

**Introgression breeding through
interspecific polyploidisation in lily:
a molecular cytogenetic study**

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Propositions

1. Fluorescence *in situ* Hybridisation (FISH) mapping of rDNA sites in the genomes of the parental species and hybrids of *Lilium* is essential for understanding the chromosomal evolutionary processes in this genus (*this thesis*).
2. Second division restitution (SDR) gametes, which are rarely produced in interspecific hybrids, demonstrate low viability (*this thesis*).
3. Somatic doubling of interspecific lily hybrids is not the suitable method, in those cases where homoeologous recombination is required, for introgression of desirable characters into a cultivar (*this thesis*).
4. Indeterminate meiotic restitution (IMR) leads to the origin of euploid 2n-gametes with disproportionate number of parental chromosomes (*this thesis*).
5. Indeterminate meiotic restitution can explain the occurrence of homozygosity in 2n-gametes produced in interspecific hybrids.
6. Indeterminate meiotic restitution can explain autosegregation as was found in obligate diplosporic apomictic species.
7. Triploids are much more useful in breeding programmes of allopolyploids than hitherto has been recognised.
8. One of the benefits of FISH for assigning DNA sequences to chromosomes is its ability to reveal discrepancies between physical and genetic maps.
9. Lily has been a **VIP** since ancient times: not in the sense of a Very Important Person but as a Very Important Plant.
10. Developing new lily cultivars with a women-friendly fragrance should be an important goal in future breeding programmes.
11. One of the interesting characteristics of the behaviour of the Koreans is that they always try to finish with "hurry up". This is considered a strong point of them but also needs retrospection occasionally.

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Abstract

This thesis describes the introgression breeding through mitotic and meiotic polyploidisation of lily hybrids analysed by genomic *in situ* hybridisation (GISH) and fluorescence *in situ* hybridisation (FISH). The karyotypes of *L. longiflorum* and *L. rubellum* species were made by using conventional and molecular cytogenetic techniques. Microscopic observations of meiotic chromosome behaviour of *L. longiflorum* × Asiatic interspecific hybrids revealed a novel type of nuclear restitution, the so-called, 'Indeterminate Meiotic Restitution' (IMR). First Division Restitution (FDR), Second Division Restitution (SDR) and IMR were compared in relation to their genetic consequences. Introgression of homoeologous chromosomes and chromosome segments from donor species into recipients was analysed through chromosome painting techniques. The systematic methods of breakthroughs of sterility of F_1 interspecific hybrids and their BC individuals were discussed.

Abbreviations

C-bands	<u>C</u> onstitutive heterochromatin bands
DAPI	4',6-d <u>a</u> minido-2-phenylindole
DNA	<u>d</u> eoxyribo <u>n</u> ucleic <u>a</u> cid
dUTP	<u>d</u> eoxy- <u>u</u> ridine-5'- <u>t</u> riphosphate
EMCs	<u>E</u> gg <u>M</u> other <u>C</u> ells
FDA	<u>f</u> luorescein <u>d</u> iacetate
FDR	<u>F</u> irst <u>D</u> ivision <u>R</u> estitution
FISH	<u>F</u> luorescence <i><u>i</u>n <u>s</u>itu</i> hybridization
FITC	<u>f</u> luorescein isothiocyanate
GISH	<u>G</u> enomic <i><u>i</u>n <u>s</u>itu</i> hybridization
,	<u>U</u> nivalent(s)
"	<u>B</u> ivalent(s)
IMR	<u>I</u> ndeterminate <u>M</u> eiotic <u>R</u> estitution
LSV	<u>L</u> ily <u>S</u> ymptomless <u>V</u> irus
LVX	<u>L</u> ily <u>V</u> irus <u>X</u>
NORs	<u>N</u> ucleolar <u>O</u> rganizer <u>R</u> egions
pg	<u>p</u> ico grams
PI	<u>P</u> ropidium <u>I</u> odide
PMCs	<u>P</u> ollen <u>M</u> other <u>C</u> ells
Q-banding	<u>Q</u> uinacrine banding
rDNA	<u>r</u> ibosomal <u>D</u> N <u>A</u>
RNase A	<u>R</u> ibonuclease <u>A</u>
SDR	<u>S</u> econd <u>D</u> ivision <u>R</u> estitution
SDS	<u>S</u> odium <u>D</u> odecyl <u>S</u> ulfate
SSC	<u>S</u> tandard <u>S</u> aline <u>C</u> itrate
TBV	<u>T</u> ulip <u>B</u> reaking <u>V</u> irus
VBV	<u>V</u> ereniging van <u>B</u> loemenveilingen in <u>N</u> ederland

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To my wife and lovely two daughters

1 General introduction

The genus *Lilium* L.

The lily has an ancient heritage dating back some 36 centuries. It can be traced back to the Middle Minoan IIIA-B period (ca. 1750-1675 B.C.) when Cretan vases and frescoes celebrated its beauty, pure white colour and elegant fragrance (Evans 1921, 1930; Woodcock and Stearn 1950). It belongs to the genus *Lilium* of the family Liliaceae, which comprises over 80 species (Comber 1949, De Jong 1974). All species are dispersed in the Northern Hemisphere (10° to 60°), mainly in Asia, North America and Europe. Today, the lily occupies a prominent place in horticulture as a cut flower, pot and garden plant. About 1500 million bulbs are produced around the world; most commonly in the Netherlands, Japan and the United States of America but more recently also in the Southern Hemisphere lands such as Australia and Chile. As a cut flower, lily is now ranked as the fourth most important crop in the Netherlands (statistic data from VBN 2000).

The genus *Lilium* was classified into seven sections by Comber (1949) and later revised by Lighty (1968) and De Jong (1974). The seven sections are Martagon, Pseudolirium, *Lilium* (Liriotypus), Archelirion, Sinomartagon, Leucolirion and Daurolirion, a classification based upon 15 morphological and physiological characters by Comber (1949). Because of its wide range of flower shape, size, colour and morphological characteristics in some sections, the Leucolirion, Sinomartagon and Archelirion sections are the most important groups economically. Interspecific hybrids within the sections, especially Asiatic hybrids, have been bred since the early 1800s (Shimizu 1987). The distinctive characters of three important hybrids groups for cut flowers are:

- 1) **The Longiflorum hybrids (L-genome)** group in the Leucolirion section has trumpet-shaped, pure white flowers, a distinctive fragrance, year-round forcing ability and mostly outward-facing flowers;
- 2) **The Asiatic hybrids (A-genome)** group in the Sinomartagon section possesses a range of flower colours (orange, yellow, white, pink, red, purple and salmon), mostly upright-facing flowers with early (*L. cernuum* Komarov) to late (*L. callosum* Sieb. et Zucc.) flowering;
- 3) **The Oriental hybrids (O-genome)** group in the Archelirion section has large, pink or white flowers, a strong fragrance, sturdy stem, wide dark-green leaf shape and early (*L. rubellum* Baker) to late flowering (*L. nobilissimum* Makino).

The major commercial cultivars are Asiatic hybrids originating from interspecific crosses between species of the Sinomartagon section, Oriental hybrids derived from crosses in the Archelirion section, and *Longiflorum* hybrids obtained from *L. longiflorum* Thunb. or crosses of *L. longiflorum* and *L. formosanum* Wallace.

As many as 12 species of the Sinomartagon section are involved in the present cultivars of the Asiatic hybrids: *L. amabile* Palibin (Korean lily; orange or yellow colour), *L. bulbiferum* Linn. (orange, upright-facing), *L. cernuum* (early flowering, white or pink colour), *L. concolor* Salisb. (small flower, early, upright-facing), *L. dauricum* Ker-Gawler (early flowering, upright-facing, hairy and *Fusarium* resistant), *L. davidii* Elwes (orange with black spots), *L. lancifolium* Thunb. (strong stem, vigorous, hairy and bulbil formation) *L. lankongense* Franchet (pink and fire spots), *L. leichtlinii* Hook. (citron-yellow or orange with hairy buds), *L. maculatum* Thunb. (upright-facing) and *L. pumilum* D.C. (orange or yellow flower colour and dwarf) (Woodcock and Stearn 1950).

At least five species of the Archelirion section - *L. alexandrae* Wallace, *L. auratum* Lindley, *L. nobilissimum*, *L. rubellum* and *L. speciosum* Thunb. - have been intercrossed (Beattie and White 1993). Their hybrids are referred to as Oriental hybrids. *L. japonicum* Thunb. was also used as parent for the Oriental hybrids (McRae 1998).

Trends in lily breeding

About 7,000 lily cultivars were registered since 1960 (Leslie 1982). Active lily breeding work was seen in Japan between the 1920s and 1940s, in Australia and New Zealand during the 1950s and 1960s, in the United States of America from the 1960s to 1970s. Moreover, it has been since 25 years predominantly carried out in the Netherlands. Due to the release of many tetraploid clones from Plant Research International (former IVT, CPRO-DLO) to Dutch commercial breeders, the number of polyploid cultivars has steadily increased during the last decade (Van Tuyl et al. 1991; Schmitzer 1991). Especially in the case of Asiatic hybrids many of the diploid cultivars have been replaced by the tri- and tetraploid and the LA-hybrids are mainly triploids. In contrast, all of the commercial *L. longiflorum* and Oriental hybrids are still diploid (Van Tuyl, personal communication).

Interspecific polyploid cultivars have been produced as a result of the recent employment of interspecific hybridisation. Examples include, respectively, LA-, LO-, OA- and OT-hybrids derived from *L. longiflorum* (L) and Asiatic hybrids (A), *L. longiflorum* (L) and Oriental hybrids (O), Oriental hybrids (O) and Asiatic hybrids (A), and Oriental hybrids (O) and Trumpet hybrids (T; Leucolirion section).

Modern lily breeding aims at combining the three distinctive hybrid groups; Longiflorum-, Asiatic- and Oriental-hybrids. For example, LA-hybrids have become popular in the market over the past 10 years because of their flower shape and size, upright-facing, sturdy-long stem, early flowering and scent which was not available in Asiatic hybrids. By expanding interspecific hybridisation between LO-hybrids and OT-hybrids, new types of interspecific hybrids will soon be on the market together with OA-hybrids. OLA-hybrids derived from merging the three hybrid groups are also available to become cultivars.

Interspecific hybridisation in lily

Many valuable horticultural characters are present in the different species of the genus *Lilium*. Commercially important characters include:

- 1) Resistance to diseases such as bulb rot (*Fusarium*), *Botrytis* and several viruses (TBV, LSV and LVX);
- 2) Phenotypic characteristics such as flower shapes, sturdy stem, new colours and fragrance;
- 3) Physiological characteristics such as low-light intensity and heat tolerance, leaf scorch, year-round forcing ability, long-term storage ability and bulb growth speed.

Some well-known examples of valuable characters among species are the following:

- 1) *L. auratum* (Archelirion section) for large flower size;
- 2) *L. candidum* (Lilium section) for low-temperature and low-light intensity tolerance;
- 3) *L. concolor* (Sinomartagon section) for flower shape and upright-facing small flower size;
- 4) *L. dauricum* (Sinomartagon section) for *Fusarium* resistance;
- 5) *L. henryi* (Leucolirion section) for its virus resistance;
- 6) *L. longiflorum* (Leucolirion section) for year-round forcing ability;
- 7) *L. nepalense* Don and *L. primulinum* Baker (Leucolirion section) for pea-green flower colour with dark-purple throat;
- 8) *L. rubellum* (Archelirion section) for early flowering.

Van Tuyl et al. (1986) summarised the aims of interspecific hybridisation in *Lilium* as follows:

- 1) Introduction of desirable characters from alien species into the cultivars directly or indirectly (i.e., bridge crosses);

- 2) Creation of new forms and types of lilies;
- 3) Overcoming F_1 -sterility and polyploidisation to increase flower size and sturdiness;
- 4) Generation or expansion of the knowledge regarding taxonomic relationships and inheritance mechanisms and introgression of specific genes.

Both pre-fertilisation and post-fertilisation barriers hinder interspecific hybridisation between the different sections (Van Tuyl et al. 1991). Several techniques, such as the cut-style method (Asano and Myodo 1977a,b), the grafted-style method and *in vitro* pollination techniques have been developed to overcome pre-fertilisation barriers (Van Tuyl et al. 1991). However, even if fertilisation is successful, the growth of hybrid embryos can be hampered by post-fertilisation barriers (Van Tuyl et al. 1991). *In vitro* pollination and rescue methods such as embryo culture (Skirm 1942; North and Wills 1969; Ascher 1973a; Asano and Myodo 1977a,b; Asano 1978, 1980a), ovary-slice culture and ovule culture have been developed to circumvent these barriers (Van Tuyl et al. 1991). The plants cultured from embryos crossed between *L. henryi* × *L. regale* were rescued by Skirm (1942). North and Wills (1969) and North (1970) reported the successful culture of embryos from seeds without endosperm originating from interspecific crosses involving *L. lankongense*. Ascher (1973a,b) succeeded in growing embryos of *L. 'Damson'* × *L. longiflorum*. Asano and Myodo (1977b) reported the culture of immature hybrid embryos between *L. longiflorum* × *L. 'Sugehime'* and *L. 'Shikayama'* × *L. henryi*. Asano (1980a,b) produced many interspecific hybrids between *L. longiflorum* × *L. dauricum*, *L. longiflorum* × *L. amabile*, *L. longiflorum* × *L. pumilum*, *L. longiflorum* × *L. candidum*, *L. auratum* × *L. henryi*, *L. 'Sasatame'* × *L. henryi*, *L. 'Royal Gold'* × *L. speciosum* and *L. regale* × *L. leichtlinii maximowiczii*.

Later, Van Tuyl et al. (1988, 1991 and 2000) succeeded in making numerous new combinations between many sections of the genus *Lilium* by the use of various pollination and embryo rescue methods. Examples include *L. longiflorum* (Leucolirion section) × *L. monadelphum* (Lilium section), *L. longiflorum* × *L. lankongense* (Sinomartagon section), *L. longiflorum* × *L. martagon* (Martagon section), *L. longiflorum* × *L. candidum* (Lilium section), *L. henryi* (Leucolirion section) × *L. candidum*, *L. longiflorum* × *L. rubellum* (Archelirion section), *L. longiflorum* × Oriental hybrid, Oriental × Asiatic hybrid, *L. longiflorum* × *L. canadense* (Pseudolirium section) and Oriental hybrid × *L. pardalinum* (Pseudolirium section). The crossing polygon given in Figure 1 shows the crossing compatibility within and between the sections achieved by our research group so far (Van Tuyl et al. 2000).

