the Asiatic chromosomes were counter-stained with DAPI (blue fluorescence). In the case of recombinant chromosomes, the centromeres are taken into account for estimating the number of chromosomes of each genome. (**a-b**) The triploid complement of 022538-1, showing 12 O + 24 A, with seven recombinant chromosomes. (**c-d**) Late metaphase of the triploid complement of 022538-7 showing both chromatids of each 13 O + 23 A, with six recombinant chromosomes. (**e-f**) The triploid complement of 0022538-15 showing 11 O + 25 A, with four recombinant chromosomes. *Bar represents 10 μm*.

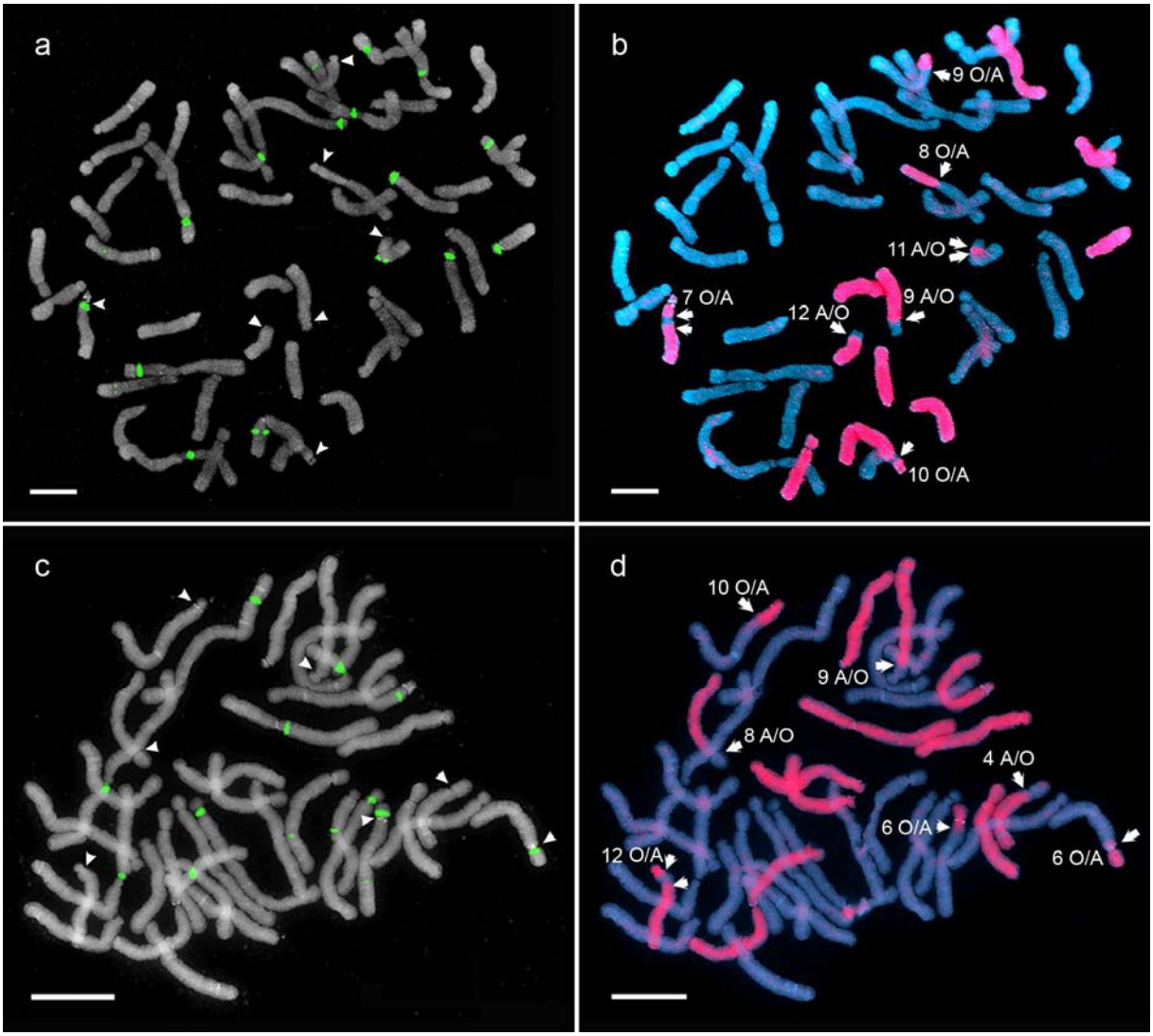

Figure 4.2. Chromosome identification and detection of intergenomic recombination in two BC_1 progenies. In both cases, FISH images (left), show 45S rDNA, probed with digoxigenin and detected with the anti-digoxigenin detection system (green fluorescence). The centromeres are marked with arrow heads for only the relevant chromosomes. In all GISH images (right) the respective recombinant chromosomes are mentioned appropriately (e.g., O/A or A/O) and the arrows indicate the recombinant segments. The biotin-labeled Oriental DNA was detected with the Cy3-streptavidin system (pink fluorescence) and the Asiatic chromosomes were counter-stained with DAPI (blue fluorescence). In the case of recombinant chromosomes, the centromeres are taken into account for estimating the number of chromosomes of each genome. (**a-b**) The tetraploid complement of 022605-3 showing 12 O + 36 A, with seven recombinant chromosomes. (**c-d)** The triploid complement of 022605-9 showing 12 O + 24 A, with seven recombinant chromosomes. *Bar represents 10 μm*

Chromosome preparation

For the study of somatic metaphase chromosomes, root tips were collected early in the morning, incubated in saturated α-bromonaphthalene solution in ice-water overnight and fixed in the ethanol acetic acid solution (3:1) for at least 12 h, stored at –20°C until use. The root tips were incubated in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase

Y23, 0.2% (w/v) cellulase RS and 0.2% (w/v) cytohelicase in 10 m*M* citrate buffer (pH 4.5) at 37°C for about 2 h. Squash preparations were made in a drop of 50% acetic acid and frozen in liquid nitrogen; the cover slips were removed by using a razor blade. Slides were dehydrated in absolute ethanol and air-dried.

DNA probe preparation

Fluorescent *in situ* hybridization (FISH) was performed using two different probes, i) clone pTa71 which contains the *EcoRI* fragment of 45S ribosomal DNA from wheat (9 kb) (Gerlach and Bedbrook 1979); ii) a synthetic telomeric probe that was generated by PCR according to Cox et al. (1993) with minor modifications. In brief, two oligomer primers 1fw (5'-TTTAGGG-3')$_5$ and 1rev (5'-CCCTAAA-3')$_5$ were synthetized by Isogen Life Science, the Netherlands. Reactions were amplified in the absence of template DNA. Each 100 µl reaction comprised 10 µl of 10x *Taq* buffer (Promega,) 1.5 mM $MgCl_2$, 2 units of *Taq* polymerase (Promega), 2.5 mM dNTPs and 10 pmol of each primer 1fw and 1rev. Temperature cycling was performed according to Ijdo et al. (1991) with the final extension step of 10 min at 72°C.
For the genomic *in situ* hybridization (GISH), sonicated genomic DNA (1-10 kb) from Oriental cultivar 'Sorbonne' was used as a probe. All probes were labelled with either biotin-16-dUTP or digoxigenin-11-dUTP by nick translation according to manufacturer instructions (Roche, Germany).

In situ *hybridization*

FISH was performed in slides with metaphase chromosomes of 10 different BC_1 progeny plants by incubating in RNase A (100 µg/ml) for 1 h and pepsin (5 µg/ml) for 10 min, both at 37°C, followed by paraformaldehyde (4%) for 10 min at room temperature, dehydrated with 70%, 90% and absolute ethanol for 3 min in each and air dried. Hybridization followed using a mixture consisting of 20x SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS and 25-50 ng/ml of each probe. The DNA was denatured by heating the hybridization mixture at 70°C for 10 min and then placed on ice for at least 10 min. For each slide, 80 µl hybridization mixture was used. The preparations were denatured at 80°C for 10 min. After overnight hybridization at 37°C in a humid chamber, slides were washed at room temperature in 2x SSC for 15 min and 0.1x SSC at 42°C for 30 min. Biotin-labelled probes were detected with Cy3 labelled streptavidin (Amersham Biosciences, UK), and amplified with biotinylated goat-antistreptavidin (Vector laboratories, Burlingame, CA); digoxigenin-labelled probes were detected with anti-digoxigenin-fluorescein (Roche, Germany) and amplified with fluorescein anti-sheep and fluorescein anti-rabbit (Vector laboratories, Burlingame, CA).

Chromosomes were counterstained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector Laboratories. Brulingame, CA) was added for its examination under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescent illumination, filter sets of DAPI, FITC and Cy3. Images were captured as will be explained later. The probes were removed from the preparations and reprobed, as described by Schwarzacher and Heslop-Harrison (2000), with genomic DNA from Oriental cultivar 'Sorbonne'. Autoclaved DNA (100-500 bp) from the Asiatic cultivar 'Connecticut King' was utilized for blocking the non-hybridized sequences. The following GISH protocol was carried out according to Chapter 2. For the other BC_1 progeny plants the same GISH protocol was utilized. Selected images were captured by a Photometrics Sensys 1,305 × 1,024 pixel CCD camera and processed with Genus Image Analysis Workstation software (Applied Imaging Corporation). The DAPI images were sharpened with a 7×7 High Gauss spatial filter. DAPI fluorescence was displayed in grey value for the FISH analysis and pseudo-coloured for the GISH analysis. For both analyses the probes fluorescence was pseudo-coloured with either red or green colour. Optimal brightness and contrast were achieved with Adobe Photoshop image processing.

Chromosome analysis and flow cytometry

Chromosomes measurements were made using the freeware computer application MicroMeasure (Reeves & Tear, 1997) and arranged in sequence of decreasing short arm length according to Stuart (1947) taking into account the position of the 45S hybridization signals. Leaves from BC_1 plants were collected in order to determine DNA values which reflected the ploidy levels as described by Van Tuyl & Boon (1997).

Results

Ploidy levels of BC_1 progeny

In all 38 BC_1 progeny plants were monitored through GISH for their ploidy levels, the number of chromosomes from O and A genomes and the number of recombinant chromosomes. Out of 38 BC_1 plants, there were 33 triploids (2n=3x=36), four tetraploids (2n=4x=48) and a hexaploid (2n=6x=72) (Table 4.1). A notable feature was that all BC_1 plants, regardless of ploidy level, were euploid.

In GISH preparations it was possible to clearly identify the chromosomes of parental genomes; including recombinant segments (Figures 4.1 and 4.2). In a majority of the triploid BC_1 progeny plants, 12 chromosomes of O genome and 24 of A genome were clearly identified (Table 4.1). This was according to the expectation because, in a backcross, the *2n* gamete from the F_1 hybrid had contributed one set each of O and A genomes. However, there

were five genotypes in which the number of chromosomes of O and A genomes did not strictly conform to the expected number of 12 and 24, respectively (Table 4.1 asterisks). As will be explained later, the *2n* gametes in these cases had failed to transmit 12 chromosomes each of O and A genomes.

Out of three tetraploids, one (022544-2) had 24 chromosomes of each of O and A genomes. On the other hand three tetraploids, 022605-3, 022605-13 and 022605-15, had originated through the functioning of *2n* eggs from the female parent and thus contributed two sets of A genome. Remarkably, one of these plants had 11 O + 37 A instead of 12 O + 36 A constitution (Table 4.1). The only hexaploid that was found had 36 O and 36 A constitution implying the allotetraploid female parent had contributed the *2n* egg.

Homoeologous recombination

Out of the 38 BC_1 progenies derived from sexual polyploidization, 25 plants (65.8%) possessed recombinant chromosomes (Table 4.1). There was, however, a clear difference between the two groups of progenies within this population with regard to the number of plants with recombinant chromosomes. This difference was dependent on the genotype of the F_1 OA hybrid that was used as the parent in the cross. Thus, in the 14 plants derived from crossing 952400-1 as a parent, there were only five plants (35.7%) with recombinant chromosomes. On the other hand, among the 24 plants that originated from crossing with the OA hybrid, 951502-1, as a parent, there were 19 plants (79.1%) with recombinant chromosomes. Thus, there was an obvious difference between the two genotypes of OA hybrids with regard to the contribution of recombinant chromosomes to the BC_1 progeny plants. Furthermore, the range of recombinant chromosomes varied from 0-2 in the crosses of 952400-1 whereas in the case of 951502-1, it ranged from 0-7. And, whereas mostly single crossovers had occurred in the case of the former, there was evidence for the occurrence of double or more crossovers per chromosome in the latter. A notable feature of homoeologous recombination was that the crossovers were unevenly distributed within the genome. The larger chromosomes had fewer crossovers compared to the smaller chromosomes of the F_1 chromosome complement (data not presented).

Chromosome constitution of BC_1 plants

As was mentioned earlier, the chromosome constitution of some of the BC_1 plants deviated from the expected 12 O + 24 A chromosomes. In order to investigate such plants more critically as well as to determine the frequency and the types of recombinant chromosomes that were transmitted, 10 BC_1 plants were analyzed by combining GISH and FISH techniques and five of these are illustrated (Figures 4.1, 4.2). The FISH technique was used for the

identification of individual chromosomes by using 45S rDNA sites and a synthetic telomeric probe as markers (Figures 4.1, 4.2). With one exception (022605-3), all the genotypes that were investigated had triploid chromosome constitution (Figures 4.1, 4.2, 4.3, and 4.4) and only one was a tetraploid (Figures 4.2a-b and 4.4). In all the ideograms, the chromosomes derived from the backcross parent are shown at the left and the two chromosomes derived from the F_1, OA hybrid are shown at the right side for each individual chromosome.

Among the 10 plants, six (022538-1, -3, -8; 022605-3, -8 and -40) had received the full complement of 12 O + 12 A from the OA hybrid. However, in the case of recombinant chromosomes only the centromeres were taken into account to asses the chromosome complement. Hereafter, to avoid confusion, the recombinant chromosomes will be mentioned in the text only as centromeres. In this sense, all the above mentioned six plants had received 12 individual chromosomes/centromeres each of O and A genomes from the *2n* gametes of OA hybrids. This implied that in all these six cases FDR gametes were functional. In four cases, viz., 022538-7, -9, -15 and 022605-9, there was evidence that not all the individual chromosomes/centromeres of either O or A genome were transmitted through *2n* gametes to the BC_1 progenies. For example, the chromosome 5 in 022538-7 was represented by a pair of one each of O genome centromere/chromosome instead of one each of both A and O genomes (Figure 4.3, marked as SC). Because of this anomalous situation, this plant possessed 13 O + 23 A instead of the expected 12 O + 24 A chromosome constitution. Similarly, in the case of 022538-9, chromosome 8 was represented by a pair of O centromere/chromosome rather than one each of O and A chromosomes. The consequence was that it led to a chromosome constitution of 13 O + 23 A as in 022538-7 (Figure 4.1 c-d). Just as the presence of a pair O genome chromosome/centromere instead of a single chromosome or centromere, led to 13 O +23 A chromosome/centromere constitution. There were also instances in which one of the O genome chromosome/centromere was missing in the BC_1 plant. One example was 022538-15 in which the chromosome 8 was represented by two centromeres of A rather than one each O and A. This resulted in a chromosome constitution of 11 O + 25 A (Figure 4.1 e-f). Although the unbalanced chromosome/centromere constitution of 13 O and 23 A and 11 O + 25 A resulted from the transmission of duplicate chromosomes/centromeres through *2n* gametes to BC_1, it always produced the same result. For example, in the case of 022605-9 there were duplicate chromosomes/centromeres for chromosome 4 and 6 (Figure 4.2 c-d and 4.4). This resulted in a chromosome constitution of 12 O and 24 A. apparently; this gave the impression that the two complete sets of O and A genomes were transmitted by the *2n* gamete.

Table 4.1. Genotypic information on ploidy level and number of Oriental, Asiatic and recombinant chromosomes of a BC_1 population originated through crossing of *2n* gametes producing OA hybrids with three parental diploid Asiatic cultivars and two 4x–OA genotypes.

Genotype *	Parents		Cross	Ploidy	Genome composition		Number of recombinant chromosomes
	Female	Male			O ($^{O}/_{A}$)	A ($^{A}/_{O}$)	
022215-1	952400-1	'Mont blanc'	OA × A	3x	12	24	0
022217-1	'Gironde'	952400-1	A × OA	3x	12 (1)	24 (1)	2
022217-2	'Gironde'	952400-1	A × OA	3x	12	24	0
022217-3	'Gironde'	952400-1	A × OA	3x	12 (1)	24 (1)	2
022217-4	'Gironde'	952400-1	A × OA	3x	12	24	0
022217-5	'Gironde'	952400-1	A × OA	3x	12	24 (2)	2
022218-1	'Gironde'	952400-1	A × OA	3x	12	24	0
022218-3	'Gironde'	952400-1	A × OA	3x	12	24	0
022218-4	'Gironde'	952400-1	A × OA	3x	12	24 (1)	1
022218-5	'Gironde'	952400-1	A × OA	3x	12	24	0
022218-7	'Gironde'	952400-1	A × OA	3x	12	24	0
022218-10	'Gironde'	952400-1	A × OA	3x	12	24	0
022218-11	'Gironde'	952400-1	A × OA	3x	12	24	0
022219-2	'Mont blanc'	952400-1	A × OA	3x	12	24 (1)	1
022538-1[1,3]	'Amarone'	951502-1	A × OA	3x	12 (4)	24 (3)	7
022538-3[3]	'Amarone'	951502-1	A × OA	3x	12 (4)	24 (2)	6
022538-5	'Amarone'	951502-1	A × OA	3x	12 (3)	24 (2)	5
022538-7[1,3]	'Amarone'	951502-1	A × OA	3x	13 (3)	23 (3)	6**
022538-8 [3]	'Amarone'	951502-1	A × OA	3x	12 (2)	24 (2)	4
022538-9 [3]	'Amarone'	951502-1	A × OA	3x	13 (2)	23(2)	4**
022538-14	'Amarone'	951502-1	A × OA	3x	12 (3)	24 (2)	5
022538-15[1,4]	'Amarone'	951502-1	A × OA	3x	11 (1)	25 (3)	4**
022544-2	991110	951502-1	OA × OA	4x	24	24	0
022604-6	'Gironde'	951502-1	A × OA	3x	12	24	0
022604-9	'Gironde'	951502-1	A × OA	3x	12 (1)	24	1
022604-10	'Gironde'	951502-1	A × OA	3x	12	24	0
022605-1	'Amarone'	951502-1	A × OA	3x	12 (1)	24 (1)	2
022605-3[2,4]	'Amarone'	951502-1	A × OA	4x	12 (4)	36 (3)	7
022605-7	'Amarone'	951502-1	A × OA	3x	12 (3)	24(3)	6
022605-8[4]	'Amarone'	951502-1	A × OA	3x	12 (2)	24 (1)	3
022605-9[2,4]	'Amarone'	951502-1	A × OA	3x	12 (4)	24 (3)	7**
022605-13	'Amarone'	951502-1	A × OA	4x	11 (1)	37 (1)	2**
022605-15	'Amarone'	951502-1	A × OA	4x	12	36	0
022605-38	'Amarone'	951502-1	A × OA	3x	12 (1)	24 (1)	2
022605-40[4]	'Amarone'	951502-1	A × OA	3x	12 (2)	24 (2)	4
022605-42	'Amarone'	951502-1	A × OA	3x	12 (1)	24 (3)	4
022611-4	'Gironde'	951502-1	A × OA	3x	12 (1)	24 (3)	4
022631	991108	951502-1	OA × OA	6x	36 (1)	36 (2)	3

* The superscripts [1,2,3,4] refer to the number(s) of figure(s) where more information is presented

** Genomic composition originated through the functioning of IMR gametes

A closer look, however, revealed that the chromosomes/centromeres of 4 and 6 of O and A genomes, respectively, were not transmitted by the *2n* gamete to the progenies. These four anomalous chromosome constitutions could be explained as due to the occurrence of *2n* gametes through IMR.

Substitution of chromosome segments

One notable fact that emerged from the analysis of the BC_1 plants with recombinant chromosomes was the occurrence of substitutions for some of the segments of chromosomes. Assuming that a crossing over occurred between two non-sister chromatids in a bivalent in the OA hybrid, the four chromosomes that can result may be indicated as O, A, $^{O}/_{A}$ and $^{A}/_{O}$, the latter two being recombinant chromosomes where O represents the centromere of the O genome and A the recombinant segment of Asiatic chromosome and vice versa. In the majority of BC_1 plants the recombinant chromosomes had single crossovers but there were also instances of two strand double, three strand double and four strand double crossovers (see later). In the event of a single crossover, FDR being the mechanism, the following three situations with regard to the recombinant chromosomes were observed: $^{O}/_{A}$, $^{A}/_{O}$; O, $^{A}/_{O}$ and A, $^{O}/_{A}$. The first of these three situations consisted of both the reciprocal products of a crossover (marked as RP in Figures. 4.3 and 4.4) and the latter two consisted of non-reciprocal products of a crossover (marked as NRP in Figures 4.3 and 4.4). Among these, the A, $^{O}/_{A}$ combination of chromosomes produced substitutions for the distal recombinant segment of the $^{O}/_{A}$ chromosome. Such substitutions were observed in eight of 10 plants that were analyzed (Figures 4.3 and 4.4 asterisks below). In two cases, despite the presence of recombinant chromosomes, either both reciprocal products were present or the recombinant chromosome was $^{A}/_{O}$ (e.g., chromosome 3 of 022538-7).

Besides single crossovers, there were cases in which the recombinant segments were interstitial, mostly resulting from two strand double type of crossovers (Figures 4.3, 4.4, chromosome 5 of 022538-3; chromosome 9 of 022538-9; chromosome 7 and 11 of 022605-3; and chromosome 12 of 022605-9). Out of these five cases, there were substitutions in three cases that possessed $^{O}/_{A}$ recombinant chromosomes but not the other ones. There were also instances of multiple crossovers (e.g. chromosome 8 and 9 of 022538-8; chromosome 11 of 022538-9) in which there were substitutions in two cases (asterisks in Figures 4.3 and 4.4).

In addition to FDR mechanisms where all univalents or half bivalents divide equationally, we observed four cases of IMR derived BC_1 plants (022538-7, 022538-9, 022538-15 and 022605-9) in which *2n* gametes had contributed sister chromosome/centromere (marked as SC in Figures 4.3 and 4.4). In some of these cases substitutions had occurred in proximal as well as

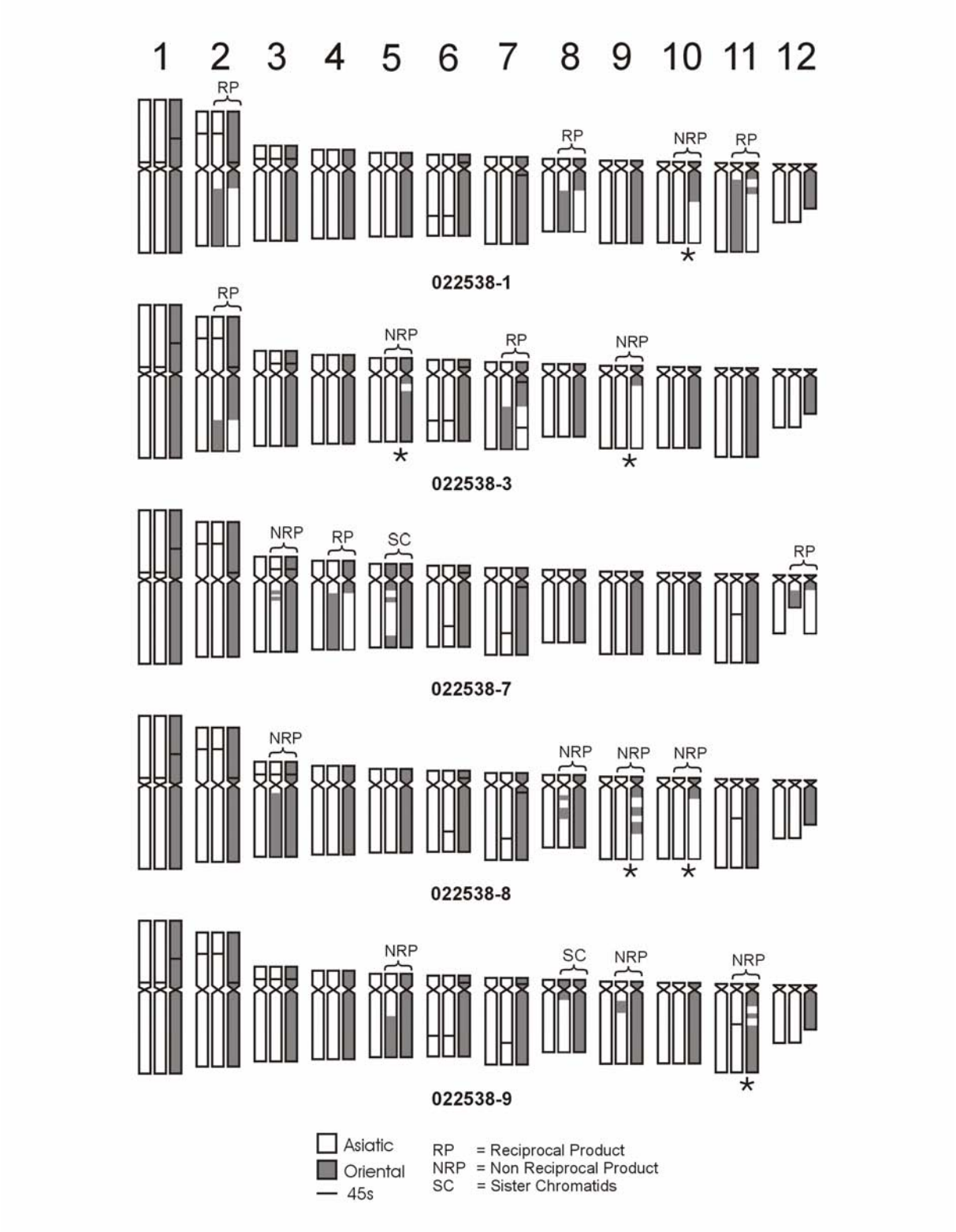

Figure 4.3. Ideograms of 5 BC_1 progeny plants showing recombinant chromosomes and 45S rDNA sites. The reciprocal and non-reciprocal products of recombinant chromosomes are marked as RP and NRP respectively. Sister centromeres are marked as SC in all cases. Substitutions for recombinant segments, when present, are marked with asterisks at the bottom of each group of chromosomes.

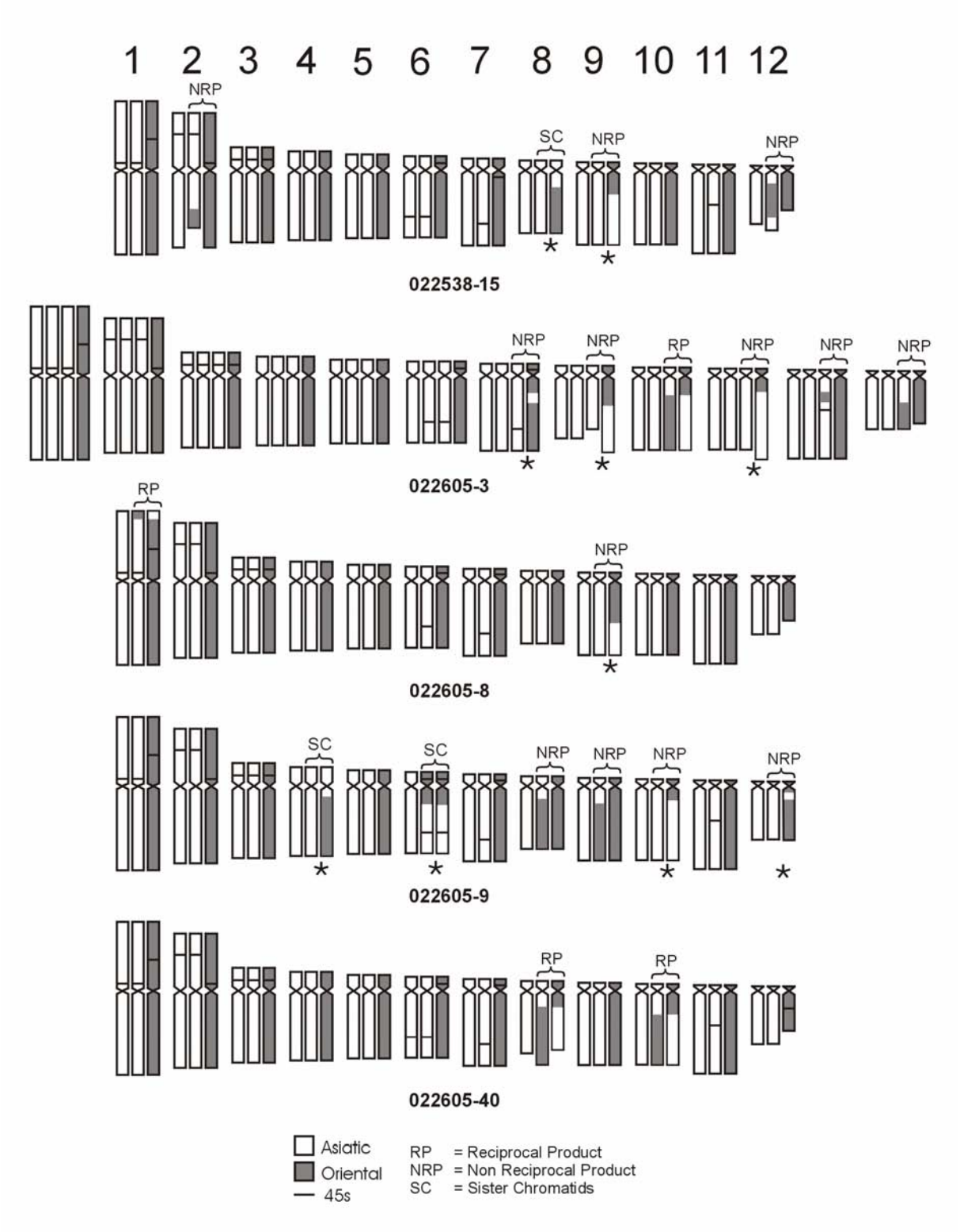

Figure 4.4. Ideograms of 5 BC_1 progeny plants showing recombinant chromosomes and 45S rDNA sites. The reciprocal and non-reciprocal products of recombinant chromosomes are marked as RP and NRP respectively. Sister centromeres are marked as SC in all cases. Substitutions for recombinant segments, when present, are marked with asterisks at the bottom of each group of chromosomes.

the distal regions of the recombinant chromosomes. Examples of proximal substitutions were chromosome 8 in 022538-15; chromosome 4 in 022605-9, whereas in the case of chromosome 6 of 022605-9 it was a distal substitution of the recombinant segment. The latter situation could be explained as due to a four strand double crossing over followed by IMR, where the sister chromatids of half-bivalent were included in the same *2n* gamete.

Discussion

In many respects, OA hybrids used in backcrosses in the present investigation conform to distant species hybrids in numerous other plant taxa. It is difficult to produce F_1 hybrids, they are highly sterile, the chromosomes of alien genomes are highly differentiated, and when allotetraploids are produced they behave as "permanent hybrids". In view of these considerations, when traditional approach of chromosome doubling of OA hybrids is used, the progress could be slow and laborious. The use of *2n* gametes is advantageous for the following reasons: 1) F_1 sterility can be readily overcome, 2) intergenomic recombination can be accomplished and, most importantly, 3) genetic variation can be obtained instantly in the BC_1 generation. All these three aspects will be discussed below.

In OA hybrids F_1 sterility is indeed a great impediment. However, through large scale screening it was possible to select some genotypes of OA hybrids with reasonably high frequencies of *2n* pollen that are fertile (Chapters 2 and 3). This is similar to the success that has already been achieved in the LA hybrids that were used for the production of a large number of backcross progenies (Lim et al., 2003a) and numerous cultivars by the breeders (in preparation). The fertility in OA as well as in LA hybrids has been shown to result mainly from two main mechanisms of *2n* gamete formation, viz., FDR and IMR (Lim et al., 2001; Chapter 2). Cytological analysis in previous studies on LA hybrids and the present investigation have clearly established that both types of *2n* gametes are functional and BC_1 populations can be produced (Chapter 3).

With regard to intergenomic recombination, there is a difference between the two genotypes of OA hybrids, viz., 952400-1 and 951502-1. Whereas only 35.7% of BC_1 plants possessed recombinant chromosomes when 952400-1 was the *2n* gamete donor, 79.1% of the BC_1 progenies possessed recombinant chromosomes when 951502-1 was the parent. This clearly reflects the difference between the two genotypes with regard to the extent of crossing over that occurs previous to the restitution nucleus formation (Chapter 2). In a recent survey of diploid LA hybrids we have observed a clear quantitative difference among genotypes showing complete failure of chromosome pairing to almost normal pairing (in preparation). Because intergenomic recombination can be crucial for generating genetic variation in BC_1

progenies (see later), it might be desirable to screen diverse populations of OA hybrids for high frequency of chromosome pairing and chiasma formation.

An important feature of genetic recombination in OA as well as in LA hybrids is that both homoeologous crossing over as well as chromosome assortment is accomplished. Chromosome assortment is not expected to occur in the case of FDR because the sister chromatids, as a rule, move to opposite poles during equational division of the nucleus. However, in the case of IMR some of the half-bivalents disjoin and the two sister chromatids are included in one of the two restitution nuclei that result form a germ cell. This forms the basis of chromosome assortment (Lim et al., 2001). In the present study there were at least five BC_1 plants that had originated through the functioning of IMR gametes (Table 4.1 asterisks). In order to achieve substitutions for recombinant segments or whole chromosomes, FDR and IMR gametes serve different purposes.