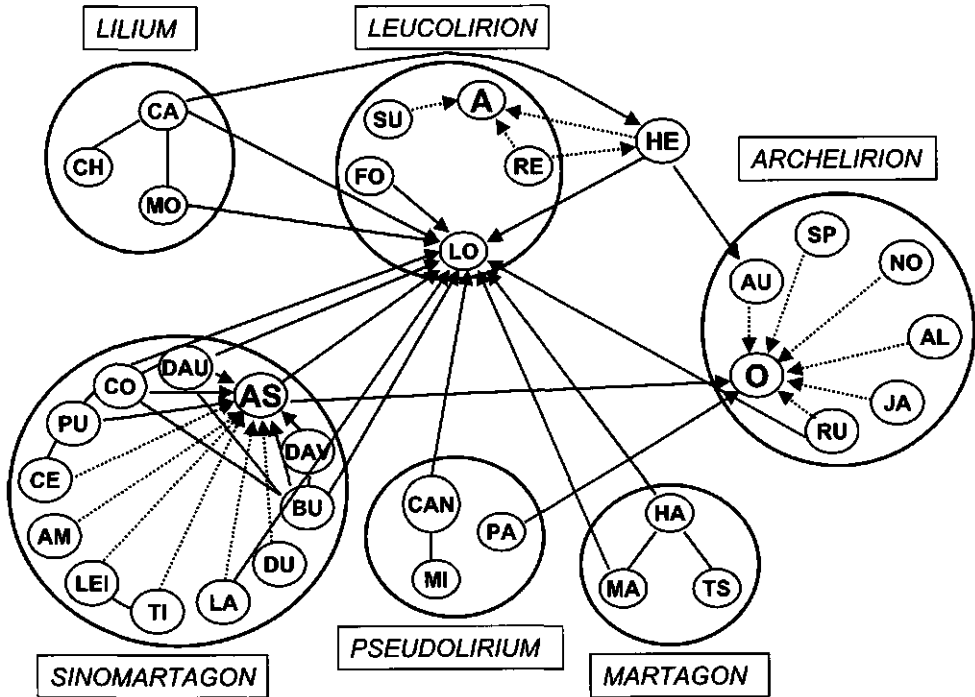


Figure 1. A crossing polygon of the genus *Lilium* including all successful crosses of species between different sections of the genus *Lilium* developed at Plant Research International, The Netherlands. In this figure the connection between the Asiatic, Aurelian and Oriental hybrid groups (large ellipses) 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. chalcidonicum*; 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. leichlinii*; 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* (Van Tuyl et al. 2000).

Pollen fertility of interspecific lily hybrids

The next bottleneck in achieving introgression by backcrossing is the sterility of the interspecific hybrids. This can be due to several reasons such as chromosome aberrations, genetic incongruity (genic sterility) or other unknown factors (Asano 1982a). Meiotic division of the wide interspecific hybrids is often disturbed due to factors such as unbalanced chromosome separation, chromosome bridges, chromosome lagging during anaphase I and II, time discrepancy between chromosome movement and cytokinesis (Asano 1982a). Any pollen generated

through the aforementioned disturbances is lethal. Although the chromosomes of two distantly related genomes have high levels of chromosome association, the pollen will be predominantly sterile or unbalanced owing to the random distribution of homoeologous chromosomes during meiotic division (Asano 1982a; Hermesen 1984; Ramanna, personal communication). Unreduced gametes formation is an exception in this type of material, circumventing these balance disturbances. This phenomenon was demonstrated by 83.6% of restituted pollen in the hybrid of *L. auratum* (Archelirion section) \times *L. henryi*, and 52 % in *L. longiflorum* (Leucolirion section) \times *L. leichtlinii* (Sinomartagon section) (Asano 1982a), *L. 'Connecticut Yankee'* (Sinomartagon section) \times *L. longiflorum* (Leucolirion section), *L. aurelianense* (Leucolirion section) \times *L. longiflorum* (Ascher 1973a,b and 1977).

n vs. 2n gametes

Theoretically, as has been identified through microscopic observation, interspecific hybrids can produce four different types of sporads: monads (4n), dyads (2n + 2n), triads (2n + n + n) and tetrads (n + n + n + n). All these euploid spores can be fertile and can fertilise with n-gamete of the female (or male) parent to produce pentaploid (5x), triploid (3x) and diploid (2x) offspring, respectively.

Fertile n- and 2n-gametes have been produced in interspecific lily hybrids (*L. 'Enchantment'* \times *L. pumilum*) within the Sinomartagon section (Van Tuyl et al. 1989). The fertile n-gametes are also confirmed in interspecific *Allium* and *Alstroemeria* hybrids with homoeologous recombinations analysed by GISH detection (Khrustaleva and Kik 1998, 2000; Kamstra et al. 1999a). In fact, fertile n-gametes with homoeologous recombination are ideal for introgression breeding without increasing the ploidy level of the following generation. However, in most cases, the unreduced (2n) gametes are predominantly produced. Unreduced gametes via FDR (first division restitution) and SDR (second division restitution) have been used for sexual polyploidisation in *Alstroemeria* (Ramanna 1991, 1992; Kamstra et al. 1999a,b), Orchid (Lee 1987), potato (Den Nijs 1977; Mendiburu and Peloquin 1977; Peloquin 1982; Ramanna 1983; Iwanaga et al. 1989; Hutten et al. 1994; Tai and De Jong 1997), wheat (Alonso and Kimber 1984; Liu et al. 1994; and many others) and other crops (see Veilleux 1985). In a few cases, interspecific hybrids produce viable SDR (fertile) pollen (Ramanna, personal communication). FDR and SDR have different genetic consequences as far as chromosome segregation and heterozygosity are concerned (Hermesen 1984; Barone et al. 1995).

The meiotic division of wide interspecific hybrids can be categorised as follows:

- 1) **Normal meiosis:** If all steps of meiotic division are normal, n-gametes are formed in the tetrad stage. Since homoeologous chromosomes are not close to

pair, the movement of all homoeologous univalents will be disturbed due to random distribution at anaphase I. Most of these gametes in wide interspecific hybrids, however, will be unbalanced and lethal.

- 2) **FDR:** The meiotic prophase I is the same as in a normal division. However, metaphase I stage takes a little longer than that for normal cells (Wagenaar 1968). It then goes directly to the anaphase II, without cytokinesis I, giving rise to dyads. FDR mechanism gives rise to balanced gametes with genetically identical nuclei (or nearly identical depending on the level of crossing-over) resulting in fertile gametes. The $2n$ -FDR gametes consist of non-sister chromatids indicating a maximum heterozygosity as in their F_1 interspecific hybrid (see Fig. 3 in Chapter 4).
- 3) **SDR:** As a very rare case, viable SDR $2n$ -gametes in interspecific hybrids can originate after high frequency of homoeologous chromosome association followed by normal disjunction at anaphase I. The two $2n$ -gametes that originate from a SDR mechanism are sterile, unbalanced and genetically different from each other and the parental genotype from which they derived.

Mitotic vs. meiotic polyploidisation for introgression breeding

Most of interspecific hybrids between distantly related *Lilium* species are, as indicated above, highly sterile. Therefore, even after successful interspecific hybridisation between diploid species ($2n=2x=24$), the sterility of interspecific hybrids imposes a significant bottleneck for introgression breeding. Polyploidisation can solve this problem, which can be distinguished into mitotic and meiotic polyploidisation. The former is obtained through artificial chromosome doubling by treatment of vegetative tissue with spindle inhibitors such as colchicine (Blakeslee and Avery 1937; Emsweller and Brierley 1940) or oryzalin (Van Tuyl et al. 1992). Meiotic polyploidisation is the use of $2n$ -gametes that occur spontaneously through abnormal meiosis (Mok and Peloquin 1975; Vorsa and Bingham 1979; Ramanna 1979; Veilleux et al. 1982; Lim et al. 2000). In both cases, the chromosome number of the gametes is diploid ($2n$) instead of haploid (n).

The traditional method of doubling the chromosome number of the F_1 -hybrids through oryzalin or any other chemical reagent treatment has been successful to a certain extent in lilies. The plants derived from mitotic chromosome doubling often showed not only restoration of fertility but also vigorous growth habit with larger and thicker organs (except for the plant height that become normally shorter). Nevertheless, the production of the amphidiploids in this way is not ideal for introgression breeding because due to preferential chromosome association hardly any intergenomic recombination is found. An alternative and more preferable

method of utilising interspecific hybrids involves the use of 2n-gametes that occur occasionally in interspecific hybrids of lilies (Van Tuyl et al. 1989). The plants derived from crosses with 2n-gametes often show one of the parental characteristics that are more diverse, genetically more homozygous (SDR effect) or more heterozygous with some degree of heterozygosity depending on recombinations (FDR effect) (Hermesen 1984). An important feature of 2n-gametes is that, depending on the mode of origin, a certain level of intergenomic recombination can occur during the meiosis I division. The occurrence of genetic recombination during the 2n-gametes formation has been demonstrated (Douches and Quiros 1988; Buso et al. 1999; Karlov et al. 1999; Kamstra et al. 1999a,b).

Importance of the introgression breeding

Introgression is one of the main aims in interspecific hybridisation for introducing a restricted number of traits from the donor species to the recipient. As in lily, the Oriental hybrid group (Archelirion section), for example, has no orange flower colour and is susceptible to *Fusarium*, although it is resistant to *Botrytis*. The Asiatic hybrids (Sinomartagon section), however, are available in a range of colour, but they are mostly susceptible to *Botrytis* and resistant to *Fusarium*. From these examples it is clear that interspecific hybridisation is considered to be the best way for combining desirable traits as compared to alternative methods such as genetic transformation or mutation breeding.

At the chromosome level, homoeologous cross-over is an essential event for the introgression of species-specific genes. Little research has thus far been carried out in *Lilium* on the occurrence and frequency of homoeologous chromosome association and crossing-over between two or three parental genomes depending on the degree of relationship between species. The frequency of chromosome association in interspecific hybrids often reflects the genetic relationship between the parental species (Asano 1984).

Although interspecific hybridisation is the first step towards achieving the successful introgression of desirable genes, several backcrossing steps to the appropriate recipient are also needed for introgression of the desirable trait(s). During subsequent backcross generation, the number of chromosomes from the donor species will diminish, ultimately leading to plants with single alien chromosomes or chromosome segments. Two types of introgression can be distinguished as whole chromosome(s) or as chromosome segment(s). The former, used when no homoeologous recombination in backcrossing occurs, transmits all genes present on the same chromosome to the next generation. This type of introgression resulting in a monosomic addition or chromosome substitution can be

achieved in the BC₂ or next generation. In case homoeologous recombination occurs, a restricted number of gene(s) present on a chromosome segment is transmitted. In this case, introgression might already occur in the BC₁ generation.

Since 2n-gametes are considered being fertile pollen with homoeologous recombination, significant efforts have been invested into the use of 2n-gametes for introgression breeding. Such 2n-gametes are very rarely produced spontaneously in nature. Only a few F₁ interspecific lily hybrids produce 2n-gametes with some extent of pollen fertility (see Chapter 4). Although the possibility of artificial doubling of meiotic chromosomes exists, only few reports are available on the use of high solar level (Ortiz and Vuylsteke 1995; Negri and Lemmi 1998) and low temperature treatment (Stein 1970).

Cytogenetics of lily

The genome size of Liliaceae such as *Fritillaria*, *Hyacinthus*, *Lilium*, *Tulipa* is the largest in the plant kingdom. Many cytogeneticists since Strasburger (1880) have used lily as a model plant for chromosome studies including the description of meiotic stages, duration of female meiotic stages (Bennett and Stern 1975), crossing-over (Fogwill 1958; Stern and Hotta 1977), synaptonemal complexes (Stern and Hotta 1977; Holm and Rasmussen 1984; Anderson et al. 1994), fertility of interspecific hybrids (Brock 1954; Asano 1984), chromosome morphology (Sato 1932; Stewart 1947; Noda 1978; Stack 1991 and many others) and chromosome banding (Holm 1976; Son 1977; Song 1987; 1991). The genome size of *Lilium* species varies from 69.5 pg/2C (*L. henryi*) up to 96 pg/2C (*L. parryi*) (Bennett and Smith 1976, 1991; Van Tuyl and Boon 1997). The basic chromosome number of all species in the genus *Lilium* is $x=12$ with 2 metacentric and 10 acro- or sub-acrocentric chromosomes. The basic nomenclature of *Lilium* karyotypes is based on the order of chromosomes in sequences of decreasing short-arm length, with 1 representing the longest short-arm and 12 the shortest (Stewart 1947; Lim et al. 2000). All the species are diploid with exception to *L. lancifolium* in which both diploids and triploids were found (Sato 1932; Stewart and Bamford 1943; Noda 1966).

Chromosome analysis of interspecific hybrids provides:

- 1) Discrimination of parental chromosomes through banding techniques and/or GISH analysis;
- 2) Determination of the genome composition in BC progenies;
- 3) Identification of the individual chromosome numbers;
- 4) Tracing of the recombinant chromosome(s) or chromosome segment(s);
- 5) Determination of the mechanism of genetic inheritance.

Table 1. List of species with identified nucleolar-bearing chromosomes (Stewart 1947; Brandram 1967; Ogiwara 1968; Noda 1973; Son and Song 1978; Von Kalm and Smyth 1984; Song 1987; Yamaguchi et al. 1990; Lim et al. 2000).

Long 1987, Panigrahi et al. 1990, Eml et al. 2000).

Section		Species	Chromosome number												Total
			1 A	2 B	3 C	4 D	5 E	6 F	7 G	8 H	9 I	10 J	11 K	12 L	
1	Sinomartagon	<i>L. amabile</i>	1					1	1						3
2		<i>L. callosum</i>	1		1			1	1		1				5
3		<i>L. cernuum</i>		1		1									2
4		<i>L. concolor</i>	1	1				1			1		1		5
5		<i>L. dauricum</i>	1	1	1			1	1						5
6		<i>L. davidii</i>	1			1		1	1						4
7		<i>L. duchartrei</i>		1	1				1						3
8		<i>L. lankongense</i>				1	1								2
9		<i>L. leichtlinii</i>	1	1					1						3
10		<i>L. pumilum</i>	1	1	1	1		1							5
11		<i>L. tigrinum</i>	1	1					1						3
12		<i>L. wardii</i>	1			1									2
13	Martagon	<i>L. hansonii</i>		1	1	2	1		1			1	1		8
14		<i>L. martagon</i>	1	1	1			1					1		5
15		<i>L. tsingtauense</i>			1	1		1				1			4
16	Lilium	<i>L. candidum</i>				1		1			1				3
17		<i>L. monadelphum</i>			1	1	1	2	1						6
18	Pseudolirium	<i>L. canadense</i>					1		1						2
19		<i>L. catesbaei</i>		1	1								1	1	5
20		<i>L. columbianum</i>		1							1	1		1	5
21		<i>L. grayi</i>			1								1		2
22		<i>L. michiganense</i>					1		1						2
23		<i>L. occidentale</i>		1							1	1		1	5
24		<i>L. pardalinum</i>									1	1		1	3
25		<i>L. parryi</i>									1	1		1	3
26		<i>L. philadelphicum</i>				1		1					1	1	4
27		<i>L. roezlii</i>									1	1		1	3
28		<i>L. superbum</i>			1	1						1	1		4
29	Leucolirion	<i>L. brownii</i>				1		1	1						3
30		<i>L. formosanum</i>			1	1			1						3
31		<i>L. henryi</i>	1					1							2
32		<i>L. leucanthum</i>	1	1	1	1	1								5
33		<i>L. longiflorum</i>			1	1			1						3
34		<i>L. myriophyllum</i>	1	1	1	1	1								5
35		<i>L. nepalense</i>	1	1					1						3
36		<i>L. regale</i>	1	1	1	1	1								5
37		<i>L. sargentiae</i>	1	1	1	1	1								5
38		<i>L. sulphureum</i>	1	1		1	1								4
39	Archelirion	<i>L. alexandrae</i>	1			1								1	3
40		<i>L. auratum</i>	1	1	1	1								1	5
41		<i>L. japonicum</i>		1		1								1	3
42		<i>L. nobilissimum</i>	1												1
43		<i>L. rubellum</i>	1	2	1	1		1						1	7
44		<i>L. speciosum</i>	1		1		1							1	4
Total			22	22	20	24	11	15	15	5	8	3	12	9	

Ag-NOR staining, C-, N- and Q-banding techniques have been applied for karyotype analysis to discriminate between the NOR-bearing satellite chromosomes or the other chromosomes (Table 1). Ag-NOR staining and C-banding was used to confirm that it was a real interspecific hybrid for the analysis of *L. 'Black Beauty'*, obtained from *L. speciosum* and *L. henryi*, (Von Kalm and Smyth 1980; Song 1987) and for karyotype portraits of the species (Smyth et al. 1989). However, these techniques were not appropriate to determine the whole parental genome at individual chromosome level. The recently developed molecular cytogenetic approaches such as fluorescence *in situ* hybridisation (FISH) have opened the possibilities for distinguishing between parental genomes, detection of the position of translocation breakpoints and sites of homoeologous recombinations. These approaches are applicable to mitotic as well as meiotic chromosomes and enable cytogeneticists to elucidate the meiotic behaviour and genetic transmissions of individual chromosomes in the F_1 and BC generations.

Since the development of non-isotopic *in situ* hybridisation techniques, a powerful tool has been added to plant cytogenetics. Thus, FISH has been employed in many plant species or their interspecific hybrids such as *Allium* (Hizume 1994; Keller et al. 1996; Khrustaleva and Kik 1998, 2000), *Aloe* (Takahashi et al. 1997), *Alstroemeria* (Kamstra et al. 1999a,b), *Arabidopsis* (Maluszynska and Heslop-Harrison 1993; Fransz et al. 1996, 1998), banana (Osuji et al. 1997; D'Hont et al. 2000a,b), *Brassica* (Nagpal et al. 1996; Snowdon et al. 1997), *Coffea* (Barre et al. 1998), grass (Bailey et al. 1993), *Lilium* (Karlov et al. 1999; Lim et al. 2000), *Lolium* (Thomas et al. 1994; Pasakinskiene et al. 1997), maize (Poggio et al. 1998), *Nicotiana* (Parokonny et al. 1992), potato (Jacobsen et al. 1993; Garriga-Caldere et al. 1997), rice (Fukui et al. 1997; Yan et al. 1999), soybean (Shi et al. 1996; Zhu et al. 1996), tomato (Parokonny et al. 1997; Zhong et al. 1998), and wheat (Schwarzacher et al. 1992; King et al. 1993; Miller et al. 1994; Chen et al. 1995). Monitoring of the introgressed alien chromosome segments in the subsequent generation is an important tool in assessing the processes of meiotic recombination, chromosome transmission and genetic analysis methods for confirming the best way of gene flow.

The detection of NORs can be an important tool for identification of individual chromosomes. Ribosomal DNA probes such as 5S and 45S have been frequently used together with GISH analysis to detect nucleolar-bearing chromosomes in various crops (Mukai et al. 1991a,b; Leitch and Heslop-Harrison 1992; Jiang and Gill 1993, 1994; Iwano 1998). Species-specific probes have been used to identify the number of parental chromosomes for instance in *Alstroemeria* hybrids (Kamstra et al. 1999a).

With the differentiation of the parental chromosomes, identification of NOR-bearing chromosomes through rDNA detection by FISH gives important information on specific chromosomes. Multicolour GISH can be used to identify three or more genome origins simultaneously (Mukai et al. 1993; Khurstaleva and Kik 2000). Monitoring of chromosome behaviour during meiosis of interspecific hybrids and their derivatives is necessary to understand the chromosome composition of the subsequent gametes.

Scope of the thesis

The aims of this research were:

- 1) to detect and describe chromosome characteristics which enable the identification of various genomes and individual chromosomes using both conventional and molecular cytogenetics;
- 2) to establish the chromosome karyotypes, especially with reference to parental chromosomes and intergenomic recombination events, in F_1 -hybrids between species and their backcross progenies using GISH;
- 3) to create allopolyploids from *Lilium* interspecific hybrids through somatic doubling as well as through the use of 2n-gametes (meiotic polyploidisation), and to analyse the chromosome composition of these hybrids and their backcross progenies;
- 4) to elucidate the modes of origin of 2n-gametes in *Lilium* interspecific hybrids by GISH and FISH, and to assess their consequences for sexual polyploidisation.

In order to realise these aims, interspecific hybrids were available, e.g., *L. longiflorum* × Asiatic hybrids (LA) and *L. longiflorum* × *L. rubellum* (LR), respectively. The two different types of crosses, LA and LR, were analysed for evaluation of the occurrence of intergenomic recombination. Homoeologous recombination was taken into account together with meiotic nuclear restitution as revealed by GISH and FISH analyses. The parental chromosome composition of the sexual polyploid progenies, derived from LA-hybrids and backcross derivatives (ALA) were critically analysed to determine the involvement of 2n-gamete types. Finally, the BC_1 (ALA) plants were crossed with various parents with different ploidy level (2x, 3x and 4x) and the progenies were evaluated for ploidy level and, in some cases, for chromosome composition.

Chapter 2 provides basic information on the karyotypes of *L. longiflorum* and *L. rubellum* species. The physical localisation was completed by using C-banding, DAPI-

banding, PI-banding, Ag-NORs staining and FISH detection of 5S and 45S rDNA probes to the somatic chromosome complement of both species.

Chapter 3 presents a GISH study of an amphidiploid F_1 -hybrid (LLRR) derived from mitotic chromosome doubling. Its BC_1 (LLR) and BC_2 (LLLR) plants were analysed for their parental chromosome composition.

In Chapter 4, the Indeterminate Meiotic Restitution (IMR) mechanism was demonstrated, as found in the PMCs of some LA-hybrids. Microscopic observation of the PMCs has been performed, and the FISH technique was employed to monitor the chromosome behaviour of the parental chromosomes at the anaphase I stage. The new type of meiotic restitution (IMR) and other 2n-gametes-producing mechanisms (FDR and SDR) were detected via the FISH and GISH analysis.

In Chapter 5, BC_1 (ALA) plants were analysed using GISH to confirm the meiotic nuclear restitution mechanisms. In addition, the frequency of homoeologous chromosome recombination as well as 2n-gametes producing mechanisms in F_1 hybrids was taken into account in mitotic chromosome complements of the BC_1 progenies. The genetic consequences of FDR with/without crossing-over and the other new meiotic nuclear restitution mechanism 'IMR' were discussed.

In Chapter 6, an analysis is presented of the BC_2 individuals that were produced by crossing a BC_1 plant (ALA; 921238-1) as female with males parents having different genome compositions and ploidy levels. DNA measurement by flowcytometry and GISH analysis of the BC_2 plants were performed to determine their ploidy level and genome composition.

In chapter 7, the genetic consequences of mitotic and meiotic polyploidisation of the interspecific F_1 -hybrids and the application of the different meiotic nuclear restitution mechanisms for introgression breeding have been discussed.

2

Karyotype analysis of *Lilium longiflorum* Thunb. and *Lilium rubellum* Baker by chromosome banding and fluorescence *in situ* hybridisation (FISH)

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Abstract

Detailed karyotypes of *Lilium longiflorum* and *L. rubellum* were constructed on the basis of chromosome arm lengths, C-banding and Fluorescence *in situ* Hybridisation (FISH) with the 5S and 45S rDNA sequences as probes. The chromosomes of *L. longiflorum* range from 34.4 to 18.1 μm with a total length of 286.1 μm . These values are slightly larger than those of *L. rubellum*, which are 33.8 – 16.9 μm long and have a total complement length of 269.9 μm . The small difference in chromosome length is also reflected by the total DNA contents, which amounts 77.1 ± 0.3 pg/2C and 73.6 ± 0.6 pg/2C for the two lily species, respectively. Constitutive heterochromatin shown by the standard BSG technique revealed completely different distributions for the C-bands: in *L. longiflorum* we observed a total of eleven small C-bands on the chromosomes 1, 3, 4, 7, 8, 9, 11 and 12, whereas in *L. rubellum* twelve C-bands occurred on the chromosomes 2, 3, 4, 6, 8 and 12, most of them of different sites. Fluorescence *in situ* hybridisation of the 5S and 45S rDNA probes showed in *L. longiflorum* overlapping signals at the proximal positions of the short arms of the chromosome 4 and 7. Chromosome 3 showed one 5S-rDNA segment on the secondary constriction and one 45S-rDNA region adjacent to 5S-rDNA signal on the sub-distal part of the long arm. In *L. rubellum*, we observed co-localisation of the 5S and 45S rDNA sequences on the short arm of chromosomes 2 and 4, and on the long arms of the chromosomes 2, 3 and two adjacent bands on 12. In addition, single 45S rDNA sites were found on the chromosomes 1 and 6. Among them, the chromosome 2 contained two 45S rDNA sites both at the short arm and long arm and the chromosome 12 possessed two 5S rDNA sites, whereas the chromosomes 1, 3, 4, 6 and 12 showed only single sites. Most C-bands co-localised with the 45S rDNA segments. Staining of the nucleoli and the nucleolar organiser regions (NORs) in *L. longiflorum* and *L. rubellum* by AgNO_3 gave a highly variable number of signals in the interphase nuclei. Only few mitotic metaphase chromosomes showed faint silver deposits at their NORs. Staining with a mixture of the DNA-specific fluorochromes propidium iodide (PI) and DAPI, alone or in combination, the chromosomes revealed 3 minor, the so-called, reverse PI/DAPI bands on the chromosomes 1, 4 and 7 in *L. longiflorum* and six bands on the chromosomes 2, 3, 4 and 12 of *L. rubellum*. Single PI bands always matches the rDNA sites, whereas single DAPI bands generally correspond with C-bands. The combination of chromosome morphology, silver staining, rDNA FISH and reverse PI/DAPI bands revealed karyotypes for *L. longiflorum* and *L. rubellum*, in which most chromosomes can unequivocally be identified. Comparison of the karyotypes of these two species also demonstrated striking dissimilarities between these two lily

species suggesting complex genome dynamics during the evolution of these lily genomes, at least with respect to these repeat families.

Key words: *Lilium* • fluorescence *in situ* hybridisation (FISH) • 5S rDNA • 45S rDNA • karyotype analysis • C-banding • Ag-NOR staining • reverse PI/DAPI banding

Introduction

The species of the genus *Lilium* not only have one of the largest genomes in plant kingdom (Bennett and Smith, 1976, 1991), but also feature very long chromosomes at mitotic and meiotic divisions. Strasburger (1880) already recognised lily species as particularly suitable for chromosome research. Since then many studies followed, especially on chromosome morphology in *L. longiflorum* Thunb. (Stewart 1947), banding pattern (Holm 1976; Son 1977; Von Kalm and Smyth 1984), detection of Nucleolar Organiser Regions (Von Kalm and Smyth 1980, 1984) and genome size (Bennett and Smith 1976, 1991; Van Tuyl and Boon 1997). Surprisingly, little attention was drawn to the chromosomes of *L. rubellum* Baker, an economically important species used for interspecific hybridisation with *L. longiflorum*. Although phenotypic characters are very different for the two species, their chromosome portraits are pretty much alike (Noda 1991) and nuclear DNA content differ less than 2 % (Van Tuyl and Boon 1997). However, relatively high frequency of meiotic recombination between homoeologues at metaphase I of *L. longiflorum* × *L. rubellum* hybrids suggests chromosome homology between the parental genomes (unpublished observations).

The increased use of *L. longiflorum* and *L. rubellum* in lily breeding requires detailed knowledge of their chromosome portraits for cytogenetic studies of their interspecific hybrids and breeding programs. The ability to identify individual chromosomes will be helpful in localising translocation breakpoints and positions of homoeologous recombination in metaphase sets of the interspecific hybrids and backcross individuals. In addition, the karyotypes will also be indispensable for mapping genes on the chromosome arms and trace these chromosome segments during the consecutive backcross generations.

The basic karyotypes of lily species generally display the chromosomes of a mitotic metaphase set, ordered in sequence of decreasing short arm length, and with the heterochromatin segments as revealed with the C-, N- and Q-banding and other chromosome differentiation techniques. Satellite chromosomes can be described by the presence of their micro- or macro-satellites, or by Ag-NOR staining, a technique that specifically demonstrates sites of metabolically active nucleolar

organiser regions (Funaki et al. 1975; Linde-Laursen 1975; Gerlach 1977). More recently, the introduction of fluorescence *in situ* hybridisation (FISH) tools using repetitive or single copy sequences complement the arsenal of banding technologies, along with the use of DNA specific fluorochromes (Peterson et al. 1999). The most common application of FISH for karyotype analysis is the localisation of ribosomal DNA repeat families. The 45S rDNA components were shown to be part of the nucleolar organiser region of the satellite chromosomes, whereas the 5S rDNA generally occurred on one of the other chromosomes (Gerlach and Dyer 1980; Kamstra et al. 1997; Takeda et al. 1999; Mukai et al. 1991a,b; Leitch and Heslop-Harrison 1992; Jiang and Gill 1994; Fukui et al. 1998). One other important application of FISH for chromosome identification was the detection of microsatellites (Cuadrado and Schwarzacher 1998), satellite DNAs (Pederson et al. 1996; Kamstra et al. 1997) and other repeat families.

In the standard karyotype of *Lilium* species most chromosomes (4-6, 7-9, 10-12) are morphologically too similar to be identified unequivocally without additional diagnostic landmarks. Previous C-banding studies (Holm 1976; Son 1977; Kongsuwan and Smyth 1978; Smyth et al. 1989) showed small constitutive heterochromatin bands in the *L. longiflorum* chromosomes 1, 3, 4, 7, 8, 9 and 12, whereas NOR detection by isotopic *in situ* hybridisation and silver staining demonstrated nucleoli and active ribosomal sites on the chromosomes 3, 4 and 7 (Von Kalm and Smyth 1980, 1984). Fluorescence *in situ* hybridisation (FISH) detection of the ribosomal DNA sequences has already reported in other crops (Mukai et al. 1991a,b; Leitch and Heslop-Harrison 1992; Jiang and Gill 1994; Fukui et al. 1998), but so far, no report of this technique in lily appeared.

Previous studies on fluorescence and genomic *in situ* hybridisation showed that counterstaining the chromosomes with a mixture of the DNA specific fluorochromes DAPI (4', 6-diamidino-2-phenylindole) and PI (propidium iodide) produced small bands on specific regions of the chromosomes (Schweizer 1980; Peterson et al. 1999; Andras et al. 2000), the so-called, "CPD". This reversed PI/DAPI bands (CPD bands) in the lily species and interspecific lily hybrids were found to co-localise in the NOR regions of a few chromosomes (see Fig. 1c and f in Lim et al. 2000).

The aim of this study was an accurate comparative description of the karyotypes of these two lily species, *L. longiflorum* and *L. rubellum*, using morphometric data, of C-banding, Ag-NOR staining, FISH detection of the 45S rDNA and 5S rDNA sequences, and the reverse PI/DAPI banding.