In the case of FDR gametes with crossing over, the non-sister chromatids may consist of reciprocal products ($^{O}/_{A}$; $^{A}/_{O}$), or non-reciprocal products (O, $^{A}/_{O}$ or A, $^{O}/_{A}$). From the point of view of producing substitutions for crossover segments in a backcross, involving A × OA parents, it is always the $^{O}/_{A}$ recombinant that is useful (asterisks in Figures 4.3 and 4.4) but not the $^{A}/_{O}$ recombinant. The substituted segment is invariably the distal one. Had it been a backcross involving O parent, i.e., O × OA cross, only $^{A}/_{O}$ recombinant would have been useful. In the case of IMR, because both the sister-chromosomes/centromeres are included in the *2n* gamete, mostly substitutions occur for the proximal segment.

The importance of substitutions of recombinant segments cannot be overemphasized. This is because the recessive genetic loci in the substituted regions can attain nulliplex condition (aaa). This forms the basis of genetic variation that can be observed in the BC_1 generation itself. With a traditional approach of producing an allotetraploid from a hybrid such as OA, it would be a formidable task to achieve nulliplex genotypes through backcrossing.

Because intergenomic recombination is one of the important attributes of sexual polyploidization, this process appears to have been utilized extensively by horticultural breeders. There are numerous examples in which hybridization between distantly related species has been made and the fertile *2n* gametes are inadvertently used by the breeders to produce polyploid cultivars. Some of the examples are *Narcissus* (Brandham, 1986), *Alstroemeria* (Ramanna, 1992) and several others (reviews, Van Tuyl et al., 2003; Ramanna & Jacobsen, 2003). In this context, the phenomenon observed in lilies, OA hybrids as well as LA hybrids (Lim et al., 2003a; Van Tuyl et al., 2002a), may be consider as repetition of what might have occurred in some of the other horticultural crops.

Furthermore, some of these triploid hybrids have shown to be fertile and have been backcrossed in many directions. From such crosses, progeny was obtained revealing the value for breeding of these triploid hybrids and that introgression of segments of the recombinant chromosomes can be transmitted to further generations (see later in Chapter 5).

Finally, the occurrence of small recombinant segments due to multiple crossover events in OA hybrids auger well for transferring specific horticultural traits into the cultivars. If only large blocks of recombinant segments or whole chromosomes were to be transferred through introgression, many undesirable traits may be added to the cultivars along with the desirable ones (the so-called 'linkage drag'). Fortunately, there appears to be possibilities for avoiding the addition of undesirable traits during the process of introgression.

Chapter 5

Utilization of allotriploid BC_1 progenies of Oriental × Asiatic lilies (*Lilium*) in introgression breeding – an assessment based on GISH analysis

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Abstract

With the aim of utilizing allotriploid (2n=3x=36) lily hybrids (*Lilium*) in introgression breeding, two types of crosses were made. Using diploid Asiatic lilies (2n=2x=24), reciprocal crosses (3x – 2x and 2x – 3x) were made with AOA allotriploids. Secondly, the AOA allotriploids were crossed with allotetraploid (OAOA, 2n = 4x = 48), in 3x – 4x combination. Two types of triploids were used as parents in both types of crosses, derived from: a) mitotic polyploidization and b) sexual polyploidization. Ploidy of 82 progeny plants was determined by estimating nuclear DNA values through flowcytometry and in many cases verified by chromosome counting. The aneuploid progeny plants from 3x – 2x and reciprocal crosses had approximate diploid levels and in 3x – 4x crosses the progeny had approximate tetraploid levels. Balanced euploid gametes (x, 2x and 3x) were formed in the AOA genotypes. Non-recombinant and recombinant chromosomes were found in the progenies of 3x – 2x as well as 3x – 4x crosses through genomic *in situ* hybridization (GISH) analyses. Recombinant chromosomes occurred in the F_1 OA hybrid when the AOA hybrid was derived from sexual polyploidization, but not through mitotic polyploidization. Those recombinant chromosomes were transmitted to the progenies in variable frequencies. Furthermore, in two cases recombination occurred in the triploid hybrids derived from mitotic polyploidization.

Introduction

Oriental and Asiatic lilies *(Lilium*) belong to two different taxonomic sections, viz., Archelirion and Sinomartagon, respectively. The cultivars of each of these two groups consist of diploid (2n=2x=24) hybrids of species within the taxonomic series. In order to produce new cultivars, desirable horticultural characters from the cultivars, or species, from the two groups of lilies is an important goal in breeding. Because of their taxonomic distance, however, it is difficult to hybridize the cultivars between the two different sections. Hybridization can be accomplished through special techniques (Asano, 1978; 1980a; Van Tuyl et al,. 1988; 1991; 2000). However, intersectional interspecific hybrids such as Oriental × Asiatic lilies (OA) are totally sterile due to genome differentiation and failure of chromosome pairing. Such sterile F_1 hybrids can be utilized in breeding only after chromosome doubling through colchicine or oryzalin treatment (Van Tuyl et al., 1992) to produce allotetraploids (2n=4x=48) or through the use of *2n* gametes (Van Tuyl & De Jeu, 1997; Karlov et al., 1999; Lim et al., 2001). Both of these approaches have been used to produce a number of backcross (BC_1) progenies by crossing with Asiatic lilies (AA). One consequence of this approach is that in both cases allotriploid (AOA) BC_1 progenies are produced and these are in general sterile. In order to utilize these triploids in further introgression breeding, a critical assessment was necessary so that they could be used in

breeding. In the past, both auto- and allotriploids have been successfully used in crossing (reviews, Kuspira et al., 1986; Brandham, 1982; Ramsey & Schemske, 2002; Ramanna & Jacobsen, 2003) in various plant species. In lilies, allotriploids of Longiflorum × Asiatic hybrids (ALA) have been successfully used for producing BC_2 progenies (Lim et al., 2003a). A notable feature is that the triploids can be crossed with both diploid and tetraploid parents so that the aneuploid progenies with near–diploids or near– tetraploids to pentaploid can be obtained. In the present investigation we used allotriploid BC_1 plants derived from two approaches: 1) by crossing somatically doubled allotetraploids (OAOA = 4x-OA) to AA parent; 2) by using *2n* gametes from OA hybrids for crossing with AA parents. In both cases, the allotriploids were crossed with diploids, i.e. 3x – 2x (or reciprocal) and 3x – 4x combinations. The progenies from these crosses were analyzed through fluorescent and genomic *in situ* hybridization (FISH and GISH) in order to determine the genome composition of the progenies as well as the extent and transmission of chromosomes with intergenomic recombination. The implications of using allotetraploids for introgression are discussed.

Material and Methods

Plant material used for making F_1 hybrids and production of BC_1 progenies has been described earlier in Chapter 3. A detailed account of the origin of the progenies used in this investigation is given in Figure 5.1. Because the parents involved in making the initial and subsequent crosses were cultivars, which are interspecific hybrids, species names are avoided. All the plant material was grown in greenhouses with standard procedures and utilized for crossing work.

Ovule and embryo rescue

Swollen fruits were collected 40 to 60 days after pollination, surface sterilized by submerging them in 80% ethanol and flamed. The ovules that were successfully fertilized were easily recognizable by their increased size, the embryo-sacs containing embryos were excised from the ovules and placed in enriched media as well as the embryo itself when it could be removed without damage according to Van Tuyl et al. (1991).

Chromosome preparation

Root tips were collected early in the morning, incubated in saturated α-bromonaphthalene solution in ice-water overnight, fixed in the ethanol - acetic acid solution (3:1) for 12 h and stored at –20°C until use. The root tips were incubated in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase Y23, 0.2% (w/v) cellulase RS and 0.2% (w/v) cytohelicase

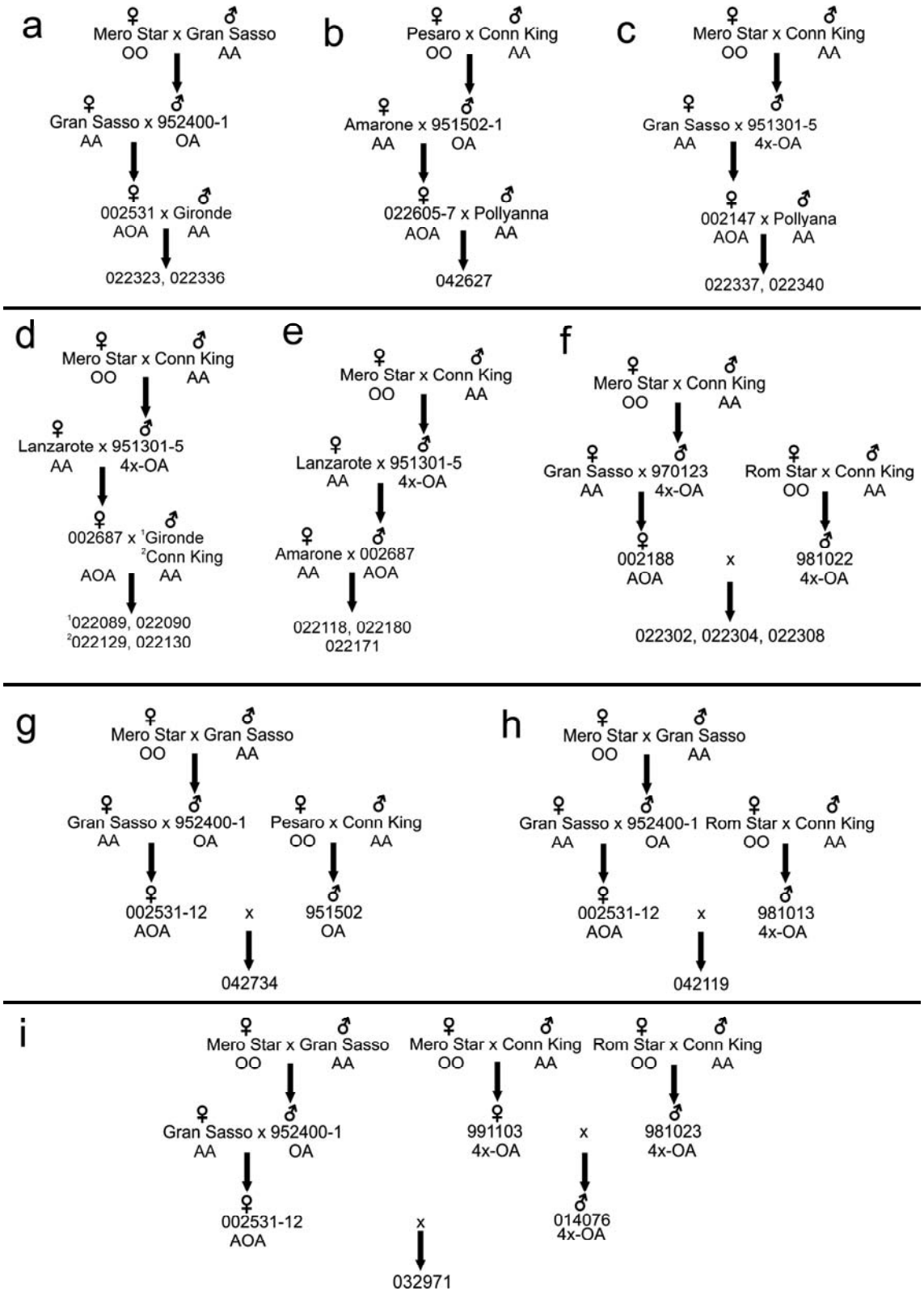


Figure 5.1. Origin of the progenies. (**a-b**) Progeny from 3x – 2x crosses obtained through meiotic polyploidization; (**c-d**) mitotic polyploidization and (**e**) its reciprocal 2x – 3x crosses. (**f**) Progeny from 3x – 4x crosses obtained by mitotic polyploidization; (**g**) meiotic polyploidization and (**h-i**) both, meiotic and mitotic polyploidization. O = Oriental, A = Asiatic, Conn King = Connecticut King, Rom Star = Romero Star, OA= *2n* gamete producer, 4x-OA= Former sterile F_1 hybrid, whose fertility was restored by chromosome doubling, used for mitotic polyploidization.

in 10 m*M* citrate buffer (pH 4.5) at 37°C for about 2 h. Squash preparations were made in a drop of 50% acetic acid and frozen in liquid nitrogen; the cover slips were removed by using a razor blade. Slides were dehydrated in absolute ethanol and air-dried.

DNA probe preparation

For the chromosome identification in 022605-7 and its progeny 45S ribosomal DNA from wheat (9 kb) (Gerlach & Bedbrook, 1979) was used as a probe (see Chapter 4). For the GISH analysis in all the slides, sonicated genomic DNA (1-10 kb) from Oriental cultivar 'Sorbonne' was used as a probe. Both probes were labelled by nick-translation with biotin-16-dUTP according to manufacturer instructions (Roche, Germany).

In situ *hybridization*

Slides were incubated at 37°C in RNase A (100 μg/ml) for 1 h, and incubated in pepsin (5 μg/ml) during 10 min, followed by paraformaldehyde (4%) for 10 min at room temperature, between every step the slides were briefly rinsed in 2x SSC and finally dehydrated with 70%, 90% and absolute ethanol for 3 min in each and air dried. Hybridization followed using a mixture consisting of 20x SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS, 25-50 ng of the probe and 3 μg per slide of autoclaved DNA (100-500 bp) from the Asiatic cultivar 'Connecticut King'. The hybridization mixture was heated at 70°C for 10 min and then placed on ice for at least 10 min. For each slide, 40 μl hybridization mixture was used. The preparations were denatured at 80°C for 10 min and incubated overnight at 37°C in a humid chamber. Slides were washed at room temperature in 2x SSC for 15 min and in 0.1x SSC at 42°C for 30 min. The probe was detected with Cy3 labelled streptavidin (Amersham Biosciences, UK), and amplified with biotinylated goat-antistreptavidin (Vector laboratories, Burlingame, CA). Chromosomes were counterstained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector Laboratories. Burlingame, CA) was added for its examination under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescent illumination, filter sets of DAPI and Cy3. Images were captured as will be explained later.

In the case of the slides of 022605-7 and its progeny, the 45S rDNA probe was removed from the preparations by removing the coverslide and washing the slides in 4x SSC containing 0.2% (v/v) of Tween 20, during 1 h followed by dipping the slides in 40% formamide in 1x SCC at 72°C. The slides were dehydrated in ice-cooled ethanol series, air dried and reprobed with the genomic DNA as described previously.

Selected images were captured by a Photometrics Sensys 1,305 × 1,024 pixel CCD camera and processed with Genus Image Analysis Workstation software (Applied Imaging Corporation). The DAPI images were sharpened with a 7×7 High Gauss spatial filter and pseudo-coloured in blue. The probe fluorescence was red pseudo-coloured. Optimal brightness and contrast were achieved with Adobe Photoshop image processing.

Flow cytometry

Leaves from BC_1 plants were collected in order to determine DNA values which reflected the ploidy levels as described by Van Tuyl & Boon (1997). As internal standard, *Alstroemeria* was used for calibration.

Results

As a result of extensive crossing (Figure 5.1) followed by *in vitro* ovule and embryo culture, a number of progenies were obtained in 3x – 2x (reciprocal) and 3x – 4x crosses. Whereas the reciprocal crosses were successful in the case of 3x – 2x combinations, this was not the case for 3x – 4x reciprocal combinations. Because the allotriploid AOA plants were expected to produce a range of aneuploid gametes (x+1 to 3x-1) as well as euploid gametes (x, 2x and 3x), the ploidy levels were monitored by measuring DNA values through flowcytometry in different types of progenies (Table 5.1). In order to verify whether the DNA values obtained by flowcytometry reflected the chromosome numbers reliably, root tip chromosome numbers were also counted in a number of cases (Table 5.1). Despite some discrepancies in a few cases, the ploidy levels of the progenies were generally concurrent with the actual chromosome counts.

Ploidy levels of the progenies

A total of 82 progeny plants were examined for their ploidy levels through flowcytometry. These included 46 plants derived from 3x – 2x crosses, 10 from 2x – 3x crosses and 24 from 3x – 4x crosses (Table 5.1). From these data the following conclusions were made: 1. the progenies of 3x – 2x, and reciprocal crosses, consisted predominantly of plants with circa diploid chromosome numbers (range 24-32); 2. Progenies of 3x – 4x crosses contained largely circa tetraploid to circa pentaploid chromosome numbers (range, 38-62). Although almost all plants were aneuploids, there were a few cases of euploids (2n = 2x = 24 in 022118-7; 022180-2, 2n = 4x = 48 in 022302-3 and 2n = 5x = 60 in 042734-1). Because the actual chromosome counts were not made in all plants, these four instances of euploids are probably an underestimate.

Table 5.1. DNA content, ploidy level and chromosome number of aneuploid progeny plants derived from 3x-2x and 3x-4x crosses.

Cross	Genotype	Parents		DNA content	Ploidy C-level	Chromosome Number *
		Female	Male			
3*x* (MP) – **2x**						
AOA × AA	022089-1	002687-12	'Gironde'	135.70	2.8	34 ^{e}
AOA × AA	022089-2	002687-12	'Gironde'	104.06	2.2	26 ^{e}
AOA × AA	022089-3	002687-12	'Gironde'	129.75	2.7	32 ^{e}
AOA × AA	022089-4	002687-12	'Gironde'	122.45	2.6	31 ^{e}
AOA × AA	022089-5	002687-12	'Gironde'	109.75	2.3	27 ^{e}
AOA × AA	022089-6	002687-12	'Gironde'	105.63	2.2	26 ^{e}
AOA × AA	022089-9	002687-12	'Gironde'	131.86	2.8	33 ^{e}
AOA × AA	022090-1	002687-13	'Gironde'	125.52	2.6	31 ^{e}
AOA × AA	022090-2	002687-13	'Gironde'	116.17	2.4	29 ^{e}
AOA × AA	022090-3	002687-13	'Gironde'	112.89	2.4	28 ^{e}
AOA × AA	022090-4	002687-13	'Gironde'	119.38	2.5	30 ^{e}
AOA × AA	022090-5	002687-13	'Gironde'	105.84	2.2	26 ^{e}
AOA × AA	022090-6	002687-13	'Gironde'	108.00	2.3	27 ^{e}
AOA × AA	022090-8	002687-13	'Gironde'	107.86	2.3	27 ^{e}
AOA × AA	022090-9	002687-13	'Gironde'	99.49	2.1	25 ^{e}
AOA × AA	022090-10	002687-13	'Gironde'	118.26	2.5	30 ^{e}
AOA × AA	022129-1	002687-29	'Conn King'	121.19	2.5	30 ^{e}
AOA × AA	022129-2	002687-29	'Conn King'	124.08	2.6	31 ^{e}
AOA × AA	022129-3	002687-29	'Conn King'	127.26	2.7	32 ^{e}
AOA × AA	022130-1	002687-36	'Conn King'	124.75	2.6	31 ^{e}
AOA × AA	022130-3	002687-36	'Conn King'	109.82	2.3	27 ^{e}
AOA × AA	022130-6	002687-36	'Conn King'	129.70	2.7	32 ^{e}
AOA × AA	022130-7	002687-36	'Conn King'	123.49	2.6	31 ^{e}
AOA × AA	022337-1	002147-12	'Pollyanna'	107.79	2.2	27 ^{e}
AOA × AA	022337-2	002147-12	'Pollyanna'	110.45	2.5	28 ^{e}
AOA × AA	022337-3	002147-12	'Pollyanna'	139.68	2.9	35 ^{e}
AOA × AA	022337-4	002147-12	'Pollyanna'	111.63	2.3	28 ^{e}
AOA × AA	022337-5	002147-12	'Pollyanna'	116.79	2.5	29 ^{e}
AOA × AA	022340-1	002147-16	'Pollyanna'	122.03	2.6	30 ^{e}
AOA × AA	022340-2	002147-16	'Pollyanna'	111.77	2.3	28 ^{e}
AOA × AA	022340-3	002147-16	'Pollyanna'	120.28	2.5	30 ^{e}
3*x* (*2n*) – **2x**						
AOA × AA	022323-1	002531-3	'Gironde'	108.60	3.3	27 ^{e}
AOA × AA	022336-1	002531-12	'Gironde'	108.35	2.3	27 ^{e}
AOA × AA	022336-2	002531-12	'Gironde'	122.17	2.6	30 ^{e}
AOA × AA	042627-1	042605-7	'Pollyanna'	103.78	2.1	26 ^{e} /25 ^{o}
AOA × AA	042627-2	042605-7	'Pollyanna'	138.10	2.8	34 ^{e} /32 ^{o}
AOA × AA	042627-3	042605-7	'Pollyanna'	120.35	2.4	30 ^{e} /31 ^{o}
AOA × AA	042627-4	042605-7	'Pollyanna'	129.98	2.6	32 ^{e} /30 ^{o}
AOA × AA	042627-5	042605-7	'Pollyanna'	114.67	2.3	29 ^{e} /29 ^{o}
AOA × AA	042627-6	042605-7	'Pollyanna'	120.56	2.4	30 ^{e} /29 ^{o}
AOA × AA	042627-7	042605-7	'Pollyanna'	127.08	2.5	32 ^{e} /32 ^{o}
AOA × AA	042627-8	042605-7	'Pollyanna'	103.89	2.1	26 ^{e}
AOA × AA	042627-9	042605-7	'Pollyanna'	129.91	2.6	32 ^{e}
AOA × AA	042627-10	042605-7	'Pollyanna'	138.25	2.8	35 ^{e}
AOA × AA	042627-11	042605-7	'Pollyanna'	114.56	2.3	29 ^{e}
AOA × AA	042627-12	042605-7	'Pollyanna'	111.91	2.2	28 ^{e}
2*x* × **3*x*** (MP)						
AA × AOA	022118-1	'Amarone'	022687-8	102.77	2.2	26 ^{e}
AA × AOA	022118-2	'Amarone'	022687-8	99.21	2.1	25 ^{e} /27 ^{o}
AA × AOA	022118-3	'Amarone'	022687-8	98.79	2.1	25 ^{e}
AA × AOA	022118-4	'Amarone'	022687-8	103.68	2.2	26 ^{e} /27 ^{o}
AA × AOA	022118-5	'Amarone'	022687-8	99.42	2.1	25 ^{e}
AA × AOA	022118-6	'Amarone'	022687-8	99.91	2.1	25 ^{e}
AA × AOA	022118-7	'Amarone'	022687-8	99.77	2.1	25 ^{e} /24 ^{o}
AA × AOA	022118-8	'Amarone'	022687-8	102.63	2.2	26/ ^{e} 27 ^{o}
AA × AOA	022118-9	'Amarone'	022687-8	102.98	2.2	26 ^{e}
AA × AOA	022118-10	'Amarone'	022687-8	103.47	2.2	26 ^{e}

* ^{e} = Expected chromosome number, ^{o} = Observed chromosome number
(MP) = Obtained through mitotic polyploidization
(*2n*) = Obtained through *2n* gametes

Table 5.1 cont. DNA content, ploidy level and chromosome number of aneuploid progeny plants derived from 3x-2x and 3x-4x crosses.

Cross	Genotype	Parents		DNA Content	Ploidy C-level	Chromosome Number *
		Female	Male			
3x (MP) × **4x**						
AOA × 4x-OA	022308-1	022188-25	981022	235.75	5.0	59 ^{e}
AOA × 4x-OA	022308-2	022188-25	981022	228.01	4.8	57 ^{e} /49 ^{o}
AOA × 4x-OA	022308-3	022188-25	981022	166.92	3.5	42 ^{e}
AOA × 4x-OA	022308-4	022188-25	981022	233.10	4.9	58 ^{e} /62 ^{o}
AOA × 4x-OA	022308-5	022188-25	981022	174.43	3.5	44 ^{e}
AOA × 4x-OA	022308-6	022188-25	981022	221.03	4.5	55 ^{e}
AOA × 4x-OA	022308-7	022188-25	981022	237.78	4.8	59 ^{e}
AOA × 4x-OA	022308-8	022188-25	981022	167.73	3.4	42 ^{e} / 44 ^{o}
AOA × 4x-OA	022308-9	022188-25	981022	179.24	3.6	45 ^{e} / 44 ^{o}
AOA × 4x-OA	022308-10	022188-25	981022	182.66	3.7	46 ^{e}
3x (*2n*) × **4x**						
AOA × 4x-OA	032971-1	002531-12	014076	178.02	3.6	44 ^{e} / 38 ^{o}
AOA × 4x-OA	032971-2	002531-12	014076	186.29	3.8	46 ^{e} /46 ^{o}
AOA × 4x-OA	032971-3	002531-12	014076	149.59	3.0	37 ^{e} /44 ^{o}
AOA × 4x-OA	032971-5	002531-12	014076	166.68	3.4	42
AOA × 4x-OA	032971-6	002531-12	014076	189.29	3.8	47
AOA × 4x-OA	032971-7	002531-12	014076	154.68	3.1	39
AOA × 4x-OA	032971-8	002531-12	014076	159.56	3.2	40
AOA × 4x-OA	032971-9	002531-12	014076	193.26	3.9	48
AOA × 4x-OA	042119-1	002531-12	981013	147.70	3.0	37
AOA × 4x-OA	042119-2	002531-12	981013	184.54	3.7	46
AOA × 4x-OA	042119-4	002531-12	981013	168.83	3.4	42 ^{e} /42 ^{o}
AOA × 4x-OA	042119-5	002531-12	981013	175.79	3.5	44 ^{e} /45 ^{o}
AOA × 4x-OA	042119-7	002531-12	981013	191.80	3.8	48 ^{e} /49 ^{o}
AOA × 4x-OA	042119-8	002531-12	981013	187.47	3.7	47
3x (*2n*) × **2x****						
AOA × OA	042734-1	002531-12	951502-1	n.a.	n.a.	60 ^{o}
AOA × OA	042734-2	002531-12	951502-1	145.89	2.9	36 ^{e} /38 ^{o}

* ^{e} = Expected chromosome number, ^{o} = Observed chromosome number
** *2n* gamete producer
(MP) = Obtained through mitotic polyploidization
(*2n*) = Obtained through *2n* gametes

Nevertheless, it is an indication that euploid gametes (x, 2x and 3x) are produced by the allotriploid AOA in some cases.

Chromosome constitution and intergenomic recombination

There were two types of AOA plants that were used as parents: 1) those derived through mitotic polyploidization and 2) those which had originated through sexual polyploidization (Figure 5.1). In the case of 2x – 3x crosses (Table 5.2), all the triploid genotypes used as male parents had originated by mitotic polyploidization. Out of six plants, none of them possessed recombinant chromosomes (Table 5.2). On the contrary, all plants that were analyzed from 3x – 2x crosses, in which the female parent was of sexual polyploid origin, possessed at least one recombinant chromosome (Table 5.2). Obviously, this was an indication that sexual polyploidization was the most suitable approach for inducing intergenomic recombination. Another difference between the progenies of 2x – 3x and 3x – 2x crosses was the transmission of the extra chromosomes. Whereas only one out of six progeny plants of 2x – 3x crosses

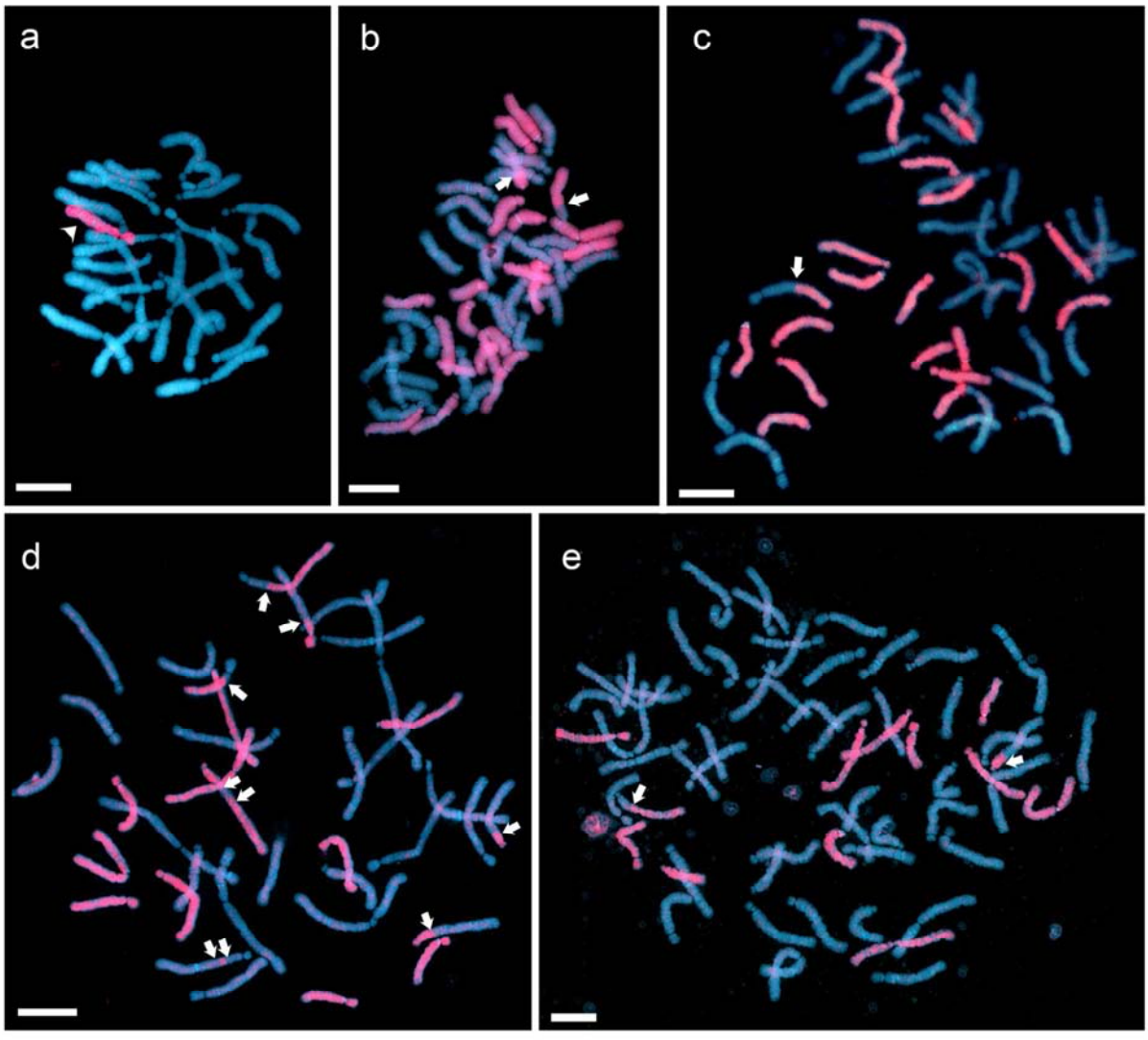
possessed an extra chromosome of the O genome (Figure 5.2a, Table 5.2), all the progenies of 3x – 2x crosses had extra chromosomes originated from this genome. This implied that the extra chromosomes were transmitted to the progeny at a higher frequency when triploids were used as female parents. It must be noticed that the results from Table 5.2 compare a population from the 2x – 3x crosses derived from mitotic polyploidization and the progeny plants analyzed in the 3x – 2x crosses had originated through sexual polyploidization.

Table 5.2. Genomic composition of progeny plants derived from 3x – 2x, 2x – 3x and 3x – 4x crosses. The number of recombinant chromosomes and the possible chromosome contribution of the parental gametes analyzed through GISH.

Cross (Triploid origin)	Genotype	Ploidy Level	Genome composition		Number of recombinant chromosomes	Chromosome contribution of the gametes			
						♀		♂	
			O (O/A)	A (A/O)		O	A	O	A
2x – 3x (MP)									
AA × AOA	022118-2	2x+3	-	27	-		12	-	15
AA × AOA	022118-4	2x+3	-	27	-		12	-	15
AA × AOA	022118-7	2x	-	24	-		12	-	12
AA × AOA	022118-8	2x+3	-	27	-		12	-	15
AA × AOA	022171-1	2x+1	1	24	-		12	1	12
AA × AOA	022180-2	2x	-	24	-		12		12
3x (*2n*) **– *2x***									
AOA × AA	042627-1	2x+1	1	24(1)	1	1	12		12
AOA × AA	042627-2	2x+8	8(1)	24(1)	2	8	12		12
AOA × AA	042627-3	2x+7	7(1)	24(1)	2	7	12		12
AOA × AA	042627-4	2x+6	6(1)	24(1)	2	6	12		12
AOA × AA	042627-5	2x+5	5(1)	24(2)	3	5	12		12
AOA × AA	042627-6	2x+5	5(1)	24(2)	3	5	12		12
AOA × AA	042627-7	2x+8	8(2)	24(2)	4	8	12		12
3x (MP) **– *4x-OA***									
AOA × 4x-OA	022302-3	4x	22	26	-	10	14	12	12
AOA × 4x-OA	022302-4	3x+7	18	25(1)	1	6	13	12	12
AOA × 4x-OA	022302-5	3x+4	16	24	-	4	12	12	12
AOA × 4x-OA	022304-1	3x+3	14	25	-	2	13	12	12
AOA × 4x-OA	022304-2	3x+7	19	24(1)	1	7	12	12	12
AOA × 4x-OA	022304-3	3x+7	18	25	-	6	13	12	12
AOA × 4x-OA	022304-4	3x+3	15	24	-	3	12	12	12
AOA × 4x-OA	022308-2	4x+1	20	29	-	8	17	12	12
AOA × 4x-OA	022308-4	5x+2	20	42	-	8	30	12	12
AOA × 4x-OA	022308-9	3x+8	19	25	-	7	13	12	12
3x (*2n*) **– *4x-OA***									
AOA × 4x-OA	032971-1	3x+2	14(1)	24	1	2	12	12	12
AOA × 4x-OA	032971-2	3x+10	22(1)	24	1	10	12	12	12
AOA × 4x-OA	032971-3	3x+8	20	24	-	8	12	12	12
AOA × 4x-OA	042119-4	3x+6	18	24	-	6	12	12	12
AOA × 4x-OA	042119-5	3x+9	19(1)	26(1)	2	7	14	12	12
AOA × 4x-OA	042119-7	4x+1	23(1)	26	1	11	14	12	12
3x (*2n*) **– *2x****									
AOA × OA	042734-1	5x	12(1)	48(1)	2	-	36	12	12
AOA × OA	042734-2	3x+2	14(4)	24(3)	7	2	12	12	12

* *2n* gamete producer
(MP) = Obtained through mitotic polyploidization, (*2n*) = Obtained through *2n* gametes

In the same way, it must be noticed that these results are somewhat exaggerated because of the small number of plants that were analyzed in both cases. But from flowcytometric data (Table 5.1) it was clear that the range of expected chromosome numbers in the aneuploid progenies of 3x – 2x crosses were higher (range 25-35) than in the progenies of 2x – 3x crosses (range 25-26). In the case of the progenies of 3x – 4x crosses, the chromosome numbers varied from 3x+2 to 5x+2 among which near tetraploids were predominant. Because the male parent in most cases was a typical allotetraploid, mostly a balanced chromosome number of 2x = 24 (12 O + 12 A) was contributed and near diploid gametes were functional from the female parent (Table 5.2). These data, based on actual chromosome counting, corresponded with the flowcytometric data (Table 5.1). Taking into consideration, however, the sample size is small and nearly 12 out of 18 progeny plants of 3x – 4x crosses had 43 – 60 chromosomes, it is evident that embryos with near tetraploid and higher constitution survived in these crosses. As for the number of recombinant chromosomes concerned, there were only two progenies with a single recombinant chromosome when allotriploids had originated through mitotic polyploidization (022302-4, and 022304-2) (Figure 5.2c).