Material and methods

Plant materials and chromosome preparation

L. longiflorum 'Snow Queen' and *L. rubellum* (accession number 980085; originally from the mountainous area of the Fukushima prefecture, Japan) were obtained from the germplasm collection of Plant Research International, Wageningen, the Netherlands. Bulbs were grown in the greenhouse at 14 °C – 18 °C during the night and 20 °C – 25 °C during the day. Root tips were collected in the morning and pre-treated in a saturated α -bromonaphtalin solution for 2 hours at 20 °C and kept in the solution at 4 °C until the next morning. Then the material was rinsed three times in tap water before being fixed in acetic acid : ethanol = 1:3 for 2 hours. The root tips were stored at -20 °C until use. For the chromosome preparations, the tips were rinsed thoroughly and then incubated in a pectolytic enzyme mixture containing 0.3 % pectolyase Y23, 0.3 % cellulase RS and 0.3 % cytohelicase in 10mM citrate buffer (pH 4.5) for about 1 hour at 37 °C. Squash preparations were made in a drop of 60 % acetic acid. The microscopic slides were frozen in liquid nitrogen to remove the cover slips with a razor blade. Slides were then finally dehydrated in absolute ethanol, air-dried and stored in a -20 °C freezer until use.

C-banding and silver staining

Prior to the banding procedure, the preparations were baked overnight at 37 °C. The next morning the chromosomes were denatured in a 6 % Ba(OH)₂ solution for 8 min, followed by a wash step for 30 min with tap water. Slides were then re-annealed with 2x SSC (1x SSC contains 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) at 60 °C for 50 min, and stained with a 4 % Giemsa solution in 10 mM Sörensen buffer (pH 6.8) for 45 min. After a brief wash, the preparations were air-dried and mounted in Euparal.

For Ag-NOR staining, we used 200 mL of a freshly prepared 50 % AgNO₃ solution per slide, which was covered with a 24 x 36 mm piece of nylon cloth 'Nybolt 300'. The slides were left in a humidified petridish for 45 min at 60 °C until the nylon patch turned to yellowish. Slides were then washed briefly in running tap water, air-dried and mounted with Entellan-M (Merck) for microscopic observation.

Probe DNA

Clone pTa71 contains the 9 Kb EcoRI fragment of 45S ribosomal DNA from wheat (Gerlach and Bedbrook 1979) and pScT7 contains 462 bp BamHI fragment of 5S rDNA from rye (Lawrence and Appels 1986). Isolated DNA of 45S rDNA and 5S sequences from pTa71 and pScT7 were labelled with either biotin-16-dUTP or

digoxigenin-11-dUTP by nick translation for *in situ* hybridisation according to the manufacturer's manual (Boehringer Mannheim, Germany).

Fluorescence in situ hybridisation (FISH)

Slides with metaphase chromosome complements were left overnight at 37 °C. The next day the slides were treated with 1 µg/mL RNase A in 2x SSC at 37 °C for 60 min and washed three times of 2x SSC at room temperature for 5 min. Slides were then incubated with 10 mM HCl at 37 °C for 2 min, treated with 100 µL of a pepsin solution (5 µg/mL in 10 mM HCl) at 37 °C for 10 min, followed with washing two times in 2x SSC for 5 min, then with 4 % (para) formaldehyde solution for 10 min, and finally three times 5 min washed with 2x SSC. Before air-drying the slides were rapidly dehydrated in a graded alcohol solution series (70, 90, 100 %) for 3 min each. Samples of 40 µL of the hybridisation mixture containing 100 ng of the DNA isolated from pScT7 and pTa71, 2 mg of sheared herring sperm DNA (GIBCO BRL), 50 % deionised formamide, 10 % (w/v) sodium dextran sulphate (Sigma), 2x SSC and 0.25 % (w/v) SDS were denatured for 5 min at 70 °C and then directly put on ice for at least 5 min. Each slide with 40 µL of the hybridisation mix and covered with a slip of plastic sheet was denatured for 5 min at 80 °C and left overnight at 37 °C in a tightly closed humidified container. The slide was washed in 2x SSC buffer for 15 min, transferred to 0.1x SSC buffer at 42 °C for 30 min, and incubated for 60 min at 37 °C in blocking buffer (0.1 M maleic acid, 0.15 M NaCl, 1 % w/v blocking reagent from Boehringer Mannheim). Biotin and/or digoxigenin labelled probe DNA was detected by Cy3-Avidin-streptavidin detection system (Vector Laboratories) and FITC-anti-digoxigenin detection system (Boehringer Mannheim, Germany), respectively. All slides were counterstained with 10 mg/mL DAPI (4',6-diaminido-2-phenylindole) or 5 mg/mL propidium iodide (PI). Images were photographed with a Zeiss Axiophot photomicroscope equipped with epi-fluorescence illumination and single band filters for DAPI, FITC and Cy3/PI using 400 ISO colour negative film. The film was then scanned at 1200 dpi for digital processing with the Image software 'Photoshop' (version 5.0; Adobe Inc. USA).

Karyotype analysis and flow cytometric analysis of nuclear DNA

Chromosomes were measured with a ruler and arranged in sequence of decreased short arm length according to Stewart (1947). Total nuclear DNA contents of leaf interphase nuclei were stained in DAPI and measured with a Partec CA-II cell analyser. Relative DNA content was calculated by comparing the 2C peak in the flow karyogram with that of the *Allium cepa* nuclei, which were added as an internal reference (DNA content = 33.5 pg / 2C).

Results

An overview of all morphometric data, chromosome banding and FISH results are given in Table 1. The positions of the bands and FISH signals are depicted in the ideograms of Fig. 1.

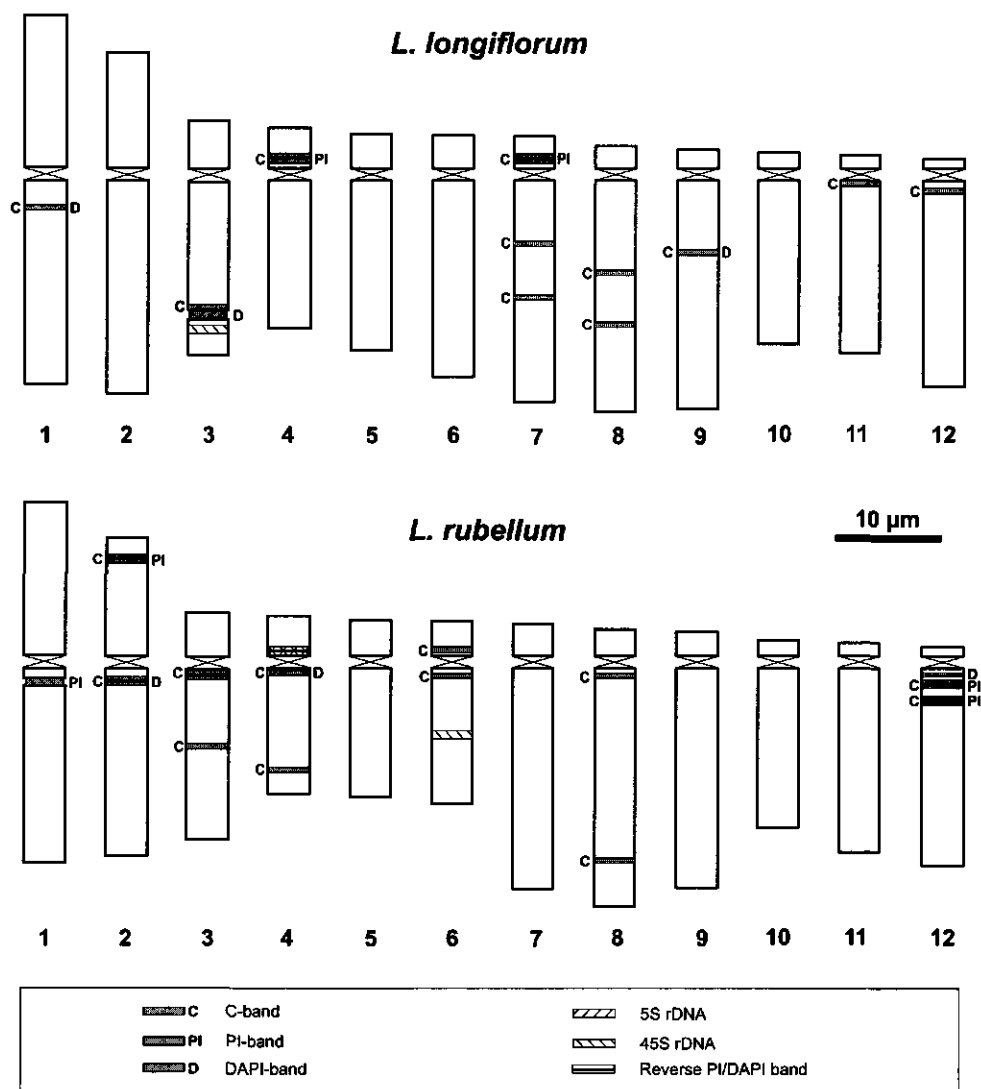


Figure 1. Ideogram of the *L. longiflorum* and *L. rubellum* indicate the positions of the C-bands, DAPI, PI, 5S rDNA and 45S rDNA sites. The reversed PI/DAPI bands were obtained from staining the chromosome preparations with a mixture of DAPI and PI, simultaneously.

Our flow cytometric analysis of DAPI stained total nuclear DNA gave 77.1 ± 0.3 pg / 2C for *L. longiflorum* and 73.6 ± 0.6 pg / 2C for *L. rubellum*, a difference of less than 5%. The karyotypes of these two species revealed a comparable small difference. Total cell complement, i.e., the lengths of all metaphase chromosomes together, amounts for 286.1 μ m for *L. longiflorum* and 269.9 μ m for *L. rubellum*. The chromosomes 1 and 2 in both species are metacentric and (sub)metacentric, respectively, and are far longer than all the other chromosomes, which make them easy to recognise in the cells. A second characteristic of their karyotypes is that the chromosomes 3 - 12 are highly asymmetrical with centromere indexes ranging from 20 to 5%. The chromosomes 4 and 7 of *L. longiflorum* and the chromosomes 2 and 4 of *L. rubellum* have macrosatellites separated from their short arms by secondary constrictions. Other secondary constrictions were observed on the long arms of chromosome 3 (subdistal) of *L. longiflorum* and long arms of the chromosomes 1, 2 and 12 (proximal).

The C-banding technique revealed 11 small bands at different positions on the chromosomes in *L.*

Table 1. Summary of the some morphometric and karyotypic data of *Lilium longiflorum* and *L. rubellum*.

	<i>L. longiflorum</i>	<i>L. rubellum</i>
DNA content		
	77.1 ± 0.3 pg / 2C	73.6 ± 0.6 pg / 2C
Chromosome length		
Longest	34.4 μ m (chr. 1)	33.8 μ m (chr. 1)
Shortest	18.1 μ m (chr. 10)	16.3 μ m (chr. 4)
Total length	286.1 μ m	269.9 μ m
Ag-NOR staining		
Signals / interphase	6	6 – 10
C-banding		
Chromosomes (bands)	1, 3, 4, 7(3x), 8(2x), 9, 11, 12	2(2x), 3(2x), 4(2x), 6(2x), 8(2x), 12(2x)
FISH		
5S rDNA alone	3	-
45S rDNA alone	3	1, 6
5S + 45S rDNA	4, 7	2(2x), 3, 4, 12
DAPI and PI bands		
DAPI alone	1, 3, 9	2, 4, 12
PI alone	4, 7	1, 2, 12
Reverse PI/DAPI bands	4, 7	2(2x), 3, 4, 12

longiflorum. We observed single proximal heterochromatin bands on the chromosomes 1, 11 and 12, a single subdistal band on chromosome 3, at the secondary constriction, and several intercalary bands on the chromosomes 7, 8 and 9. The *L. rubellum* karyotype showed a completely different pattern with a total of 12 small bands on the chromosomes 2 (2x), 3 (2x), 4 (2x), 6

(2x), 8 (2x) and 12 (2x) (Fig. 2b and d).

Ag-NOR staining of *L. longiflorum* chromosomes showed weakly stained spots on chromosome 4 only (Fig 2e). Interphase nuclei, however, displayed 6 dark silver deposits or had completely stained nucleoli. In *L. rubellum*, the Ag-NOR dots on the mitotic metaphase chromosome were even weaker or not detectable, whereas interphase cells, displayed 6 to 10 large spots.

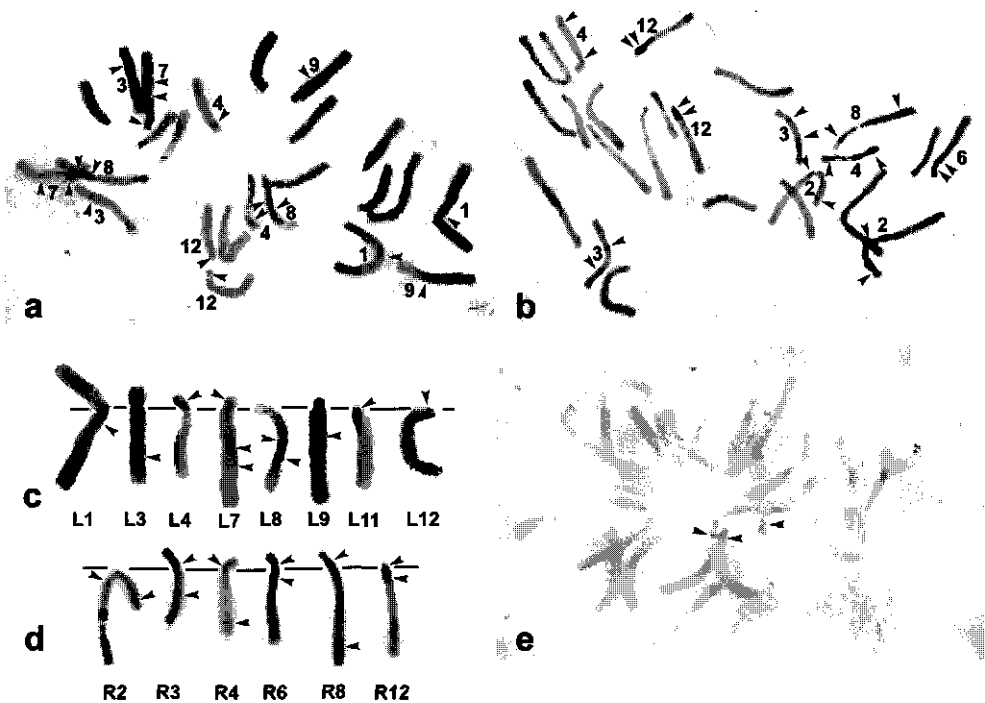
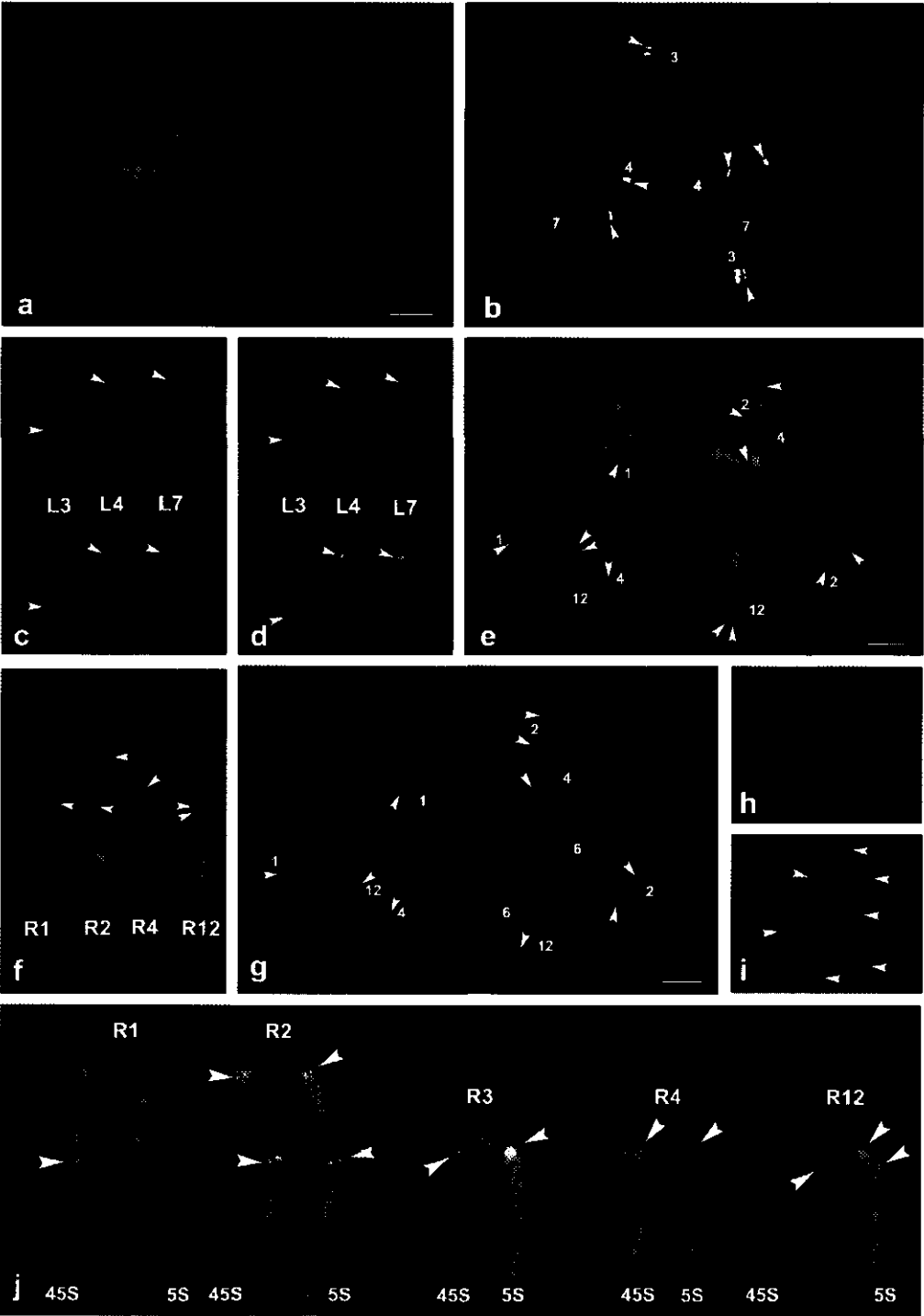