On the other hand, when the AOA was of sexual polyploid origin (032971-1,-2; 042119-5,-7), four out of six plants possessed recombinant chromosomes (Table 5.2) (Figure 5.2b). In two cases, where AOA was crossed with *2n* gamete producing OA (042734-1,-2) (Figures 5.2d-e), both progenies possessed recombinant chromosomes. The highest number of recombinant chromosomes (7) was found in the genotype 042734-2 (Figure 5.2d), which was the product of sexual polyploidization and, probably, both parents had contributed recombinant chromosomes.

Transmission of recombinant chromosomes

As expected, there were two types of recombinant chromosomes, viz., the centromere of A genome with recombinant segment of O genome ($^{A}/_{O}$) and vice-versa ($^{O}/_{A}$). With a view to determine the possible transmission of both types of recombinant chromosomes from a triploid parent to its progenies, the chromosome constitution of the parent 022605-7 and seven of its progeny plants (042627-1-7) were analyzed through FISH each chromosome was identified considering the position of the 45S rDNA signal and by length measurements (data not included). Furthermore, through GISH the chromosomes from O and A genome were identified. There were six recombinant chromosomes in 022605-7 of which three were $^{A}/_{O}$ and three were $^{O}/_{A}$ (Figure 5.3). From the seven progeny plants that were analyzed, it was evident that all the recombinant chromosomes were transmitted to the progenies, albeit in different frequencies. Among the recombinant chromosomes, chromosome 12 $^{O}/_{A}$ was present in all but one of the progenies (Figure 5.3, b-h). The others followed in the order of: chromosome 3 $^{A}/_{O}$ (4); chromosome 10 $^{A}/_{O}$ (3); chromosome 9 $^{O}/_{A}$ (2); chromosome 8 $^{O}/_{A}$ (1) and chromosome 12 $^{A}/_{O}$ (1).

Figure 5.2. Chromosome detection of intergenomic recombination and chromosome constitution in five BC_2 progenies. In all cases, the biotin-labeled Oriental (O) DNA was detected with the Cy3-streptavidin system (pink fluorescence) and the Asiatic (A) DNA was counter-stained with DAPI (blue fluorescence). (**a**) The near-diploid (2x+1) complement of 022171-1, showing 24 A + 1 O (arrowhead) chromosomes. (**b**) The near-tetraploid (3x+9) complement of 042119-5, showing 26 A + 19 O and two recombinant chromosomes (arrows). (**c**) The near-tetraploid (3x+7) complement of 022304-2, showing 24 A + 19 O and a recombinant chromosome (arrow). (**d**) The near-triploid (3x+2) complement of 042734-2, showing 24 A + 14 O with seven recombinant chromosomes (arrows). (**e**) The pentaploid (5x) complement of 042734-1, showing 48 A + 12 O with two recombinant chromosomes (arrows). *Bar represents 10 μm*

Discussion

Generally, allotriploids cannot be used easily as parents. This investigation shows that, with some effort, allotriploid AOA genotypes can be used as parents to produce a considerable number of progenies.

This confirms the results of an earlier investigation, using allotriploids of Longiflorum × Asiatic lilies (ALA), in which a number of BC_2 progenies were produced (Lim et al., 2003a). Furthermore, it also confirms the earlier observation that there is a difference in the ploidy levels of the progenies of 3x – 2x, and reciprocals, as compared with 3x – 4x crosses. Such differences in ploidy levels in the progenies of 2x – 3x and 3x – 4x crosses have been reported in certain autopolyploid crops (Brandham, 1982). Different types of progenies obtained from AOA hybrids might be useful in lily breeding for the following reason. Although diploid cultivars of lilies have been cultivated for centuries, the polyploid cultivars have appeared recently. There appears to be no assessment of the optimum threshold and selection has been made without considering the optimum ploidy levels for cultivars.

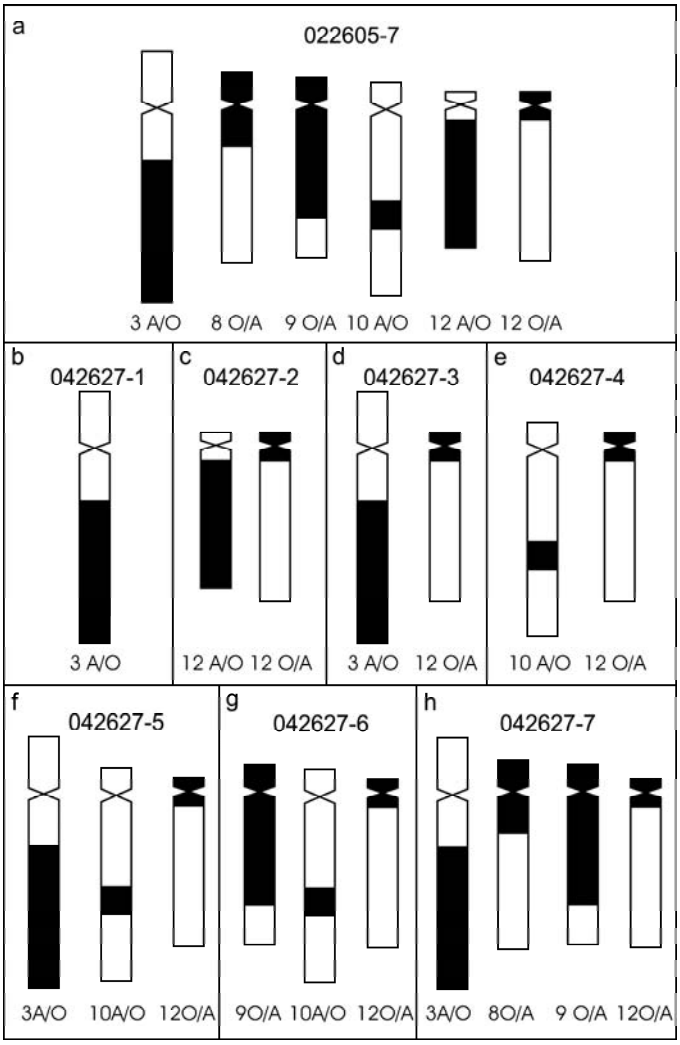


Figure 5.3. Diagrammatic representation of the six recombinant chromosomes in the BC_1 allotriploid AOA (**a**) and its segregation to seven progeny plants (**b-h**). The solid (black) parts of recombinant chromosomes represent the O genome chromatin, while the empty (white) ones – the A genome chromatin.

For example, in *Narcissus* tetraploid level has been proven to be most ideal and this has been achieved during the last century through unconscious selection (Brandham, 1986; Brandham & West, 1993; Brandham et al., 1995). By comparing DNA values of some of the horticultural plants such as *Narcissus*, *Tulipa*, *Hyacinthus*, among others, these authors have argued that DNA values from 100 pg to 120 pg are the most ideal in these cases. If these were to be true, *Lilium* species which 1C have 35 – 36 pg of DNA (Bennett & Smith, 1976; 1991), triploid levels appear to be the optimal because the approximate threshold of 120pg is attained in triploids. Some of the cultivars involving Longiflorum × Asiatic lilies that we have investigated have shown both triploid and tetraploid forms (unpublished results). It remains to be tested if triploid cultivars can predominate over tetraploid forms.

In most of the aneuploids (Table 5.2) there were variable numbers of O genome chromosomes. This was expected in view of univalent formation in AOA parents where A genomes paired regularly and univalents from O genome were distributed irregularly and segregated randomly into the gametes. There was a special case in the 3x – 4x cross 042734-1, where the female parent contributed with 36 A chromosomes (Table 5.2, Figure 5.2e), eliminating completely the chromosomes of the O genome. A clear explanation for this anomaly is not possible at this stage.

The occurrence of both recombinant and non-recombinant chromosomes can be useful for creating monosomic additions (022171-1, Figure 5.2a) or eventually disomic addition series. On the other hand, the presence of recombinant chromosomes can open the possibilities for obtaining substitution lines either for whole chromosomes or parts of them. This can be achieved in the near diploid progenies obtained from the 3x – 2x crosses that might produce haploid gametes, eliminating the rest of Oriental chromosomes. So the progeny from allotriploids derived from *2n* gametes, with recombinant chromosomes (042627) is of special importance because it might retain the segments of Oriental chromosomes in a haploid gamete.

Identification of different recombinant chromosomes transmitted to the progenies (Figure 5.3) demonstrates the usefulness of *in situ* hybridization technique in introgression breeding. In the absence of these techniques, accurate detection of introgressed chromosomes or alien segments would be impossible. It should be noted, however, that the introgression has been detected cytologically in lilies (Lim et al., 2000a; Van Tuyl et al., 2002a; Karlov et al., 1999) but the phenotypic expression has not been studied critically. However, with the use of appropriate analyses, and paying attention to well defined characters it might be possible to detect introgression phenotypically as has been done in the case of *Lycompersicon esculentum* × *Solanum lycopersicoides* and *L. esculentum* × *S. sitiens* (Pertuzé et al., 2003).

Chapter 6

Nitrous oxide (N_2O) induces *2n* gametes in sterile F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids and leads to intergenomic recombination

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Abstract

Eight different genotypes of the F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids (2n=2x=24) were treated with nitrous oxide (N_2O) gas under pressure for 24 and 48 hours. At the time of treatment, all plants possessed early meiotic stages in the anthers of the oldest flower buds. The mature flowers from treated plants were monitored for fertility through pollen germination *in vitro* as well as by using them in crosses with diploid Asiatic hybrids (2n=2x=24) both as male and female parents. In five out of the eight genotypes of OA hybrids there was evidence for the production of *2n* pollen which germinated *in vitro* from either one or both treatments. The *2n* pollen from three genotypes was successfully used in crosses. In two cases, the treated plants were successfully used as female parents which indicated the formation of *2n* (or 2x) egg cells. From an analysis of 41 sexual polyploid progenies obtained from N_2O treated plants it was shown that they were all euploids consisting of 34 triploids (2n=3x=36) and seven tetraploids (2n=4x=48). A detailed cytological analysis of 12 progeny plants through genomic *in situ* hybridization (GISH) proved that N_2O had induced first division restitution gametes in most cases and in two cases they produced gametes through indeterminate meiotic restitution. There was evidence for intergenomic recombination in three cases.

Introduction

In recent years, the use of numerically unreduced (*2n*) gametes has received considerable attention in breeding both auto- and allopolyploid crops (Mariani & Tavoletti 1992; Bretagnolle & Thompson, 1995; Ramanna & Jacobsen, 2003). For the synthesis of allopolyploids especially, *2n* gametes can be useful for the following reasons:

1. Sterile F_1 hybrids can be readily used without chromosome doubling if they produce *2n* gametes.
2. Interspecific recombination can be accomplished because the homoeologous chromosomes are “forced” to pair in diploid interspecific hybrids, e.g. *Lilium* (Karlov et al., 1999; Lim et al., 2003a) and *Alstroemeria* (Kamstra et al., 1999a).
3. Sexual polyploids can be more effective for generating genetic variation in the F_2 and BC_1 progenies (Ramanna et al., 2003; Chapter 4).

Despite these advantages, however, there is a limitation for routinely using *2n* gametes in crop breeding. At present, the genotypes that produce *2n* gametes spontaneously are selected through laborious process, and there is hardly any method available for inducing *2n* gametes in desirable genotypes. Although there are reliable methods available for doubling the chromosome numbers in somatic cells (review by Jensen, 1974), hardly any serious attempt

has been made in the past to induce *2n* gametes in plants through chemical agents. Unlike somatic chromosome doubling, where the process of mitosis is disrupted through the so-called "spindle poisons", induction of *2n* gametes requires the modification of meiosis in such a way that restitution nuclei are formed. As a consequence, instead of forming *n* micro- or megaspores, meiotic nuclear restitution should lead to the formation of *2n* spores. This requires the disruption of nuclear as well as cytoplasmic divisions that occur during the process of meiosis.

Of the many chemical agents that are known to affect mitosis, viz., colchicine, oryzalin, vinblastine and nitrous oxide (N_2O), among others, the last of these is unique in one respect. Whereas all other chemicals are used as aqueous solutions, N_2O is used as a gas under pressure. Its ability to act as a "spindle poison" was first introduced by Östergren (1954). A notable advantage of using a gas for treatment is that the toxic effects, if any, can be mitigated by simply removing the tissue from the gas chamber, a process that can not be accomplished when tissues are treated with solutions. For the purpose of somatic chromosome doubling, N_2O has been successfully applied in some plant species such as *Crepis capillaries*; *Phalaris canariensis* (Östergren, 1954; 1957) *Melanrium* (Nygren, 1955); wheat and barley (Tsunewaki, 1962; Dvorak et al., 1973); clover (Giri et al., 1983) and *Psathyrostachys juncea* (Berdahi & Barker, 1991). In many of these cases, in addition to inducing polyploids, N_2O treatment also led to aneuploid production. Cytological explanation for the occurrence of aneuploids has not been provided. In one cytological investigation on the effect of N_2O in *Tradescantia*, Montezuma-De-Carvalho (1973) reported on the inhibition of meiotic spindle at prometaphase – metaphase I stage and gave rise to nuclear restitution. Such cells, on recovery, proceeded to normal second division giving rise to dyads. The consequences of dyad formation were not investigated in this study.

With the aim of inducing *2n* gametes in completely sterile F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids, whole plants with flower buds, possessing early meiotic stages, were treated with N_2O for different durations. The successful result of producing *2n* gametes and sexual polyploids in this experiment are reported and discussed.

Material and methods

Plant material and N_2O treatment

Whole plants with flower buds ranging 0.5 to 1 cm from eight different genotypes of Oriental × Asiatic *Lilium* F_1 hybrids (2n=2x=24) were placed in a gas chamber and treated with N_2O at a pressure of 6 bars during 0h (untreated control), 24h and 48h when sufficient bulbs were available (Table 6.1) as described by Zeilinga & Schouten (1966). With two exceptions,

951502-1 and 952400-1, all others (Table 6.1) were completely sterile when tested during three consecutive seasons. The two exceptions produced low frequencies of *2n* gametes (Chapters 2 and 3). The Asiatic cultivars used were 'Vivaldi' and 'Mont Blanc'. All plants were grown in greenhouses following standard procedures normally used for lily growing.

Fertility

Two criteria were used in order to determine the fertility. a. *In vitro* pollen germination. This was carried out in artificial agar medium containing 100 g sucrose, 5 g bacteriological agar, 20 mg boric acid and 200 mg calcium nitrate per litre cultured during 24 h at 25° at 25°C. b. Embryo formation after crossing N_2O treated plants with fertile genotypes. For this, swollen fruits were collected 40 – 60 days after pollination, submerged in 80% ethanol and flamed for surface sterilization. Swollen ovules generally contained an embryo and when it was possible, the embryo was removed form the ovule and cultured in enriched media (Van Tuyl et al., 1991), otherwise the embryo-sac or the ovule containing the embryo were cultured as such.

In situ *hybridization*

Root tips from the progenies were collected early in the morning and pre-treated in saturated α-bromonaphthalene solution in ice-water overnight; fixed in ethanol acetic acid (3:1) and stored at -20°C until use. The root tips were cultured for about 2h at 37°C in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase Y23, 0.2% (w/v) cellulase RS and 0.2% (w/v) cytohelicase in 10 m*M* citrate buffer (pH 4.5). Squash preparations were made in a drop of 50% acetic acid; frozen in liquid nitrogen to remove the cover-slip with a razor blade and dehydrated in absolute ethanol and air-dried. Slides were culture at 37°C during 1h in RNase A (100 µg/ml); incubated 10 min in pepsin (5 µg/ml), followed by 10 min in paraformaldehyde (4%) at room temperature, between every step the slides were rinsed in 2x SSC. Slides were dehydrated in 70%, 90% and absolute ethanol during 3 min each and air dried.

Hybridization followed using a mixture consisting of 20x SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS, 25-50ng of probe DNA (sonicated genomic DNA (1 – 10 kb) from the Oriental cultivar 'Sorbonne' labelled with Biotin-16-dUTP by nick-translation according to manufacturer instructions (Roche,Germany)) and 3µg per slide of autoclaved DNA (100-500 bp) from the Asiatic cultivar 'Connecticut King'. The hybridization mixture was heated at 70°C for 10 min and then placed on ice for at least 10 min. 40 µl of hybridization mixture were applied to each slide followed by denaturation at 80°C for 10 min and incubation at 37°C overnight in a humid chamber. Slides were washed for 15 min at room

temperature in 2x SSC and 30 min in 0.1x SSC at 42°C. The probe was detected with Cy3 labelled streptavidin (Amersham Biosciences, UK), and amplified with biotinylated goat-antistreptavidin (Vector laboratories, Burlingame, CA). Chromosomes were counterstained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector Laboratories. Brulingame, CA) was added for its examination under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescent illumination, filter sets of DAPI and Cy3. Images were captured by a Photometrics Sensys 1,305 × 1,024 pixel CCD camera, processed with Genus Image Analysis Workstation software (Applied Imaging Corporation) and sharpened with a 7×7 High Gauss spatial filter. DAPI fluorescence was pseudo-coloured in blue and the probe fluorescence in red. Optimal brightness and contrast were achieved with Adobe Photoshop image processing.

Results

Effect of N_2O treatment on fertility

Of the eight different genotypes of OA hybrids that were treated with N_2O for durations of 24h and 48h and untreated controls, all were tested for "fertility" as well as crossability. From our extensive studies on other genotypes of OA hybrids it was established that only when large, well-filled pollen grains are formed in the F_1 hybrids, they represented *2n* pollen which germinated *in vitro* as well as they were functional when used for crossing.

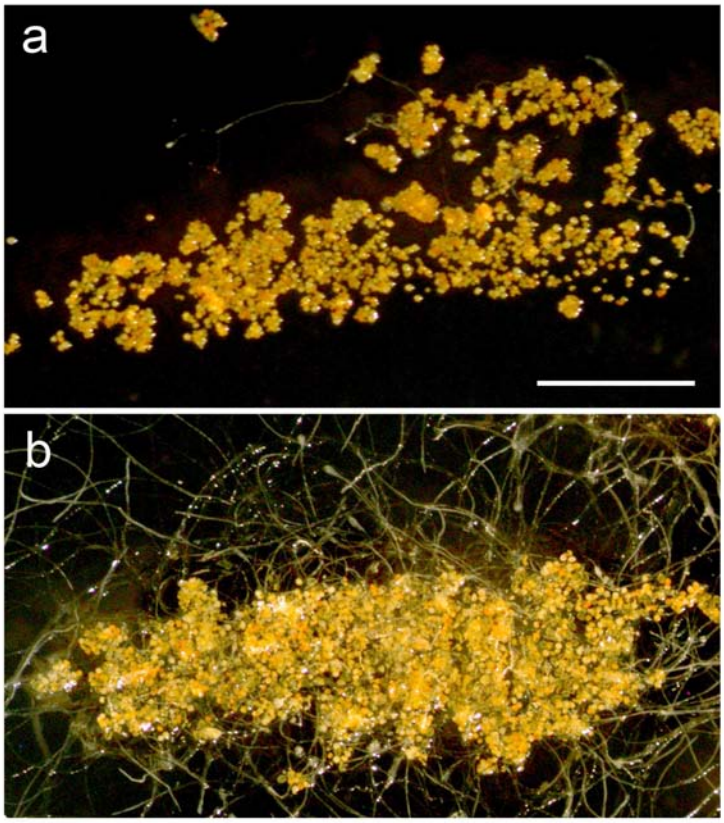

Figure 6.1. Germination of *2n* pollen from the OA hybrid 951502-1. (**a**). Untreated (0 h treatment). (**b**). After 48h N_2O treatment. *Bar represents 0.5 cm.*

In view of this, the pollen grains were monitored after staining and there was evidence for the presence of *2n* pollen in variable frequencies in some genotypes (data not included). However, a more reliable criterion was *in vitro* germination of pollen grains. In all cases, there was very little or no pollen germination in the controls (0h treatment in Table 6.1) whereas considerably high pollen germination in five genotypes that were treated with N_2O (Table 6.1). In order to calculate the frequencies of pollen germination, only the large and well-filled grains were taken into account and the numerous aborted small structures were ignored. Based on these counts, highest germination percentage was observed in 951301-5 at 24h treatment duration. The range was calculated on the basis of pollen germination among flowers within a treatment because there was considerable variation among them. Whereas more than 5% of pollen germination in five genotypes, there was very little or no germination in the other three N_2O treated plants. A dosage effect was observed in N_2O treatment for 24h and 48h when compared to the untreated controls, especially in the case of 951502-1 (Figure 6.1a and b). However, it was not possible to ascertain whether all the treated flower buds had the same or similar cell division stages at the time of treatment.

All plants that showed 5% or more of pollen germination were used as male parent to be crossed with fertile genotypes in order to determine their ability to produce embryos and progenies. Simultaneously, the fertile genotypes were also used as female parents in crosses with diploid Asiatic genotypes. In two genotypes, 951301-5 and 969023-2, embryos were obtained when they were used both as male and female parent. The other genotype, 951502-1, was successful in producing embryos when used as male parent only (Table 6.2). In the case of 952400-1 a low frequency of embryos was formed in the control (untreated) plants. 951502-1 and 952400-1 were previously known to produce a low frequency of *2n* gametes, but in the present case only 951502-1 responded to the N_2O treatments. In view of the high fertility observed in the case of 951301-5, a fairly large number of crosses were made using this genotype in order to utilize the progenies for further analyses.

Table 6.1. Pollen germination percentage of different N_2O treatments

Genotype	N_2O Treatment		
	0h	**24h**	**48h**
951301-5	0	70 (0-95)	-
951502-1	7 (0-30)	15.3 (1-55)	73.75 (55-80)
951914-1	0	0	0
952059-9	0	0	21.3 (15-25)
952400-1	4.5 (0-35)	10.6 (0-30)	36.8 (5-60)
952521-1	1.1 (0-5)	0.5 (0-2)	1.1 (0-4)
962377-1	0	0	-
969023-2	0	5.7 (1-60)	-

Table 6.2. Number of embryos obtained (number of flowers pollinated) from crosses of OA hybrids with three different N_2O treatments (hrs), with Asiatic cultivars

Genotype	Used as	N_2O Treatment		
		0h	24h	48h
951301-5	♀	*	32 (10)	-
	♂	*	176 (19)	-
951502-1	♀	0 (2)	0 (1)	-
	♂	0 (3)	7 (2)	28 (1)
952059-9	♀	-	0(1)	0 (1)
	♂	0 (1)	-	1 (4)
952400-1	♀	3 (2)	0 (1)	0 (1)
	♂	2 (5)	0 (4)	0 (1)
969023-2	♀	0 (3)	1 (10)	-
	♂	0 (2)	28 (6)	-

* Tests during three seasons confirmed complete sterility

Ploidy levels and chromosome constitution of progenies derived from N_2O treatment

A total of 41 plants were analysed for their ploidy levels (Table 6.3). Of these, 29 plants were obtained from crossing N_2O treated 951301-5 as male parent and 12 from using it as female parent. Among the progenies there were 34 triploids (2n=3x=36) and seven tetraploids (2n=4x=48). The notable feature was that all had euploid chromosome complements. The seven tetraploid progeny plants were found only when 'Vivaldi', the Asiatic cultivar, was used as the female parent but not in the reciprocal cross. It was concluded that the tetraploid progenies were the result of the functioning of *2n* eggs of spontaneous origin from 'Vivaldi'. In view of the occurrence of all euploid progeny plants, it was evident that N_2O treated 951301-5 had contributed balanced, 2n=24 chromosomes to the progenies in all cases.

Table 6.3. Ploidy level of progeny obtained from 951301-5 after N_2O treatment.

Parents		Cross	Number of progeny analyzed	Ploidy level of the progenies	
Female	Male			3x	4x
'Vivalidi'	951301-5	AA × OA	29	22	7
951301-5	'Vivaldi'	OA × AA	12	12	0

The occurrence of euploid progenies from N_2O treated genotypes when used as parents implied that the OA hybrid had contributed 12 O + 12 A genome chromosomes to the progenies. In order to verify this assumption, as well as to determine if there was any intergenomic recombination, 12 progeny plants were analysed through GISH (Table 6.4). As was mentioned in Table 6.3, all were euploids and included eight triploids and four tetraploids.

Table 6.4. Genome composition and number of recombinant chromosomes in progenies obtained from the cross of N_2O treated OA hybrid 951301-5 to Asiatic (A) parent.

Genotype	Cross	Ploidy	Genome composition		Number of recombinant chromosomes
			O ($^{O}/_{A}$)	A($^{A}/_{O}$)	
042923-1	AA × OA	3x	10	26(2)	2
042924-1	AA × OA	4x	12(1)	36(1)	2
042924-2	AA × OA	3x	12	24	0
042924-3	AA × OA	4x	12	36	0
042924-4	AA × OA	3x	12	24	0
042924-5	AA × OA	3x	12	24	0
042924-6	AA × OA	4x	13	35	0
042924-7	AA × OA	4x	12	36	0
042924-8	AA × OA	3x	12	24	0
042927-2	OA × AA	3x	12	24	0
042927-4	OA × AA	3x	12	24	0
042928-1	AA × OA	3x	12(1)	24	1

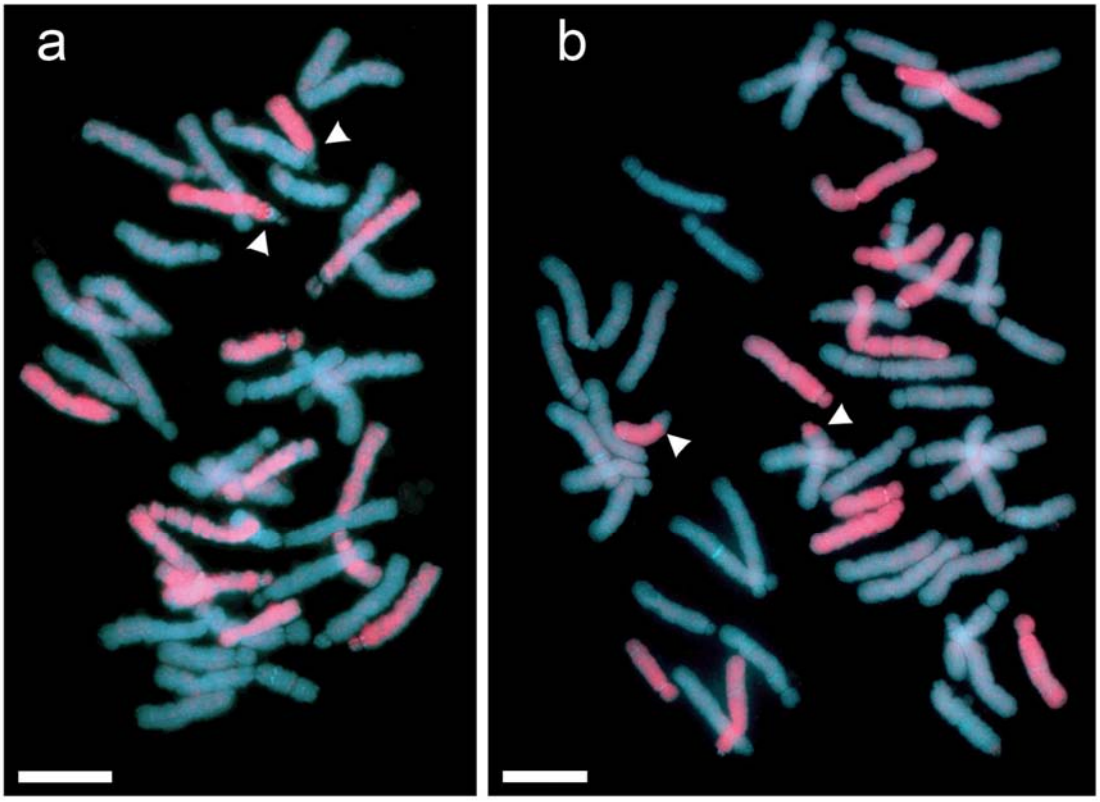

Figure 6.2. Chromosome complements of the BC_1 progenies from the cross of N_2O treated OA hybrid 951301-5 to Asiatic (A) parent showing a triploid (2n=3x=36) and a tetraploid (2n=4x=48) chromosome number. (**a**) Triploid chromosome complement of 042923-1, showing 10 Oriental and 26 Asiatic chromosomes with two recombinants (arrowheads). (**b**) Tetraploid chromosome complement of 042924-1, showing 12 Oriental and 36 Asiatic chromosomes with two recombinants (arrowheads). In both cases the biotin-labeled Oriental DNA was detected with the Cy3-streptavidin system (pink fluorescence) and Asiatic chromosomes were counter stained with DAPI (blue fluorescence). *Bar represents 10 μm.*

With the exception of one triploid (042923-1) and one tetraploid (042924-6), GISH analysis indeed confirmed that N_2O treated OA hybrids had contributed 12 chromosomes each of O and A genomes. This means, N_2O had most likely induced first division restitution gametes. In the case of two exceptional plants, 042923-1 and 042924-6, though euploids, they possessed variable numbers of O and A chromosomes (Table 6.4). In these cases, it was obvious, that indeterminate meiotic restitution had occurred. One important result of GISH was the observation of intergenomic recombination (Figures 6.2a and b) in three of the progeny plants, viz., 042923-1, 042924-1 and 042928-1 (Table 6.4). There was a clear indication that N_2O had induced restitution gametes in these cases but not chromosome doubling in pre-meiotic stages. This was because, premeiotic chromosome doubling would have resulted in tetraploids in which intergenomic recombination was expected to be very rare, if not totally absent.

Discussion

It is evident from this investigation that 'fertility' can be restored in totally sterile interspecific hybrids through N_2O treatments under pressure. Although not all eight genotypes that were treated with N_2O produced positive results, there was *in vitro* germination in five cases (Table 6.1). At least three of these genotypes could be used either as female or male parents in crosses in order to obtain embryos- and finally progenies. Production of a considerable number of embryos (208) in the case of 951301-5 shows that even highly sterile interspecific hybrids can be used in breeding.

The fact that N_2O treated plants can be successfully used both as male and female parents suggests that functional spores occur in micro- and megasporogenesis. The actual mechanisms of the origin of these functional gametes are not clear from this study because it was not cytologically analysed in treated plants. However, the chromosome constitution of the progeny plants (Table 6.4) suggests that the *2n* gametes are predominantly of FDR origin. One of the important requirements for the origin of FDR during microsporogenesis in lilies is that the half-bivalents should divide equationally (as in mitosis) before the cytokinesis and cell wall formation occurs in telophase I stage (Lim et al., 2001). If N_2O treatment is assumed to affect meiotic spindle formation during the first meiotic division, as is shown in *Tradescantia* (Montezuma-De-Carvalho, 1973), this could lead to mitosis like division of the entire chromosome complement in the treated OA hybrid. Dyads and *2n* spores that result from such division will indeed be FDR. The presence of recombinant chromosomes does indicate that they are indeed the result of FDR with crossovers. This implies that N_2O has an effect on pollen mother cells undergoing various meiotic stages. This does not exclude,

however, that N_2O might have also induced premeiotic doubling. For example, there were two progeny plants that were derived from *2n* eggs of N_2O treated OA hybrids (042927-2 and 042927-4), but did not possess recombinant chromosomes. In *Lilium*, meiotic stages in anthers initiate when flower buds reach 0.5 to 0.9 cm (Walters, 1976; 1980; Lord, 1989) and similar stages are present in the embryo sac mother cells (De Boer-De Jeu MJ, 1978). However, due to the difference in size of the flower buds at the time of N_2O treatment also premeiotic division stages could have been affected leading to premeiotic doubling. This possibility could not be answered in this study.

One aspect that deserves to be noted is that not all genotypes that were treated with N_2O responded similarly. One reason could be that not all plants used for the N_2O treatments had the same meiotic stages in their anthers. Alternatively, there might be genotypic differences. If it is only due to differences in meiotic stages, then the plants require a more stringent cytological monitoring before treatment. It has been shown that meiosis in anthers of *Lilium longiflorum* has a duration of 50 days (Taylor & McMaster, 1954). In such cases, it might be possible to determine the optimal stage for N_2O treatments and maximize the chances of inducing fertility. Although not all genotypes treated with N_2O showed fertility, the ones that became fertile showed that the procedure can be useful. This can open the way for inducing *2n* gametes in some of the desirable genotypes of OA hybrids.

Chapter 7

General discussion

Introgression of desirable characters from distantly related species is difficult and laborious. The results reported and discussed in the five experimental chapters (Chapters 2-6) on OA lily hybrids are expected to pave the way for rational approaches for introgression of characters into lily cultivars. In many ways, OA hybrids present many hurdles that may be typically encountered in utilizing distant hybrids in breeding:

1. Parental species are difficult to hybridize – special techniques are needed.
2. Once produced, they are highly sterile.
3. Homoeologous chromosomes do not pair and crossover normally.
4. Production of F_2 or BC progenies is difficult if not impossible unless special efforts are made.