Figure 2. (a-b) Giemsa C-banding pattern of the *L. longiflorum* (a) and *L. rubellum* (b) chromosomes. (c-d) Karyotype of the *L. longiflorum* (c) and *L. rubellum* (d) chromosomes. (e) Ag-NOR staining of *L. longiflorum* chromosomes.

Reverse PI/DAPI banding revealed on the secondary constrictions of the chromosomes 4 and 7 in *L. longiflorum* chromosomes. In *L. rubellum* DAPI bands appeared positions of the proximal C-bands on chromosomes 2, 4 and 12, and PI bands at proximal position of chromosome 1, subdistal position of chromosome 2, and at two adjacent sites close to the centromere of chromosome 12.

Fluorescence *in situ* hybridisations of the 45S rDNA (pTa71 probe) to the *L. longiflorum* complement revealed signals on the secondary constrictions of



chromosome 4 and 7 and near the secondary constriction on chromosome 3. The 5S rDNA (pScT7) hybridised on the long arm secondary constriction of chromosome 3 on a small band distal from the 45S rDNA site. The probe also hybridised to the secondary constrictions of the chromosomes 4 and 7 and co-localised completely with the 45S rDNA sites. The metaphase complements of *L. rubellum* showed co-localisation of the 45S rDNA and the 5S rDNA on the secondary constrictions of the chromosomes 2, 3, 4 and 12, along with a single spot of 45S rDNA in the secondary constriction of the chromosome 1 and middle of long chromosome arm of chromosome 6 (red arrowheads in Fig. 3g). Where both rDNAs hybridised on the same chromosome site, the brightness of their fluorescence signals was often different. For example, the 5S rDNA hybridised and gave stronger signal than that of the same sequence on the proximal position of the long arm in chromosome 3 of *L. longiflorum* (Fig. 3b and c).

Discussion

At first sight this study of the *L. longiflorum* (Leucolirion section) and *L. rubellum* (Archelirion section) karyotypes presents conflicting results. The nuclear DNA amounts and basic chromosome portraits of these two species suggest largely similar genomes. The striking correspondence of chromosome morphology is not restricted to the species studied, but was found to be true for other lily species as well. In his study on the karyotypes of 39 lily species, Stewart (1947) described a basic chromosome number of 12 chromosomes for almost all important lily species, consisting of two larger (sub)metacentric and 10 generally smaller, subtelocentric chromosomes. Like in the *Triticinae*, and *Alliaceae* basic karyotype can be highly conserved in a group of related species.

Looking at the chromosome portraits more accurately reveals several obvious dissimilarities. Firstly, the number of secondary constrictions are different:

Figure 3. (a) DAPI counterstaining to the metaphase chromosomes of *L. longiflorum*. (b) Simultaneous FISH detection of probes, pTa71 (45S rDNA; white arrowheads) and pScT7 (5S rDNA; red arrowheads), to the same cell of **Figure a**. Red arrowheads represent strong signals of pScT7 probe on chromosome 3. (c) Karyotype of pScT7 (5S rDNA). (d) Karyotype of pTa71 (45S rDNA). (e) Simultaneous staining of DAPI and PI on the *L. rubellum* chromosomes. Reversed PI/DAPI bands are indicated by arrowheads. (f) Detailed karyotype of reversed PI/DAPI bands of **Figure e**. (g) Detection of pTa71 probe (45S rDNA) on the *L. rubellum* chromosomes. Red arrowheads indicate the faint signals, which were detected but are invisible in this figure. (h-i) Simultaneous detection of pScT7, indicated by red arrowheads in (h) and pTa71 probe, green fluorescence indicated by white arrowheads (i) on the interphase cell of *L. rubellum*. Note that several more signals in **Figure h** were observed, however, the intensity of the signals was low. (j) Karyotype of pTa71 (45S rDNA) and pScT7 (5S rDNA). Bars in all cases represent 10 μ m.

in *L. longiflorum* 3 sites were described for the chromosomes 3, 4 and 7 (Stewart 1947) and in *L. rubellum* 5 sites on the chromosomes 1, 2, 4 and 12 (Ogihara 1968). In general, the secondary constrictions are associated with nucleolar activity and were shown to contain the 45S rDNA genes. Such a differentiation of nucleolar organisation and distribution of heterochromatin was not restricted to *L. longiflorum* and *L. rubellum*, but was also found for other species of the same section (Stewart 1947; Ogihara 1968). For example, phenotypic characteristics and chromosome morphology such as NORs and C-banding pattern of *L. longiflorum* is almost identical to those of *L. formosanum*, another representative of the Leucolirion section (Stewart 1947; Smyth et al. 1989), whereas *L. rubellum* has similar phenotypic characters and chromosome morphology with its related *L. japonicum* and *L. auratum* (Ogihara 1966, 1968).

Taking into consideration the karyotypes of all lily species studied so far, we observed that the chromosomes 1, 2, 3 and 4 are the most likely to contain a NOR, in most cases at their secondary constrictions. All these chromosomes possess single NORs, except chromosome 2 in *L. rubellum*. Two types of wild species of *L. rubellum* were found where chromosome 2 contains single NORs on long arm, or double NORs on both arms (Ogihara 1968), the latter being confirmed by our own observations (Fig. 3e, f and j).

Sites of constitutive heterochromatin (C-bands) often coincide with the secondary constrictions, but not always so. In *L. longiflorum*, the C-bands on the chromosomes 3, 4 and 7 co-localise with the NORs of these chromosomes, and so with the nucleolar organizers, whereas the remaining bands on the chromosomes 1, 7, 8, 9, 11 and 12 were not associated with these rDNA domains. This banding pattern, which is in agreement with the studies of Stewart (1947), Von Kalm and Smyth (1984), Kongsuwan and Smyth (1978) and Smyth et al. (1989), is also similar to *L. formosanum* of the same section (Smyth et al. 1989). In *L. rubellum*, most C-bands co-localise with the secondary constrictions and few beside at interstitial positions (Fig. 3d), a banding pattern, which resembles that of *L. auratum* and other species belonging to the Archelirion section (cf. Smyth et al. 1989).

The comparison of C-bands and PI/DAPI bands demonstrates that the genomes of lily species can contain molecularly different classes of heterochromatin. In *L. longiflorum*, C- / DAPI bands revealed on the chromosomes 1, 3, and 9, C- / PI bands on the chromosomes 4 and 7, and the remaining C-bands on the chromosomes 7, 8, 11 and 12 without deviant DAPI / PI fluorescence ratio (Fig. 1). A comparable situation of three C-heterochromatin classes is true for the *L. rubellum* chromosomes, although completely different chromosomes are involved. Further confirmation about the nature of the secondary constrictions can be derived from

silver staining of metabolically active NORs and FISH using the 45S rDNA as probe. Ag-NOR staining has been applied successfully to various lily species for the detection of NORs (Smyth et al. 1989; Von Kalm and Smyth 1980, 1984). However, our silver deposits on the chromosomes were quite faint in *L. longiflorum* and undetectable in *L. rubellum*, so that no reliable evidence for the sites of active NORs could be obtained. It is still questionable as to whether the less condensed chromosomes of the *in vitro* grown root tips (25 °C) used in this study has a negative effect on NOR detection by silver nitrate or that the prior transcribed ribosomal RNA genes had already disappeared from the NORs at metaphase (Von Kalm and Smyth 1984).

Our FISH detection of 5S and 45S rDNA genes clearly showed their locations and correspondence with the NORs and secondary constrictions (Fig. 3c, d and j). The following classes can be distinguished: (1) 45S + 5S rDNAs, as on the *L. longiflorum* chromosomes 4 and 7, and the *L. rubellum* chromosomes 2, 3, 4 and 12; (2) only 45S rDNA, as on the *L. longiflorum* chromosome 3 and the *L. rubellum* chromosomes 1 and 6; (3) only 5S rDNA, as on the *L. longiflorum* chromosome 3 and the *L. rubellum* chromosome 12. Only few of them could be associated with NOR activity. The 45S signals, which are located in secondary constrictions, were mostly larger and brighter than those of the 5S probe. However, the 5S rDNA spot on chromosome 3 in both *L. longiflorum* and *L. rubellum* species was stronger than that of the 45S rDNA probe (Fig. 3b, c, d and j).

All 45S rDNA sites were found to co-localise with simultaneous staining of DAPI and PI (reverse PI/DAPI band) with the exception of the long arm sites on chromosome 3 of *L. longiflorum* and chromosome 6 in *L. rubellum*, and it is assumed that the representative specific tandem repeat families intermixed with the 45S rDNA tandem repeats. Schulz-Schaeffer (1980) described that satellites are generally attached to the short arm of a nucleolus organiser chromosome. The present results confirmed that the reverse PI/DAPI bands were shown either in the secondary constriction of the short-arm or long-arm near to the centromere (Fig 1). The same type of reverse PI/DAPI bands has been demonstrated in *Lycopersicon* and *Oryza* species as well (Peterson et al. 1999; Andras et al. 2000). As a similar case of the reverse PI/DAPI bands, one of the genomic probe DNA can also hybridised to the tandemly repeated regions such as NORs of the other species chromosomes (Parokonny et al. 1997). On the other hand, genomic probe DNA was not hybridised to the position of NORs of the same species chromosomes (see Fig 1c and f in Lim et al. 2000).

DAPI is known to bind preferentially on A-T rich heterochromatic regions or as double-stranded DNA-specific dye (Trask 1999), however PI intercalates between

bases of either single- or double-stranded nucleic acid molecules (Heslop-Harrison and Schwarzscher 1996). NORs are composed of tandemly repeated G-C sequences (Macgregor and Kezer 1971; Yasmineh and Yunis 1971; Ingle et al. 1975). Therefore, DAPI-PI simultaneous staining can give a red band in NORs position. These bands position could be similar to DAPI negative bands in G - C rich region. We found that repetitive bands are located mostly at the same position with rDNA sites, and not only on the short arm but also on the long arm adjacent to the centromere (Fig 3f).

In conclusion, this study provides a most detailed karyotype analysis of *L. longiflorum* and *L. rubellum* species chromosomes. In spite of their similarity in chromosome shapes, their C-banding, DAPI/PI bands, and rDNA sites have dramatically changed positions on the putative homoeologues. Previous GISH studies of *L. longiflorum* \times *L. rubellum* hybrids also showed differences in chromosome length between two species (see Fig. 1c in Lim et al. 2000). It is likely that such genomic DNA differentiation results from entire replacements of Ty/copia or related dispersed repeat families during the evolution of lily species. New questions on the nature and molecular organisation of the C-bands, the presence and distribution of satellite DNA families, the activity and silencing of rDNA sites are next to be answered. For the breeder and geneticist it is likely more important to know as to whether these changes of chromosome morphology, banding pattern and molecular organisation also reflect large-scale chromosomal rearrangements like translocations and inversions. Preliminary observations of regular bivalent formation in metaphase I microsporocytes of *L. longiflorum* \times *L. rubellum* hybrids seem to suggest that collaboration between both homoeologous genomes still exists.

3

Introgression of *Lilium rubellum* Baker chromosomes into *Lilium longiflorum* Thunb.: a genome painting study of the F₁ hybrid, BC₁ and BC₂ progenies

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Abstract

Interspecific hybrids between *Lilium longiflorum* (L, $2n=2x=24$) and *Lilium rubellum* (R, $2n=2x=24$) were produced with the aim of transferring desirable horticultural traits from *L. rubellum* to *L. longiflorum*. All F_1 -hybrids (LR, $2n=2x=24$) and BC_1 individuals (LLR, $2n=3x=36$) were phenotypically uniform for plant height, flowering time, leaf shape and flower colour. The BC_1 plants were, in spite of their triploid nature, fertile and could be used as a female parent in backcrossings with autotetraploid *L. longiflorum* (LLLL, $2n=4x=48$). Twelve BC_2 individuals were obtained and three of them were selected for further chromosome analysis. As *L. longiflorum* and *L. rubellum* chromosomes were indistinguishable in the hybrids, genomic *in situ* hybridisation (GISH) was applied to establish the parentage of the chromosomes of the F_1 -hybrids and the BC_1 and BC_2 progenies. GISH confirmed the LLRR constitution of the doubled LR (allodiploid), and the LLR constitution of all BC_1 plants. The three selected BC_2 plants were, as expected, aneuploid, containing three complete sets of *L. longiflorum* chromosomes and six, seven or eight *L. rubellum* chromosomes, respectively. However, L/R translocation or recombinant chromosomes could not be demonstrated in the mitotic metaphase complements of the F_1 , BC_1 and BC_2 plants. In spite of the high frequencies of homoeologous recombination in the F_1 -hybrids (LR), pollen was found sterile in all cases. At metaphase I of the pollen mother cells of the BC_1 plants genome painting did not reveal any cases of homoeologous pairing and recombination between L and R chromosomes. This lack of exchange between homoeologous chromosome segments indicates complete preferential pairing of the L- and R-chromosomes in the F_1 (amphidiploid) and BC_1 plants. It seems that the preferential pairing in the F_1 and BC_1 hybrids hinders the introgression of the chromosome segments or species-specific genes into the recipient for breeding purposes.