All these difficulties have received attention in the different chapters of this thesis and in this chapter some of the problems are discussed more generally.

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 not be used in the case of OA hybrids because of the reasons mentioned previously. 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. In view of this, the use of *2n* gametes from OA hybrids would be the most logical approach, because in addition to overcoming F_1 sterility, intergenomic recombination can be accomplished. This also implies that, unlike the *Festuca* – *Lolium* hybrids, introgression in OA hybrids can be achieved only at the polyploid level. This presents an important difficulty – the expression of (recessive) characters in allopolyploids can be difficult because of "permanent hybridty". In this context, in the following paragraphs the consequences of inducing polyploids by using *2n* gametes are examined.

Implications of 2n *gametes for genetic variation*

Skiebe (1958) was the first to realize the difference between "natural polyploids", i.e., induced through *2n* gametes and colchicine doubled synthetic polyploids. Slightly before, Storey (1956) illustrated, in some detail, various mechanisms of *2n* gamete formation in orchids. These workers, however, did not point out the genetic consequences of different modes of origin of *2n* gametes. Only during the 1970's the so-called FDR and SDR mechanisms were recognized in potato and other crops (Mendiburu & et al., 1974; Mok & Peloquin, 1975; Ramanna, 1979; Bingham, 1980). These early studies were focused only in breeding autopolyploid crops and FDR gametes were shown to be the most useful for transferring parental heterozygosity to the progeny. This is because, FDR mechanisms of *2n* gamete formation involves the equational division of the entire chromosome complement and gives rise to *2n* gametes that are genotypically similar, or identical to each other as well as to the parent from which they are derived. It was also recognized that SDR gametes originate through chromosome doubling of the products of the first meiotic division and therefore, involve crossing-over and assortment of homologous chromosomes. As a result of this, SDR gametes are considered as genetically heterogeneous populations and can lead to a large genetic variation in the progenies. Therefore, not much attention was paid to SDR gametes.

Unlike in autopolyploids, *2n* gametes are useful for different reasons. In the first place, they are helpful for overcoming F_1 sterility. But more importantly, the use of *2n* gametes can facilitate intergenomic recombination. Although considerable investigations have been conducted on autopolyploids, relatively less attention has been paid to allopolyploids derived through *2n* gametes (Ramanna and Jacobsen, 2003). In a detailed investigation on the origin of *2n* gametes in Longiflorum × Asiatic lily hybrids, in addition to the FDR mechanism, a novel type of *2n* gamete formation was detected (Lim et al, 2001). The novelty of this mechanism is that it involves the features of both FDR and SDR. In the meiocyte of an F_1 hybrid that forms both univalents and bivalents during metaphase I, univalents divide and the sister chromatids move to the opposite poles. At the same time, bivalents disjoin and half-bivalents move to the opposite poles. As a consequence, the restitution nucleus receive non-sister chromatids as well as sister chromatids of half-bivalents. This is called indeterminate meiotic restitution (IMR). Because in lily interspecific hybrids both FDR and IMR occurs, the resulting genetic consequences of using *2n* gametes as well as somatically doubled polyploids are illustrated in figure 7.1.

One of the important pre-requisites for 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 (Figure 7.1b) with no 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 as illustrated in Figure 7.1a. Unlike in the triploids originating from somatically doubled allotetraploids, where it is impossible to obtain homozygosity for any of three loci considered, in the case of FDR and IMR derived triploids it is possible to achieve homozygosity for the recessive loci (Figure 7.1).

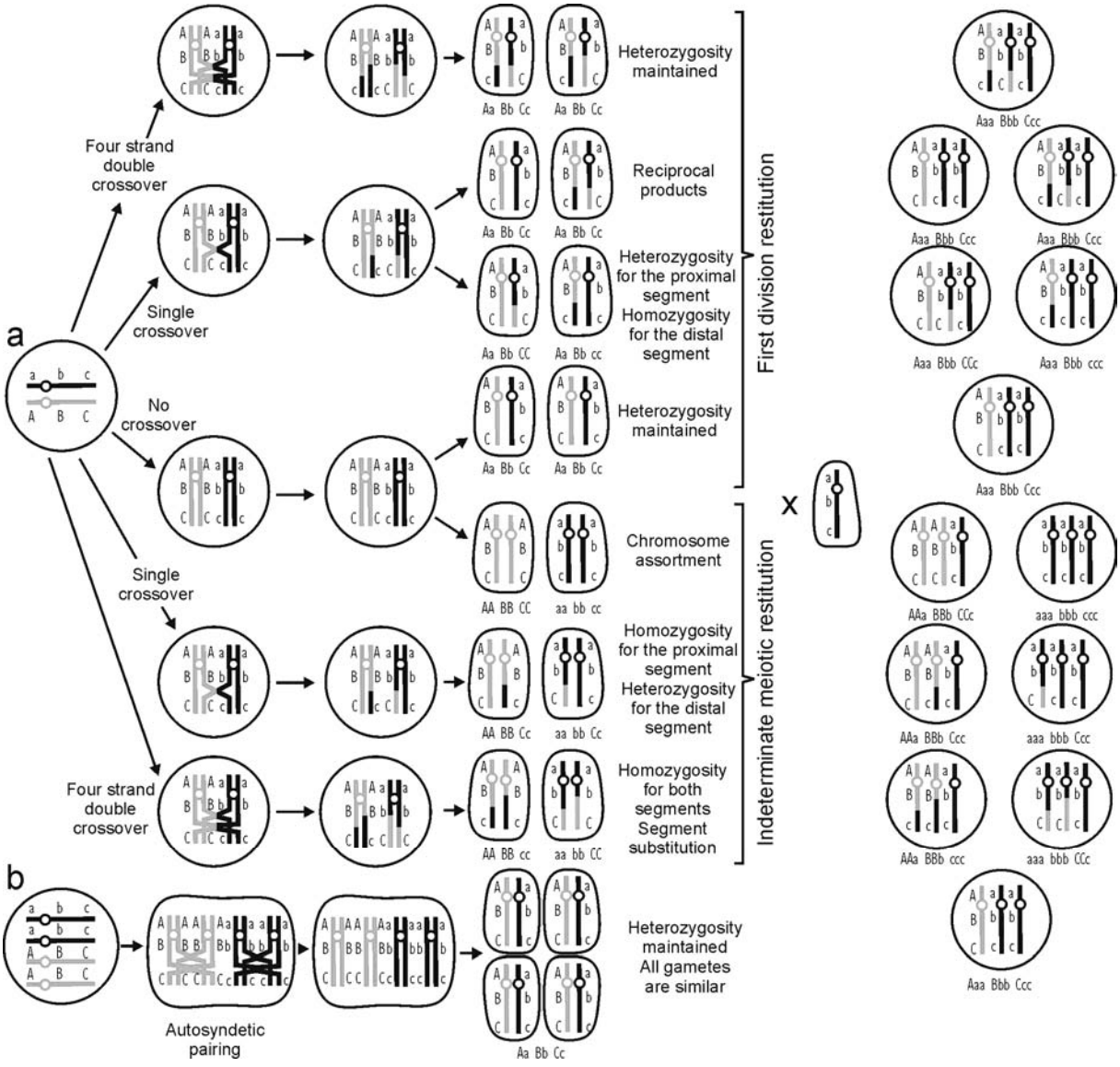

Figure 7.1. Genetic consequences of (**a**) the two meiotic restitution mechanisms found in OA lily hybrids and (**b**) somatically doubled allotetraploid. In both cases their implications for backcrossing to diploid parent are shown.

Relevance of 2n *gametes for producing backcross progenies from OA hybrids*

From the point of view of generating genetic variation in the backcross progenies of OA hybrids, the most logical approach would be to use *2n* gametes. The alternative approaches of using allotetraploid OA hybrid would lead to a situation in which recessive loci will not attain the nulliplex condition unless several backcrosses are made using the appropriate recurrent parents. On the contrary, when *2n* gametes with recombinant chromosomes can facilitate the expression of recessive loci attaining nulliplex condition as has been illustrated in Figure 7.1. This has been vindicated also by GISH analysis of BC_1 progenies in which recombinant segments are substituted both in the proximal and distal positions of chromosomes (Chapter 4). This means that BC_1 populations generated by using *2n* gametes are readily suitable for selection. It is known in the case of Longiflorum × Asiatic lily hybrids, which the breeders have succeeded in selecting cultivars directly from BC_1 populations derived from the use of *2n* gametes (unpublished results).

In spite of all the attractive features of using *2n* gametes in breeding, there is one drawback that can be a limiting factor in some cases. This is the rarity of genotypes that can produce *2n* gametes on a regular basis. Indeed they are so rare, that out of 708 OA F_1 hybrids only 12 genotypes were found to produce *2n* gametes on a regular basis, while the rest were sterile. Besides this, their production is highly subjected to the environmental conditions. Moreover, most of the genotypes produce either only *2n* pollen or *2n* eggs but not both simultaneously. In view of these difficulties, the induction of *2n* gametes through N_2O treatment (Chapter 6) is a positive development for using *2n* gametes in lily breeding. The production of *2n* gametes is not restricted anymore to those selected genotypes that produce them. Any of the more than 700 OA hybrids that were produced for this research and were discarded due to their sterility 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. In the same way, the increase of *2n* gamete production in the genotypes with low frequencies increases the chances of successful fertilization events resulting in more progeny plants per pollination. The presence of *2n* pollen originated through different restitution mechanisms and recombinant chromosomes in the progeny of N_2O treated plants makes evident the advantages of this technique to restore fertility while genetic variation is generated.

Ploidy manipulation and introgression

As was pointed out above, genotypes that produce both *2n* pollen and *2n* eggs simultaneously are rare. This is unlike in some of the other monocots where sterile hybrids or haploids

produce both types of gametes. (review, Ramanna & Jacobsen, 2003). In the absence of such genotypes, bilateral sexual polyploidization in lily interspecific hybrids has been very difficult at present. One advantage of bilateral sexual polyploidization is that fertile 4x progenies with intergenomic recombination can be obtained. Such 4x progenies can be used for generating considerable genetic variation either by selfing or crossing with other suitable genotypes as has been shown in *Alstroemeria* (Ramanna et al., 2003). Because bilateral sexual polyploidization is not yet possible in lily, the other alternative is to use unilateral sexual polyploidization as has been done in the present study (Chapters 2 and 3) where *2n* pollen producing male parent is backcrossed to one of the parental species. The obvious result of this procedure is the production of triploid BC_1 progenies. As is the case in most angiosperms, the allotriploid lilies are sterile, and generally cannot be used as parents. But some genotypes do produce fertile gametes, both male and female, consisting of predominantly aneuploid and to a low frequency euploid, x, 2x and 3x gametes (Chapter 5). The considerable number of BC_2 progenies obtained in this investigation has opened the prospects for further backcrossing. It should be pointed out, however, that the chromosomes of O genome (Oriental species) have a tendency to be eliminated in the aneuploids. However, those chromosomes that possess recombinant segments (O/A or A/O) tend to persist and are expected to be integrated into the chromosome complements of the BC progenies. There is evidence that recombinant chromosomes are transmitted regularly to the BC progenies (Figure 5.3, Chapter 5). In view of this, introgression of desirable segments can be accomplished in lilies, through hybridization of distant species and backcrossing. This is possible because *2n* gametes can be used in breeding and techniques of *in situ* hybridization facilitate the monitoring of alien chromosomes and recombinant segments in every step. These results are likely to pave the way for rational approaches for breeding new cultivars, such as molecular assisted breeding, where with the combination of molecular markers and FISH techniques it is possible to localize markers for specific genes in the chromosomes and trace them in the progeny of subsequent crosses.

In summary, *2n* gametes help to overcome F_1 hybrid sterility, facilitates intergenomic recombination leading to genetic variation and generate polyploids with introgressed chromosome segments. *2n* gametes are not restricted anymore to a small proportion of F_1 hybrids because they can be induced through N_2O treatments. Finally the triploid progenies can be readily used for further crosses and the recombinant chromosomes will be transmitted to the progeny assuring introgression of the alien chromosomes or chromosome segments.

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Summary

Besides being an important horticultural crop, lily (*Lilium*) also serves as an interesting model plant for molecular cytogenetic research for several reasons. a) The crop includes cultivars of different taxonomic species each of which possess valuable horticultural traits that need to be combined in the new cultivars. b) The genomes of different species are so well differentiated genetically that the individual chromosomes can be clearly identified in the F_1 hybrids as well as in the progenies through DNA *in situ* hybridization techniques. c) The chromosomes are so large that the number and position of homoeologous recombinant segments can be clearly detected. d) Through careful selection, or through nitrous oxide treatment, *2n* gametes can be obtained in sterile interspecific hybrids. Taking advantage of these favourable attributes of lily, a molecular cytogenetic investigation was conducted on the possibilities of introgression of characters between Oriental and Asiatic lilies through the use of *2n* gametes.

For this purpose, more than 700 genotypes of F_1 interspecific hybrid between Oriental × Asiatic lilies were produced by using special techniques of ovule and/or embryo culture techniques. All the diploid interspecific OA hybrids (2n=2x=24) were highly sterile but 12 genotypes were found to produce *2n* gametes in notable frequencies. Six of these were analyzed through GISH for intergenomic recombination as well as meiotic nuclear restitution. In all cases there was evidence for the occurrence of first division restitution (FDR) gametes through three different types of cytological abnormalities, viz., post metaphase I division (PMI); post metaphase II (PMII) and asymmetric cytokinesis (Chapter 2). The 12 genotypes of OA hybrid that produced *2n* gametes were used as parents, both as male and female, in crosses with both parental species, i.e., AA and OO, as well as the allotetraploid 4x-OA (derived from doubling the chromosome number of OA hybrids with oryzalin). From these crosses, 246 triploid and 14 tetraploid progenies were obtained. The chromosome constitution of some of the allotriploid BC_1 progenies was analysed through GISH. This confirmed the presence of both O and A genome chromosomes with some of them possessing recombinant segments. These recombinations had obviously occurred during the development of *2n* gametes in the F_1 hybrid (Chapter 3).

A selected population from the BC_1 progenies (38 plants) was analyzed by GISH and FISH techniques in order to identify chromosomes as well as to determine intergenomic recombination. These analyses indicated that most of the progeny plants had originated through FDR. However, a small proportion of the progeny originated through IMR. Three kind of plants where identified when considering the presence of the number of homoeologous chromosomes and the recombinant segments: a) plants in which both reciprocal products from a crossover were present ($^{O}/_{A}$, $^{A}/_{O}$, where O represents the

centromere of the O genome and A, the recombinant segment of Asiatic chromosome and vice versa); (b) plants in which one normal chromosome of the O genome and a recombinant chromosome where present (O, $^{A}/_{O}$) and (c) plants in which one normal chromosome of the A genome and a recombinant chromosome where present (A, $^{O}/_{A}$) (Chapter 4). Furthermore, with the aim of utilizing allotriploids (2n=3x=36) for introgression breeding two kinds of crosses were made, a) diploid Asiatic cultivars were used as female parent and allotriploids AOA hybrids as male parents and vice versa (2x – 3x and 3x – 2x); b) allotriploids were crossed with allotetraploids (4x-OA) and other *2n* gametes producers. In all these crosses two kinds of allotriploids were used, those that had originated through crosses of 4x-OA with diploid Asiatic cultivars and those that had originated from crosses using *2n* gametes of OA hybrids with diploid Asiatic cultivars. Flowcytometry analyses were used for ploidy determination and these results were confirmed by chromosome counting in some cases showing concurrence. In the case of the 2x – 3x and 3x – 2x crosses, diploid and near-diploid progeny was obtained. In the 3x – 4x crosses near-tetraploid, tetraploid, near-pentaploid and pentaploid progenies were obtained. These results indicate that the allotriploids produced aneuploid as well as euploid (x, 2x, 3x) pollen grains. Through GISH analysis the identification of the parental as well as recombinant chromosomes was possible. Those allotriploids which originated from *2n* gametes transmitted their recombinant chromosomes to the progeny achieving introgression of the recombinant segments. (Chapter 5).

Finally, through N_2O treatments the formation of *2n* gametes was induced in some of the genotypes of OA hybrids. Out of eight genotypes that were treated, six were totally sterile and two were known to produce *2n* gametes in low frequencies. In the case of the totally sterile genotypes, three of them were able to produce progeny and in one of the known *2n* gamete producers the production of *2n* pollen was considerably increased from 0-30% to 55-80%. GISH analysis in the progeny revealed intergenomic recombination between the parental genomes, indicating that FDR and IMR restitution mechanisms were responsible for the *2n* pollen formation. (Chapter 6).

These results show the advantages of the *2n* gametes to generate genetic variation and the possibility to use them to produce progeny. It also shows the possibility of using triploid hybrids for further breeding and that introgression of chromosome segments can be achieved through chromosome assortment and recombination among the parental genomes. Furthermore, ploidy levels of the progeny can be manipulated depending upon the ploidy level of the parents. The induction of *2n* gametes by N_2O treatments in sterile interspecific lily hybrids provides many possibilities to generate completely new hybrids. This investigation shows that by using sexual polyploidization and by monitoring the progenies trough FISH and GISH techniques, more accurate insights into the process of introgression can be obtained.

Samenvatting

Lelie (*Lilium*) is niet alleen een belangrijk tuinbouwgewas, maar bovendien een interessant modelgewas voor moleculaire cytogenetisch onderzoek en wel om de volgende redenen:
a) Het lelie sortiment omvat diverse taxonomische soorten die elk waardevolle tuinbouwkundige eigenschappen bezitten die gewenst zijn in nieuwe rassen. b) De genomen van de species zijn zo goed cytogenetisch te onderscheiden dat de individuele chromosomen zowel in F1-hybriden als in nakomelingschappen duidelijk geïdentificeerd kunnen worden met behulp van *in situ* hybridisatie technieken. c) De chromosomen zijn zo groot dat het aantal en de plaats van homoeologe recombinante segmenten duidelijk gedetecteerd kunnen worden. d) Door middel van nauwkeurige selectie of via lachgasbehandeling, kunnen 2n gameten in steriele interspecifieke hybriden verkregen worden.
Met deze voor lelie gunstige eigenschappen, is moleculair cytogenetisch onderzoek uitgevoerd naar de mogelijkheden van introgressie van eigenschappen tussen Oriental en Aziatische hybriden door gebruik te maken van 2n-gameten.

Hiertoe zijn meer dan 700 F1-hybriden tussen Oriental en Aziatische hybriden met behulp van speciale bestuivings- en embryorescue technieken geproduceerd. Deze diploïde interspecifieke OA-hybriden (2n=2x=24) zijn allen zeer steriel, behalve 12 genotypen die 2n gameten bleken te produceren. Zes van deze genotypen werden met behulp van GISH onderzocht op homoeologe chromosoomparing tijdens de microsporogenese en of meiotische kern restitutie plaats vindt. In alle hybriden kon het optreden van “first division restitution” (FDR) gameten worden aangetoond. Hiervoor bleken drie verschillende cytologische afwijkingen t.w. post metafase I deling (PMI), post metafase II deling (PMII) en asymmetrische cytokinese (Hoofdstuk 2). De 12 OA-hybriden die 2n-gameten vormden werden gebruikt als ouders in kruisingen met Aziatische hybriden (AA), Oriental hybriden (OO) en allotetraploïde 4x-OA hybriden (verkregen na chromosoomverdubbeling van OA-hybriden met oryzaline). Uit deze kruisingen werden 246 triploïde en 14 tetraploide nakomelingen verkregen. De chromosoomsamenstelling van enkele van de allotriploïde BC1 nakomelingen werd geanalyseerd met behulp GISH. Dit bevestigde de aanwezigheid van zowel de O als de A genoom chromosomen, waaronder enkele met recombinante chromosoom segmenten. Deze recombinatie gebeurtenissen waren kennelijk opgetreden tijdens de vorming van de 2n gameten in de F1-hybride (Hoofdsruk 3).
Een geselecteerde BC1-populatie (38 planten) werd geanalyseerd met behulp van GISH en FISH-technieken waarmee chromosoom identificatie mogelijk is en intergenomische recombinatie vastgesteld kan worden. Dit onderzoek toonde aan dat de recombinatie bij de meeste nakomelingen is ontstaan via FDR, en een klein deel via IMR. Drie typen planten

konden geïdentificeerd worden wanneer gelet wordt op de aanwezigheid van het aantal homoeologe chromosomen en de recombinante segmenten: a) planten met beide reciproke producten van een cross-over ($^{O}/_{A}$, $^{A}/_{O}$, waarbij in het geval $^{O}/_{A}$ O het O-chromosoom met het centromeer voorstelt en A het recombinante segment van het A-chromosoom en vice versa); b) planten met één normaal chromosoom van het O genoom en één recombinant chromosoom (O, $^{A}/_{O}$) en c) planten met één normaal chromosoom van het A genoom en één recombinant chromosoom (A, $^{O}/_{A}$) (Hoofdstuk 4). Vervolgens werden met als oogmerk het benutten van allotriploïden (2n=2x=36) voor introgressie veredeling twee typen kruisingen gemaakt: a) diploïde Aziatische cultivars werden gebruikt als moederen allotriploïde AOA-hybriden als vader en vice versa (2x-3x en 3x-2x); b) allotriploïden werden gekruist met allotetraploïden (4x OA) en 2n-gamete producerende OA-hybriden. In deze kruisingen werden twee typen allotriploïden genotypen, t.w. die afkomstig zijn uit kruisingen van 4x-OA met diploïde Aziatische cultivars en die afkomstig zijn uit kruisingen van diploïde OA's die 2n-gameten produceerden met diploïde Aziatische cultivars. Flowcytometrie analyses werden gebruikt om het ploïdie niveau te bepalen. Deze resultaten werden bevestigd met chromosoomtellingen. In het geval van 2x-3x en 3x-2x kruisingen werden diploïden en bijna diploïden nakomelingen verkregen. De resultaten wijzen erop dat de allotriploïden zowel aneuploïde als euploïde (x, 2x, 3x) pollen korrels produceren. Met GISH konden de ouder chromosomen en de recombinante types vastgesteld worden. De allotriploïden die afkomstig waren van 2n-gameten droegen hun recombinante chromosomen over op hun nakomelingen, waardoor introgressie van recombinante segmenten werd verkregen (Hoofdstuk 5).

Tenslotte werd door N2O behandelingen de vorming van 2n-gameten geïnduceerd in enkele OA-hybriden. Van acht behandelde genotypen, waarvan zes steriel waren en twee 2n-gameten produceren, werd de productie van 2n pollen aanzienlijk verhoogd t.w. van 0-30% tot 55-80%. GISH analyse van de nakomelingen toonde intergenomische recombinatie tussen de ouder genomen, waarbij FDR en IMR restitutie in de 2n-pollen vorming kon worden aangetoond. (Hoofdstuk 6).

Deze resultaten tonen hoe 2n-gameten de genetische variatie kunnen vergroten en hoe hiermee nakomelingen kunnen worden verkregen. Het toont bovendien de mogelijkheid om triploïdie hybriden te gebruiken voor de verdere veredeling en dat introgressie van chromosoom segmenten bereikt wordt door herverdeling van chromosomen en recombinatie tussen de ouder genomen. Het ploïdie niveau van de nakomelingen kan worden gemanipuleerd afhankelijk van het ploïdie niveau van de ouders. De inductie van 2n-gameten middels N2O behandelingen bij steriele interspecifieke lelie hybriden levert talloze mogelijkheden om volledige nieuwe hybriden te genereren. Dit onderzoek toont dat via

seksuele polyploïdisatie en door de nakomelingen via FISH en GISH technieken te onderzoeken, een nauwkeurig inzicht in het introgressie proces kan worden verkregen.

Resumen

Aparte de ser un cultivo de importancia horticultural, las lilis (*Lilium*) también sirven como un modelo interesante para investigar citogenética molecular por varias razones. a) El cultivo incluye cultivares de distintas especies taxonómicas, cada una de las cuales posee características horticulturales de gran valor que necesitan ser combinadas en nuevos cultivares. b) Los genomas de las diferentes especies se encuentran tan bien diferenciados geneticamente que los cromosomas individuales pueden ser identificados claramente en los híbridos F_1 al igual que en la progenie a través de técnicas de hibridización *in situ*. c) Los cromosomas son tan grandes que el número y las posición de los segmentos homeólogos recombinantes pueden ser claramente detectados. d) A través de una selección cuidadosa, o a través de tratamientos de oxido nitroso (N_2O), gametos *2n* pueden ser obtenidos en híbridos interespecíficos estériles. Aprovechando estos atributos favorables de las lilis, esta investigación citogenética molecular fue conducida con las posibilidades de introgresar caracteres de lilis Orientales y Asiáticos por medio de gametos *2n*.

Para este propósito, mas de 700 genotipos de híbridos interespecíficos F_1 entre lilis Orientales × Asiáticos fueron producidos con el uso de técnicas especiales de cultivo de ovulos y/o embriones. Todos los híbridos interespecíficos OA diploides (2n=2x=24) fueron altamente estériles a excepción de 12, que fueron capaces de producir gametos *2n* en frecuencias notables. Seis de los cuales fueron analizados mediante GISH para la recombinación intergenómica así como restitución meiotica nuclear. En todos los casos hubo evidencia de la presencia de gametos restituidos en la primera división (FDR) originados mediante tres tipos distintos de anomalias citológicas, viz,. División en la Post Metafase I (PMI); División en la Post Metafase II (PMII), y citocinesis asimétrica (Capítulo 2). Los 12 genotipos de híbridos OA que produjeron gametos *2n* fueron utilizados como padres, tanto masculinos como femeninos, en cruzas con ambas especies parentales, ej., AA y OO, así como alotetraploides 4x-OA (derivados del doblamiento del número cromosómico de los híbridos OA con orizalin). De estas cruzas, 246 triploides y 14 tetraploides fueron obtenidos. La constitución cromosómica de algunas progenies de los alotriploides BC_1 fueron analizadas mediante GISH. Esto confirmó la presencia de cromosomas de los genomas O y A algunos de ellos con segmentos recombinantes. Esta recombinación obviamente tuvo lugar durante el desarrollo de los gametos *2n* en el híbrido F_1 (Capítulo 3).

Se seleccionó una población de la progenie BC_1 (38 plantas) y fue analizada mediante técnicas de GISH y FISH, los cromosomas fueron identificados y se determinó la recombinación intergenómica. Estos análisis indicaron que la mayoria de la progenie se originó a través de FDR. Sin embargo, una pequeña proporción de la progenie se originó a

través de IMR. Tres tipos de plantas fueron identificadas considerando la presencia del número de cromosomas homéologos y los cromosomas recombinantes: a) plantas en las cuales ambos productos recíprocos de un sobrecruzamiento se encuentran presentes ($^{O}/_{A}$, $^{A}/_{O}$, en donde O representa el centromero del genoma O, y A, el segmento recombinante del cromosoma Asiático y viceversa; b) plantas en las cuales un cromosoma normal del genoma O y un cromosoma recombinante se encuentran presentes (O, $^{A}/_{O}$) y c) plantas con un cromosoma normal del genoma A y un cromosoma recombinante (A, $^{O}/_{A}$) (Capítulo 4). Además, con la meta de utilizar alotriploides (2n=2x=36) para la introgresión, dos tipos de cruzas fueron realizadas, a) cultivares Asiáticos diploides fueron utilizados como hembras e híbridos AOA alotriploides como machos y viceversa (2x – 3x y 3x – 2x); b) alotriploides fueron cruzados con alotetraploides y con otros productores de gametos *2n*. En todas estas cruzas dos tipos de alotriploides fueron utilizados, aquellos originados a través de cruzas con 4x-OA con cultivares Asiáticos diploides y aquellos originados de las cruzas de gametos *2n* de híbridos OA con cultivares Asiáticos diploides. Mediante análisis de citometria de flujo se determinó la ploidia y en algunos casos los resultados fueron confirmados por conteo de cromosomas mostrando coincidencia. En el caso de las cruzas 2x – 3x y 3x – 2x se obtuvo progenie diploide y circa- diploide. En las cruzas 3x – 4x se obtuvo progenie tetraploide, circa-tetraploide, pentaploide y circa-pentaploide. Los resultados indican que los alotriploides produjeron tanto polen aneuploide como euploide (x, 2x, 3x). A través de análisis mediante GISH la identificacón de los cromosomas parentales y recombinantes fue posible. Aquellos alotriploides que se originaron mediante gametos *2n* transmitieron sus cromosomas recombinantes a la progenie logrando la introgresión de los segementos recombinantes (Capítulo 5).

Finalmente, a través de tratamientos con N_2O, se indujo la formación de gametos *2n* en algunos de los genotipos de híbridos OA. De ocho genotipos que fueron tratados, seis eran totalmente estériles y dos eran conocidos por producir gametos *2n* en frecuencias bajas. En el caso de los genotipos totalmente estériles, tres fueron capaces de producir progenie y en uno de los productores de gametos *2n* la producción de gametos *2n* fue incrementada de 0-30% a 55-80%. Análisis de GISH en la progenie reveló recombinación intergenómica entre los genomas parentales, indicando que mecanismos de FDR y IMR fueron reponsables de la formación de polen (Capítulo 6).

Estos resultados muestran las ventajas de los gametos *2n* para generar variación genetica y la posibilidad de utilizarlos para producir progenie. También muestran la posibilidad de utilizar híbridos triploides para continuar con el mejoramiento y que la introgresión de segmentos cromosomicos puede ser alcanzada a través de la distribución cromosomica y recombinación

entre los genomas parentales. Además de lo anterior, el nivel de ploidia en la progenie puede ser manipulado dependiendo del nivel de los padres. La inducción de gametos *2n* con tratamientos de N_2O en híbridos interespecíficos estériles de lilis provee muchas posibilidades para generar híbridos completamente nuevos. Esta investigación muestra que con el uso de poliploidización sexual y con el monitoreo de las progenies mediante técnicas de GISH y FISH se puede comprender mas acertadamente el proceso de introgresión.

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Curriculum vitae

Rodrigo Barba-Gonzalez was born in Guadalajara, Jalisco, México on 24 June of 1977. He studied his BCs in Biology, at the Centro Universitario de Ciencias Biologicas y Agropecuarias (CUCBA), of the Universidad de Guadalajara (UdG), from 1995 to 1999 and graduated as outstanding student of the XXXIII generation. He obtained his MSc degree at the Centro Univesitario de Ciencias Exactas e Ingenierias (CUCEI), of the UdG in 2002, for this study, he was awarded with a scholarship from the Consejo Nacional de Ciencia y Tecnologia (CONACyT), Mexico. He was awarded with a second scholarship from the CONACyT and begun his PhD at Wageningen University and Research Centre. This thesis is the result of the work carried out from November 2002 up to September 2005 to obtain his PhD degree.

Related publications

Barba-Gonzalez R., Van Silfhout A.A., Visser R.G.F., Ramanna M.S. & Van Tuyl J.M. Utilization of allotriploid BC_1 progenies of Oriental × Asiatic lilies (*Lilium*) in introgression breeding – an assessment based on GISH analysis. (*submitted*).

Barba-Gonzalez R., Miller C.T., Ramanna M.S. & Van Tuyl J.M. Nitrous oxide (N_2O) induces *2n* gametes in sterile F_1 hybrids between Oriental × Asiatic lily (*Lilium*) hybrids and leads to intergenomic recombination. (*submitted*).

Lim K-B., **Barba-Gonzalez R**., Zhou S., Ramanna M.S. & Van Tuyl J.M. Meiotic polyploidization with homoeologous recombination induced by caffeine treatment in interspecific lily hybrids. Korean J of Genetics. In press

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

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

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

Lokker A.C., **Barba-Gonzalez R**., Lim K.-.B., Ramanna M.S. & Van Tuyl J.M. 2005. Genotypic and environmental variation in production of *2n*-gametes of Oriental × Asiatic lily hybrids. Acta Hort 673:453-456

Barba-Gonzalez R., Lokker A.C., Lim K-B., Ramanna M.S. & Van Tuyl J.M. 2004. Use of *2n* gametes for the production of sexual polyploids from sterile Oriental × Asiatic hybrids of lilies (*Lilium*). Theor Appl Genet. 109: 1125-1132.

Lim K-B., Shen-TsaiMu., **Barba-Gonzalez-R**., Ramanna M.S. & Van Tuyl J.M. 2004. Occurrence of SDR *2n* gametes in *Lilium* hybrids. Breeding Sci 54: 13-18.

Education Statement of

the Graduate School

Experimental Plant Sciences

1) Start-up phase	*date*
► **First presentation of your project**	
2n gametes as a breeding tool in Oriental-Asiatic hybrids	Jun 2003
► **Writing a project proposal**	Mar 2003
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day Amsterdam 2004	Jun 2004
► **EPS theme symposia**	
EPS theme 4 Symposia	Dec 2002
EPS theme 2 Symposia	Jan 2003
EPS theme 4 Symposia	Dec 2003
EPS theme 2 Symposia	Dec 2003
EPS theme 4 Symposia	Dec 2004
► **National meetings**	
► **Seminars (series), workshops and symposia**	
Frontiers in research on interactions between plants and biotic agents	Oct-Dec 2003
Workshop Metabolomics	May 2004
► **Attendance research discussions of the laboratory**	2003 through 2006
► **International symposia and conferences**	
IXth International symposium on flower bulbs 2004 Niigata Japan	19-22 Apr 2004
► **Presentations**	
Use of *2n* gametes for inducing intergenomic recombination in lily hybrids	Apr 2004
Mitotic & meiotic polyploidization in lily hybrids for transferring *Botrytis* resistance	Apr 2004
Genotypic & environmental variation in production of *2n*-gametes of Oriental x Asiatic lily hybrids	Apr 2004
► **IAB Interview**	
► **Excursion**	
Subtotal Scientific Exposure	*10.4 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD course**	
Multivariate Analysis	Jan 2004
Advanced Statistics	Feb 2004
► **Individual research training**	
Subtotal In-Depth Studies	*3.0 credits**

4) Personal development	*date*
► **Skill training courses**	
Project and time management	Mar-May 2004
Scientific Writing	Sep-Nov 2004
Advanced Photoshop	Dec 2004
Media training	Apr-May 2004
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**25.0**

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

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