Key words: *Lilium longiflorum* • *Lilium rubellum* • Interspecific hybrid • Genomic *in situ* hybridisation (GISH) • Preferential pairing

Introduction

The genus *Lilium* (Liliaceae) comprises over 80 species, which are classified into seven taxonomic sections (Comber 1949; De Jong 1974). All species are endemic to the mountainous area in the Northern Hemisphere and many of them have been used as basic materials for commercial breeding. Lily species have an exceptionally large genome size (*L. longiflorum* = 141.1 pg/cell, Bennett and Smith 1976, 1991) and

all of them are diploid ($2n = 2x = 24$), except for *L. tigrinum* of which triploids occurred as well (Stewart and Bamford 1943; Noda 1978).

The three economically most important sources for lily breeding are 1) *L. longiflorum* of the section *Leucolirion*; 2) the Asiatic hybrid group of the section *Sinomartagon* and 3) the Oriental hybrid group including *L. rubellum* of the section *Archelirion*. The transfer of desirable traits to commercial cultivars by interspecific hybridisation is difficult because of pre- and post-fertilisation barriers (Van Tuyl et al. 1991), although a few reports claim that production of interspecific hybrids is successful when the cut style pollination method (Asano and Myodo 1977a; Asano 1980a) and *in vitro* culture techniques combined with ovary, ovule and embryo cultures are applied (Asano and Myodo 1977b; Van Tuyl et al. 1991).

The production of interspecific hybrids between *L. longiflorum* and *L. rubellum* is interesting for the introduction of the pink flower colour of the latter species. The F_1 -hybrids that were obtained between *L. longiflorum* 'Gelria' and *L. rubellum*, all had pink flowers. To eliminate undesirable traits, backcrosses with *L. longiflorum* were performed. The most widely used approach in the traditional method of introgression is to double the chromosome number of a sterile F_1 plant of the interspecific hybrid in order to restore fertility and to use it as a parent in the backcrossing program.

Genomic *in situ* hybridisation (GISH), which enables the distinction of the parental chromosomes in a large number of intergeneric and interspecific hybrids (Schwarzacher et al. 1989; Anamthawat-Jonsson et al. 1990; Jacobsen et al. 1995; Takahashi et al. 1997; Kamstra et al. 1999a,b), has been applied successfully to the lily hybrids (Karlov et al. 1999) in order to establish the number of *L. rubellum* chromosomes in backcross plants. GISH also allows the detection of recombinant L/R chromosomes, which are the result of homoeologous pairing and crossing-over or of translocations between L and R chromosomes.

The aims of the present investigation were (1) to establish the number of *L. longiflorum* and *L. rubellum* chromosomes in the F_1 , BC_1 and BC_2 plants, (2) to determine whether homoeologous recombination has occurred in the F_1 hybrid and in the BC_1 plants or not and (3) to assess transmission of the *L. rubellum* chromosomes in the BC_1 and BC_2 individuals for the introgression of pink flower colour and early flowering habit.

Materials and methods

Plant material

Since F_1 -hybrids (*L. longiflorum* 'Gelria' \times *L. rubellum*) showed absolute sterility,

mitotic polyploidisation was performed by *in vitro* treatment of the bulb scales in 0.003 % oryzalin (3,5-dinitro- N^4,N^4 -dipropylsulfanilamide) solution for 3 hours. The selected amphidiploid (LLRR) plants resulting from artificial chromosome doubling were checked for pollen viability by FDA (Fluorescein diacetate) staining and pollen germination experiments. The LLRR hybrids were used as male parent in the back-cross with *L. longiflorum* 'Snow Queen' ($2n=2x=24$) and over a hundred BC_1 plants were obtained. Two BC_1 plants were selected and pollinated with tetraploid (LLLL) *L. longiflorum* (Table 1). Two F_1 , nine BC_1 and three BC_2 individuals were used for GISH analysis (Table 2). Plants were grown in a greenhouse at 20 – 25 °C during the day and 14 – 18 °C during the night.

Table 1. Genome composition and average value of several phenotypic characters of the parents (*L. longiflorum* and *L. rubellum*), F_1 hybrid and amphidiploid hybrids, BC_1 and BC_2 progenies.

Genome composition ^z	Genotype & parentage	Phenotypic characters			
		Flower colour	Leaf shape	Plant height (cm \pm SD)	Forcing time (days \pm SD)
Parents LL	<i>L. longiflorum</i> 'Gelria'	White	Narrow	101 \pm 3.7	95 \pm 4
RR	<i>L. rubellum</i>	Dark pink	Wide	ND ^y	ND
F_1 (2x) LR	<i>L. longiflorum</i> 'Gelria' \times <i>L. rubellum</i>	Pink	Wide	41 \pm 8.9	47 \pm 1
F_1 (4x) LLRR	Chromosome doubled LR-hybrid	Pink	Wide	48 \pm 4.5	53 \pm 3
BC_1 (3x) LLR	<i>L. longiflorum</i> 'Snow Queen' \times LLRR	Light pink	Intermediate	82 \pm 8.2	75 \pm 3
BC_2 LLLR	LLR \times <i>L. longiflorum</i> (4x)	Light pink or white	ND	ND	ND

^z L and R represent *L. longiflorum* and *L. rubellum*, respectively.

^y ND means not determined.

Chromosome preparation

For the study of mitotic metaphase complements, the fast growing root tips were collected in the morning and pretreated in a saturated α -bromonaphthalin solution for 2 hours at 20 °C followed by an overnight treatment at 4 °C. The material was fixed in Carnoy's solution (acetic acid : ethanol = 1:3) and stored at -20 °C until use. Anthers at the stage of meiosis were fixed directly in Carnoy's solution. Before making squash preparations, root tips and anthers were similarly incubated in a pectolytic enzyme mixture containing 0.3 % pectolyase Y23, 0.3 % cellulase RS and 0.3 % cytohelicase in 10 mM citrate buffer (pH 4.5) for about 1 – 1.5 hour at 37 °C. Squash preparations were made in 60 % acetic acid. Slides were frozen in liquid nitrogen and the cover slips were removed by using a razor blade. Slides were

finally dehydrated in absolute ethanol for a few minutes, dried and stored at -20°C until use.

DNA probes preparation

Total genomic DNA of *L. longiflorum* 'Snow Queen' was used as a probe. The probe DNA was labelled with digoxigenin-11-dUTP by nick translation according to the manufacturer's instructions (Boehringer Mannheim). Blocking DNA was obtained by autoclaving herring sperm DNA for 5 min at 121°C . The size of blocking DNA ranged from 100 to 500 bp.

Genomic in situ hybridisation

The *in situ* hybridisation protocol was carried out according to Chapter 2. Briefly, slides were pretreated with RNase A ($1\text{ }\mu\text{g/mL}$) for 1 hour and pepsin ($5\text{ }\mu\text{g/mL}$) for 10 min, both at 37°C , followed by formaldehyde (4 %) for 10 min at 20°C , dehydration with 70 %, 90 % and absolute ethanol for 3 min and air dried. Hybridisation followed using a mixture consisting of 2x SSC, 50 % formamide, 10 % sodium dextran sulfate, 0.25 % SDS, $2.0\text{ ng}/\mu\text{L}$ digoxigenin-11-dUTP labelled total genomic DNA of *L. longiflorum* 'Snow Queen' and $30\text{--}40\text{ ng}/\mu\text{L}$ herring sperm DNA for blocking. The DNA was denatured by heating the hybridisation mixture at 70°C for 10 min and then placed on ice for 10 min. For each slide, $40\text{ }\mu\text{L}$ hybridisation mixture was used. The preparations were denatured at 80°C for 10 min. After overnight hybridisation at 37°C in a humid chamber slides were washed at room temperature in 2x SSC for 15 min, 0.1x SSC at 42°C for 30 min. The digoxigenin labelled probe DNA was detected with $20\text{ }\mu\text{g/mL}$ anti-dig-FITC (fluorescein isothiocyanate; Boehringer Mannheim) and $20\text{ }\mu\text{g/mL}$ rabbit-anti-sheep-FITC (Vector Laboratory). Slides were counterstained with DAPI (4',6-diamidino-2-phenylindole) or propidium iodide (PI) and examined under the Zeiss Axiophot microscope equipped with epi-fluorescence illumination and single band filters for DAPI, FITC and PI. Images were photographed on 400 ISO colour negative film and scanned at 1200 dpi for digital processing in "Photoshop 5" (Adobe Inc.).

Results

Phenotypic and cytogenetic observations of the diploid and amphidiploid F_1 -hybrids

Phenotypic characteristics of the F_1 -hybrids and their backcross progenies were intermediate between the parents. All the F_1 -hybrids showed pink colour, which is not present in *L. longiflorum*.

As a control experiment in the F_1 -hybrids, GISH with *L. longiflorum* genomic DNA probed to the somatic metaphase chromosomes of the parental species gave bright signal on all L-chromosomes and no signal to the R-chromosomes. This indicates that the chromosome sets of the two different species in the F_1 -hybrids can be unequivocally identified.

In spite of the striking GISH differentiation between the parental genomes in the diploid (LR) F_1 -hybrids, the occurrence of homoeologous pairing between the L- and R-chromosomes was often seen in metaphase I complements (Fig 1a). We observed only rod bivalents, containing single L- and R-chromosomes and they were bound by a single chiasma. The meta- or submetacentric chromosomes were more often involved in bivalent formations than the acro- or subacrocentric chromosomes. The frequency of bivalents at metaphase I in the F_1 hybrid (LR) was $3.2_{II} + 17.6_I$.

Genome painting of both mitotic chromosomes (Fig. 1b) and meiotic chromosomes in pollen mother cells of the amphidiploid confirmed the presence of two complete L- and R-genomes apparently without any recombinations or translocations. Only bivalent formation between homologous L-L, and R-R chromosomes were observed resulting in balanced LR gametes and fertile pollen.

FDA staining of pollen of the LR hybrids was zero percent while the LLRR hybrid's ranged from 40 – 50 %, indicating the range of pollen fertility. However, this pollen only showed about 20 % of germination.

GISH analysis of BC_1 progenies

All BC_1 plants from the cross combination LLRR \times LL were phenotypically intermediate between the two parents with respect to plant height, flowering time, flower colour and leaf shape (Table 1). GISH studies showed that the chromosome

Table 2. Genome constitution and chromosome composition of the F_1 -hybrids, BC_1 and BC_2 progenies as determined by GISH.

Genome composition	Accession number	No. of individuals examined	Somatic chromosome number (2n)	Chromosome constitution	
				L	R
F_1 (2x) LR	921250	2	24	12	12
F_1 (4x) LLRR ²	940303	1	48	24	24
BC_1 LLR	961003	9	36	24	12
BC_2	LLL	982275-1	1	43	36
	LLR	982211-20	1	42	36
	LLR	982211-27	1	44	36

² LLRR was derived from artificial chromosome doubling of the LR-hybrid.

constitution was $2n=3x=36$ (LLR) in all cases (Table 2). In the triploid (LLR) BC_1 plants, there was no evidence for the presence of homoeologous chromosomes (Fig. 1c and d).

BC₂ progenies and their chromosome constitution

Three best growing BC_2 plants were selected for further cytogenetic analysis. GISH revealed that three intact L-genomes (LLL) were present plus six, seven or eight R-genome chromosomes, respectively (Fig. 1e and f). None of the three BC_2 plants displayed L/R recombinant chromosomes, indicating that as expected, homoeologous recombination was absent in the BC_1 plants. Among three BC_2 individuals, two plants were light pinks and one was white.

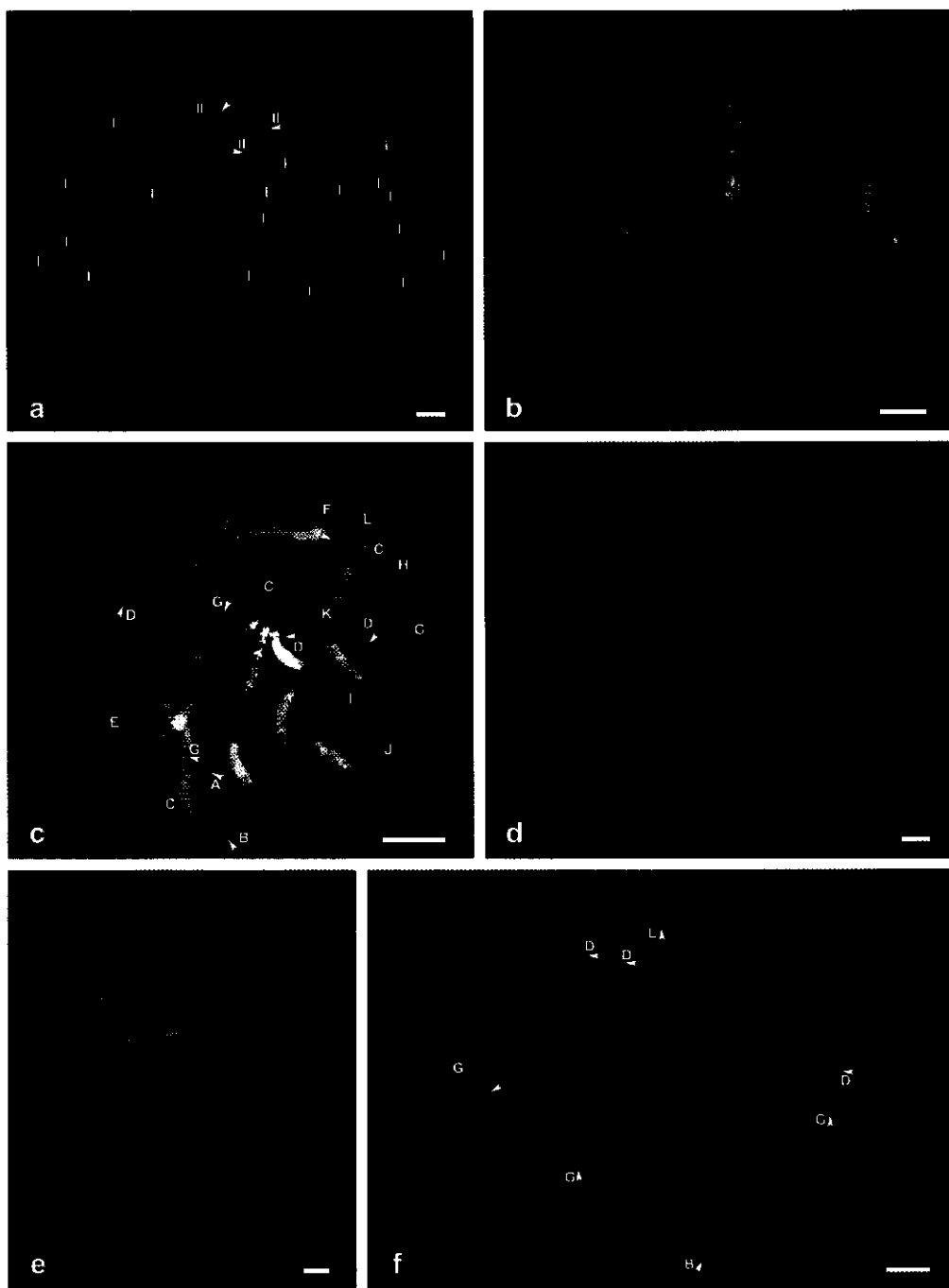
Discussion

This investigation demonstrates that GISH enables unequivocal distinction of the *L. longiflorum* and *L. rubellum* chromosomes in F_1 -hybrids, BC_1 and BC_2 progenies. Thus, the genome differentiation between *L. longiflorum* and *L. rubellum* supports the taxonomic classification into different sections, viz., Leucolirion and Archelirion.

Meiotic chromosome behaviour in many interspecific lily hybrids has been reported (Asano 1984; Richardson 1936). As a rare case, the allotriploid *L.* × 'Fire King' revealed a maximum of 12_{III} (Noda, 1971). Because of a high degree of genomic differentiation, the LR hybrid formed 3.2_{II} on average with a maximum of five bivalents. As expected, after somatic chromosome doubling, the amphidiploid (LLRR) F_1 hybrid showed only bivalent pairing between L-L and R-R chromosomes. This lack of homoeologous pairing is obviously the result of preferential pairing between L-L and R-R homologous chromosomes. The BC_1 plants (LLR) showed perfect preferential pairing which resulted to 12_{II} (L-L) + 12_I (R). Similar results have been reported in interspecific triploids between *L. longiflorum* (4x) and *L. cernuum* (2x) (Asano 1984).

One great disadvantage of the somatic chromosome doubling is the occurrence of homologous pairing in the amphidiploid, which reduces the prospects for intergenomic recombination dramatically. This explains why recombinant chromosomes are absent in all the BC_1 plants analysed. In other words, the $2x$ -gametes contributed by the amphidiploid possessed intact L- and R-genomes, i.e., without any homoeologous recombination.

If the final aim of the interspecific hybridisation program is to recombine specific horticultural traits, intra-chromosomal recombination is essential and it cannot be achieved as long as the present approach is used. At best, only intact



alien *L. rubellum* chromosomes can be introduced into *L. longiflorum*. The disadvantage of adding or substitution of alien chromosomes is that recessive traits of the alien species are not expressed in the alien addition genotypes.

In order to enable the expression of recessive phenotypes in the alien genetic background one has to substitute entire chromosome(s) by its alien homoeologue in the cultivar. This is highly laborious and depending upon the genetic load of the chromosomes involved. Unlike using amphidiploids, the use of numerically unreduced (2n) gametes produced by the diploid F_1 -hybrids can greatly enhance the possibility of obtaining homoeologous recombination as has been demonstrated recently in another interspecific lily hybrid (Karlov et al. 1999). Progeny with recombinant chromosomes often have considerable opportunity for substituting either parts or entire alien chromosomes in a species or a cultivar (Kamstra et al. 1999a,b).

The transfer of genes and chromosomes from alien species and genera has contributed a great deal in the improvement of numerous crops in the past. Because the process of introgression was laborious, time consuming and the results are not always predictable, there has been a tendency for scepticism in using wild species in breeding. The advent of molecular approaches such as GISH, have substantially contributed to render the process of introgression more efficient.

Figure 1 a-f. Genomic *in situ* hybridisation of mitotic and meiotic chromosomes of the F_1 , BC_1 and BC_2 plants. (a) Metaphase I of the PMC in F_1 -hybrid (LR, '921250-2'; *L. longiflorum* × *L. rubellum*) shows one chiasma in each bivalent of metacentric and subacrocentric chromosomes. The digoxigenin labelled *L. longiflorum* DNA was detected with anti-Dig FITC (yellow fluorescence) and counterstained with propidium iodide (red fluorescence) indicating the *L. rubellum* chromosomes. (b) Chromosome painting of the F_1 -hybrid '940303' (LLRR) derived from artificial chromosome doubling of the LR-hybrid '921250-2'. The probe DNA was used and detected the same as **Figure 1a**. (c) Thirty-six chromosomes of the BC_1 plant '961003-27' without any recombinations. *L. longiflorum* (green fluorescence) and *L. rubellum* (blue fluorescence). Letters indicate the chromosome number according to Stewart (1947). Arrowheads represent the reversed PI/DAPI bands on the NOR bearing chromosomes in both species. (d) The meiotic chromosomes of BC_1 '961003-27' with twelve bivalents (yellow fluorescence) indicating *L. longiflorum* and twelve univalents (red fluorescence) representing *L. rubellum*. (e) Aneuploid BC_2 plant '982275-1' from backcrossing of the BC_1 (LLR) to 4x (LLLL) *L. longiflorum*. Thirty-six chromosomes (three sets) of *L. longiflorum* (green fluorescence) with seven *L. rubellum* chromosomes (blue fluorescence). (f) Aneuploid BC_2 plant '982211-27' from backcrossing of the BC_1 (LLR) to 4x *L. longiflorum*. Thirty-six chromosomes (three sets) of *L. longiflorum* (yellow fluorescence) with eight *L. rubellum* chromosomes (red fluorescence). Arrowheads indicate the reversed PI bands on the *L. longiflorum* chromosomes or PI bands on the *L. rubellum* chromosomes in the position of NORs.

4

Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH

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Abstract

A detailed analysis of microsporogenesis on three diploid lily cultivars ($2n=2x=24$) and three interspecific hybrids ($2n=2x=24$) revealed how meiotic nuclear restitution mechanisms lead to $2n$ -gametes. In the cultivars, 'Gelria' (*L. longiflorum*: L-genome), 'Connecticut King' and 'Mont Blanc' (both Asiatic hybrids; A-genome), meiosis was regular resulting in haploid gametes and there was no evidence for $2n$ -gamete formation. Three interspecific hybrids between *L. longiflorum* \times Asiatic hybrid (LA) showed a variable frequency of meiotic nuclear restitution and stainable $2n$ -pollen formation ranging from 3 % to 30 %. Important features of meiotic chromosome behaviour of the LA-hybrids through *in situ* hybridisation techniques were: 1) the discrimination of univalents, half-bivalents and bivalents of parental chromosomes; 2) the observation of simultaneous consequences of equational division of univalents and normal reductional division of bivalents (half-bivalents); and 3) the determination of chromosome composition of the nuclei that resulted from meiotic nuclear restitution. Based on the meiotic chromosome composition we propose a new meiotic nuclear restitution mechanism called IMR (*indeterminate meiotic restitution*). The distinctive features of IMR are that univalents divide equationally as in FDR and that bivalents disjoin as in SDR simultaneously in the same pollen mother cell. The net result in LA-hybrids is that a low frequency of functional restitution gametes is formed in which genetic recombinations occur due to crossing-over as well as to assortment of homoeologous chromosomes.

Key words: *Lilium* • chromosome analysis • meiotic polyploidisation • first division restitution (FDR) • second division restitution (SDR) • fluorescent *in situ* hybridisation (FISH)

Introduction

It has been estimated that more than 70 % of the flowering plants are polyploids (Soltis and Soltis 1993; Masterson 1994; Leitch and Bennett 1997; Ramsey and Schemske 1998). These include many important crops such as wheat (*Triticum aestivum* L.), potato, cotton, sugarcane and others. Among horticultural crops, spontaneous polyploids have contributed greatly to the development of cultivars (Sanford and Hanneman 1982). Thus, polyploidy is an important phenomenon both for the evolution as well as in the development of new varieties. Despite the appreciation of the importance of polyploidy in plants, little attention has been paid to their mode of origin. The early belief that polyploids originated from chromosome doubling of somatic cells (mitotic polyploidisation) was seriously

questioned by Harlan and De Wet (1975). They argued that numerically unreduced (2n) gametes have played a predominant role in the origin of polyploids. Meiotic abnormalities that lead to the formation of 2n-gametes, the so-called, meiotic nuclear restitution, were described for the first time by Rosenberg (1927). Subsequently, various types of meiotic nuclear restitution mechanisms, such as semiheterotypic division, pseudohomoeotypic division, mitotised meiosis, first division restitution (FDR), second division restitution (SDR), pre- and post-meiotic doubling, have been documented (for reviews, see Ramanna 1979; Veilleux 1985). From several investigations, it is clear that meiotic nuclear restitution leading to 2n-gamete formation can occur in normal fertile plants as well as in interspecific hybrids that are otherwise sterile.

In *Lilium* ($2n = 2x = 24$), as in most of the other plant taxa, wide interspecific hybrids are in general sterile. This holds true especially for hybrids of species belonging to different sections. Sterility in these hybrids mainly results from irregular chromosome association between the parental genomes during meiosis (Asano 1982a, 1984). When completely sterile, interspecific hybrids are only useful for mutation breeding. One way of restoring the fertility of sterile diploid lily hybrids has been to double the chromosome number by chemical treatment (Van Tuyl et al. 1992). A major drawback of this approach is that, due to preferential parental chromosome association in the amphidiploid, intergenomic recombination by crossing-over is extremely decreased (Asano 1982; Lim et al. 2000). This approach is obviously not favourable for introgression breeding.

Fortunately, certain interspecific lily hybrids produce relatively high frequencies of numerically unreduced (2n) gametes (Asano 1984; Van Tuyl et al. 1989). Such hybrids offer the prospect of using the diploid hybrid genotypes directly for introgression breeding without the need for mitotic chromosome doubling. In lily, this possibility has already been demonstrated in chromosome analysis of some progenies derived from the functioning of 2n-gametes (Karlov et al. 1999). In this report, two types of 2n-gametes were functional in the backcross (BC₁) progenies of lilies. These included FDR gametes with and without recombinant chromosomes.

Although both FDR and SDR mechanisms have been shown to occur in some plant species, the cytological evidence of the origin is still rare. Half-tetrad analysis (Mok and Peloquin 1975) and genetic or molecular markers (Bastiaanssen et al. 1996) have been used to elucidate the restitution mechanisms. FDR 2n-gametes comprise the non-sister chromatids of each homoeologous chromosome in an interspecific hybrid resulting in 12 chromosomes from each parent. However, SDR 2n-gametes contain the sister chromatids from one parent and the homoeologous sister chromatids will be included in the counterpart 2n-gamete within a dyad (if the

composition of parental chromosome of one gamete is 6A+8B then the counterpart should be 8 A+6 B).

Microsporogenesis in interspecific hybrids of *Lilium* was investigated using *in situ* hybridisation to elucidate the mechanisms of meiotic nuclear restitution and their consequences. This study has led to the discovery of a new mechanism of restitution gamete formation. This mechanism (IMR: *indeterminate meiotic restitution*) has been illustrated and discussed in relation to other results.

Material and methods

Plant material

Bulbs of *L. longiflorum* Thunb 'Gelria' (L-genome) and two Asiatic hybrids 'Connecticut King' and 'Mont Blanc' (A-genome) were used from a commercial stock. Three 2n-gamete producing diploid ($2n=2x=24$) interspecific LA-hybrids of *L. longiflorum* 'Gelria' \times Asiatic hybrid 'Whilito' were developed at Plant Research International (Van Tuyl et al 1989; Karlov et al 1999). These interspecific hybrids were indicated LA₁ to LA₃. All F₁ LA-hybrids were known to produce viable 2n-pollen. Bulbs for collecting root tips and anthers were planted in pots with peat based soil mixture and grown in a greenhouse with day and night temperature at 18 – 25°C/14 – 18°C, respectively.

Cytology

For the analysis of microsporogenesis, young anthers with prophase I through telophase II stages were fixed in a freshly prepared solution of 3:1 (v/v) ethanol : acetic acid for 1-2 h at room temperature. Parts of fixed anthers were squashed in a drop of 2 % acetocarmine to determine appropriate meiotic stage and usable slide preparations were prepared for GISH analysis. In order to study the process of cytokinesis and the sporad stages, we mounted pollen mother cells in a drop of lactophenol acid-fuchsin under a microscope. Similarly, pollen from fully opened flowers was used for stainability and size classification as small (n) and large (2n). Pollen size was determined using a calibrated micrometer.

Anthers were incubated in a pectolytic enzyme mixture containing 0.3 % pectolyase Y23, 0.3 % cellulase RS and 0.3 % cytohelicase in 10mM citrate buffer (pH 4.5) at 37 °C for about 1 – 1.5 hour. Preparations were squashed in a drop of 60 % acetic acid and frozen in liquid nitrogen; the cover slips were removed by using a razor blade. Slides were dehydrated in absolute ethanol for a few minutes, dried and stored at -20 °C until use.

Genomic and fluorescence in situ hybridisation

The GISH protocol was according to Chapter 2 with minor modifications. For detection of 45S rDNA, we used a pTa71 probe (18S-5S-25S rDNA sequence; Gerlach and Bedbrook, 1979) after labelling with biotin-16-dUTP using the nick translation protocol (Boehringer Mannheim, Germany). The hybridisation mixture was denatured at 70 °C for 10 min and placed on ice for about 5 min, and 40 µL of hybridisation mixture was applied to each slide, denatured at 80 °C for 5 min and incubated overnight at 37 °C with high humidity. After hybridisation, slides were washed in 2x SSC for 15 min at room temperature, followed by 0.1x SSC for 30 min at 42 °C. Digoxigenin-labelled probe DNA was detected with the FITC-anti-digoxigenin detection system (Boehringer Mannheim, Germany). Biotin-labelled probe DNA was detected with the avidin-streptavidin detection system. All slides were counterstained with DAPI (4',6-diaminido-2-phenylindole) or PI (propidium iodide). Images were photographed on 400 ISO colour negative film and scanned at 1200 dpi for digital processing in the software program, Photoshop 5.0 (Adobe Systems Inc).

Chromosome numbering

A revised method of nomenclature of *Lilium* chromosomes in karyotypes has been proposed by Lim et al. (2000) in which the chromosomes were numbered from 1 for the longest short-arm chromosome to 12 for the chromosome with the shortest short-arm. The genomes of the species are indicated by L and A following the individual chromosome number for *L. longiflorum* and the Asiatic hybrids, respectively.

Results

Microsporogenesis was analyzed in the three diploid cultivars ($2n=2x=24$), 'Gelria', 'Connecticut King' and 'Mont Blanc' and in three diploid LA-hybrids. In all three cultivars, chromosome association was normal with the formation of 12 bivalents (data not shown). Following this, anaphase I disjunction was normal and the notable feature was that at the end of telophase I the resulting nuclei were separated by the formation of the cell wall, the so-called reductional wall. After the pro-, meta- and anaphase II stages, the equational wall was formed at telophase II giving rise to a tetrad. Thus, in *Lilium* microsporogenesis is of the "successive type" as far as cell wall formation is concerned. In view of regular meiotic divisions in all

the three diploid cultivars, pollen fertility was expected to be high as shown in Table 1.

In the three LA-hybrids pollen stainability was lower, i.e., 4 %, 40 % and 10 %. The size of 3 %, 30 % and 7 % of the pollen was large indicating 2n-gametes, of which <1 %, 12 % and 8 %, respectively, germinated on an artificial germination medium (Table 1). This indicates that LA₁ 2n-gametes behave differently in comparison with those of LA₂ and LA₃.

Table 1. Percentage (%) of stainable, large and small pollen grains and germination in six different genotypes of *Lilium*.

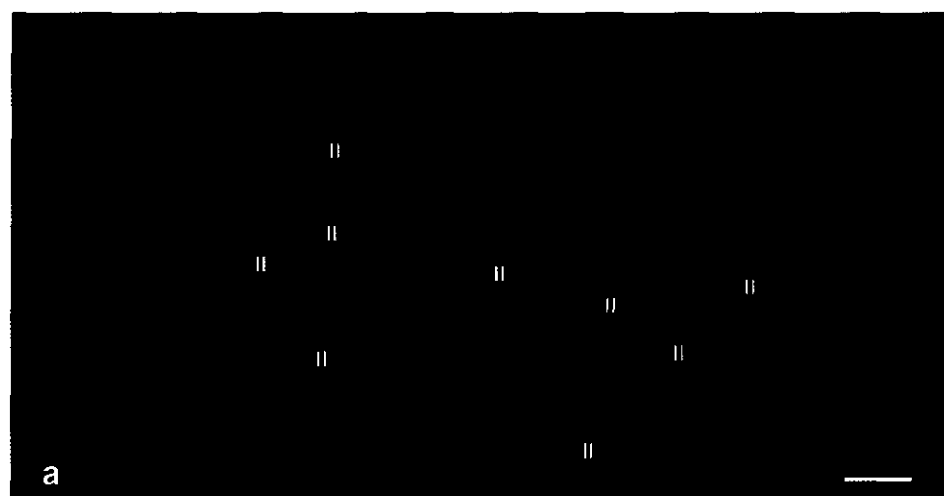
Genome, cultivar or accession	Total	Number (%) of stainable pollen counted				Germination ^c (%)
		Stained pollen ^a	Large (2n)	Small (n)	Size (µm) ^b	
L-genome, 'Gelria'	1187	761 (64)	0 (0)	761 (64)	89±7	54
A-genome, 'Connecticut King'	942	610 (65)	1 (0)	609 (65)	59±6	53
A-genome, 'Mont Blanc'	1021	883 (86)	2 (0)	881 (86)	56±4	60
LA-hybrids, '88542-24'	1403	58 (4)	45 (3)	13 (1)	98±9	<1
'88542-52'	568	227 (40)	171(30)	57 (10)	102±13	12
'88542-69'	838	80 (10)	62 (7)	18 (2)	106±15	8

^a Pollen stained by lactophenol acid-fuchsin.

^{b,c} Pollen size and germination (%) of the LA-hybrids indicate 2n-gametes only.

Abnormal meiosis was observed in all three LA-hybrids with regard to chromosome association at anaphase I. In many cases, an unbalanced anaphase I disjunction of the chromosomes was observed leading to absence of the second meiotic division. At metaphase I, there was clear evidence for homoeologous

Figure 1a-c. Early and late metaphase I stage in the pollen mother cells of the F₁ plants of *L. longiflorum* × Asiatic hybrid (LA). In Fig a and b, the chromosomes are hybridised with total genomic DNA of *L. longiflorum* detected with anti-dig FITC (yellow) and counterstained with propidium iodide (Asiatic genome being red). Bars in all cases represent 10 µm. (a) Metaphase I showing 9_{II} + 6_I. On the left, a pair of bivalents is with homoeologous recombinations. Note: number of univalents corresponds with the expectation- three of *L. longiflorum* (yellow) and three of Asiatic (red). (b) A late metaphase I showing univalents and half-bivalents, corresponding to the expected 24 bodies. Some of the univalents and all the half-bivalents, which could be identified are indicated appropriately. The half-bivalents are indicated with the respective chromosome (centromere) first followed by the homoeologous chromosome, which has contributed the recombinant segment. For example, 9L/A-9A/L and so on with respective coloured letters. Italic figures and letters indicated half-bivalents according to suspected chromosome number based on the length of the short-arm. Recombination break points are shown by arrowheads. (c) The same cell as in Fig. 1b but hybridised with the 45S rDNA probe (pTa 71). Note the clear hybridisation sites, which serve as markers for the identification of seven different chromosomes in the LA-hybrid - 3 chromosomes of L-genome and 4 chromosomes of A-genome.



chromosome association (Fig. 1a). The frequency of chromosome association varied from 24/0_{II} – 6/9_{II}. One of the difficulties for estimating the frequency of univalents and bivalents at metaphase I was that meiosis was highly asynchronous within each anther. Because of this, some of the bivalents were already precociously disjoined contributing to underestimation of the frequency of bivalents. Precocious separation of bivalents was evident from the presence of half-bivalents (Fig. 1b) in addition to univalents at metaphase I. Due to the presence of recombinant segment(s) in the chromatid(s), half-bivalents could be distinguished clearly from the univalents. However, it was likely that some of the half-bivalents with very small recombinant segments might have escaped GISH detection. Table 2 shows the frequency of chromosome association at metaphase I of the LA-hybrids. Relatively high frequency of chromosome association was observed ranging from 19.5/2.2_{II} – 14.8/4.6_{II} of PMCs among the three LA-hybrids (Table 2).

Table 2. Chromosome associations at late metaphase I of the three 2n-gametes producing interspecific hybrids (2n=2x=24). PMCs=pollen mother cells.

Genome & accession	No. of PMCs analysed	Chromosome configuration (%)	
		Univalent	Bivalent
LA ₁ '88542-24'	30	14.8 (62)	4.6 (38)
LA ₂ '88542-52'	103	19.5 (81)	2.2 (19)
LA ₃ '88542-69'	33	17.2 (72)	3.4 (28)

A unique feature in the three LA-hybrids was that meiosis appeared to be delayed in some of the PMCs with sporad stages occurring alongside chromosomal division stages. Invariably, the univalents and half-bivalents in pollen mother cells with delayed division resembled somatic chromosomes more than meiotic chromosomes (compare Fig. 1b with Fig. 1a).

Because the centromere positions of both L- (yellow) and A-genomes (red) were clearly determined for many of the chromosomes, univalents and half-bivalents could be identified (marked with L, A, L/A, A/L in Fig. 1b). For further identification of those chromosomes, FISH detection of 45S rDNA sites with probe pTa71 (Gerlach and Bedbrook, 1979) was employed (Fig. 1c). Four chromosomes of the A-genome (chromosomes 1, 2, 6 and 7) and three of the L-genome (chromosomes 3, 4 and 7) possessed 45S rDNA sites. These served as markers for identification of NORs bearing univalent and half-bivalent chromosomes (see Fig. 2c).

The salient features of the behaviour of univalents and half-bivalents in later meiotic stages (Fig. 1a for metaphase I, Fig. 1b and Fig 2a for late metaphase I, and Fig. 2b for modified anaphase stage) were that;

- a) the chromatids and the centromeres were more pronounced than in earlier stages (Fig. 1b, compare to Fig. 1a);
- b) most of the univalents, half-bivalents and bivalents were oriented at the equatorial plate of the cell (Fig. 2a);
- c) all univalents divided equationally through centromere division (Fig. 2b);
- d) bivalents (when present) disjoined reductionally in all cases (Fig. 2b).

The occurrence of the aforementioned chromosome features was shown in all LA-hybrids. The result was that two groups of chromatids with an imbalanced parental chromosome number of the complete genome ($2n=24$) could be found in later division stages of a modified anaphase (Fig. 2b).

The chromosomal orientation and equational division is representing a modified form of meiosis where only one division of chromosomes occurs. The successive cytokinesis normally occurring after telophase I in these cells was totally absent. An important feature was that it was possible to determine the distribution of chromatids to the two poles during the anaphase. This observation was obvious from GISH and FISH. The simultaneous reductional separation of bivalents and equational division of univalents could be detected in modified anaphase stages (Fig. 2a and 2b). This phenomenon was also confirmed by FISH for those chromosomes containing 45S rDNA sites (Fig. 2c). Thus, the division of univalents could be discriminated from those of half-bivalents. In the case of univalents the division was strictly equational, but the half-bivalents divided in two alternative ways:

- 1) Two non-recombinant and two recombinant chromatids of a pair of half-bivalents (two half-bivalents; they were one bivalent in metaphase I) moved to each pole (no data shown)
- 2) A non-recombinant and a recombinant chromatid of a pair of half-bivalents moved to opposite pole (for example, 7 L/A and 7 A/L in Fig 2b).

There was also evidence for the inclusion of two sister chromatids of a half-bivalent at the same pole (for example, chromosomes #4, #7, #8 and #9 in Fig 2b). In order to summarize the consequences of these abnormal chromosomal distributions for the composition of $2n$ -gametes, the possible mechanisms of meiotic restitution have been schematically illustrated in Fig. 3.

Based on the analysis of sporads (Table 3) it was possible to determine the type of cytokinesis that had occurred in each of the pollen mother cells (PMCs). Accordingly, two equational walls (following anaphase II) had formed in two

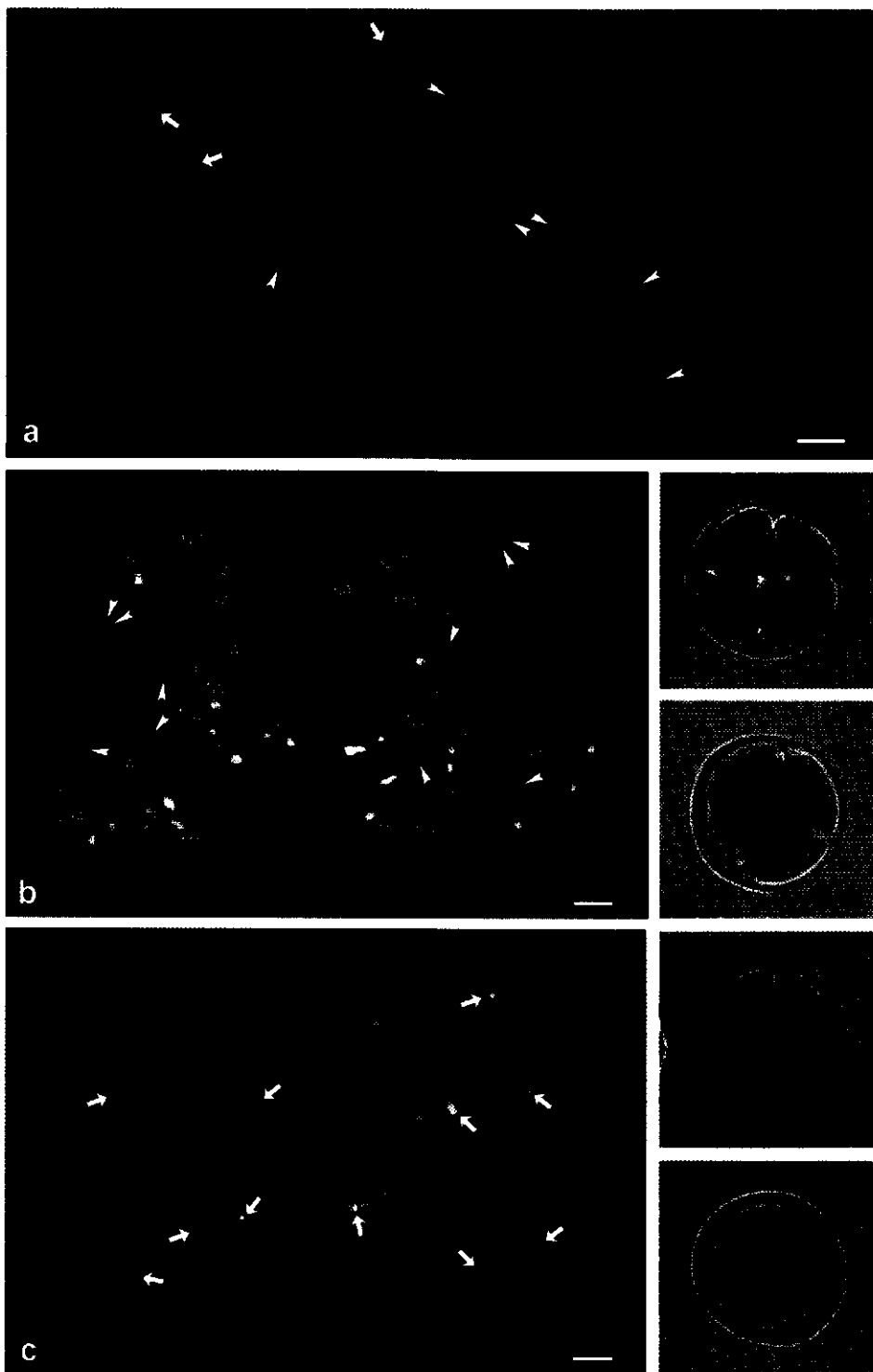


Table 3. Number (percentages) of different types of sporads in LA-hybrids.

Accession	PMCs					Total
	Monad	Dyad	Triad	Tetrad	Others	
LA ₁ '88542-24'	40 (17)	58 (25)	41 (17)	86 (36)	11 (5)	236 (100)
LA ₂ '88542-52'	48 (20)	99 (40)	16 (7)	68 (28)	12 (5)	243 (100)
LA ₃ '88542-69'	2 (1)	46 (11)	70 (17)	281 (66)	19 (5)	418 (100)

separate cells, giving rise to a tetrad. In all three LA genotypes tetrads were formed varying from 28 % to 67 % (Table 3). Obviously, the four spores were unbalanced leading to sterility. There was, however, a considerable percentage of triads (7-17 %) that indicated the occurrence of a reductional wall and of an equational wall in one of the cells (Table 3 and Fig 2e). In the rare event of normal anaphase I separation, the presence of triads indicated the potential of second division restitution (SDR). The dyad was the other most abundant class, which varied in frequency from 11 - 41 %. In this case, considering the chromosomal divisions in pollen mother cells with delayed meiosis, dyad formation was due to one equational wall formation. The high frequency of dyad formation in genotype, '88542-52' was in agreement with the higher frequency of 2n-pollen recorded (Table 1 and 3).

Figure 2a-g. "Division" of chromosomes in the stage after late metaphase I and sporad formation in LA-hybrid. Bars represent 10µm in all cases. (a) Late meta/anaphase I showing "division" of bivalents and univalents. Note: some of the bivalents, which have already disjoined are represented by two half-bivalents, viz., 2A/L-2L/A; 3A/L-3L/A and 4A/L-4L/A. Centromere of the univalents indicated by arrows have already divided. Arrowheads indicate the breakpoint of half-bivalents. The figures in some of the chromosomes are given the corresponding chromosome number. (b) Distribution and division of half-bivalents and univalents following late metaphase I. In this modified anaphase stage, equational division of univalents as well as disjunctional separation of the half-bivalents are confirmed through GISH. Some of the half-bivalents that could be identified as according to Fig. 2c are marked, e.g., 4A/L-4L/A, 7A/L-7L/A, 8A/L-8L/A and 9A/L-9L/A. All the univalents that have divided equationally are also marked with numbers and letters. All the numbers combined with letter stand for the exact chromosome number of both genomes. Italic numbers combined with letter represent doubtful identification. Nevertheless, based on their positioning in the two poles the numbers are helpful for the placement of the respective counterparts of the dyad. (c) The same cell as in Fig. 2b but reprobred with 45S rDNA sequences (pTa 71) to identify the counterpart chromosomes at both poles. The presence of half-bivalents at the poles is confirmed for 7A/L-7L/A and 4L/A based on FISH detection. Similarly, the equational division of univalents is also confirmed for 1A, 2A, 3L and 6L. (d-g) Sporad stages showing a tetrad (d), triad (e), dyad (f) and monad (g).

Discussion

As a genetic consequence of abnormal meiotic division, two types of $2n$ -gametes are known, viz., FDR and SDR (Fig. 3a and b). Several mechanisms such as semi-heterotypic division (Rosenburg 1927), mitotitized-meiosis (Stebbins 1932), pseudo-homoeotypic division (Gustafsson 1935), aberrant cytokinesis (Ramanna 1974), parallel spindles (Mok and Peloquin 1975) and fused spindle (Ramanna 1979) that produce FDR $2n$ -gametes have been identified (Ramanna 1979; Vorsa and Bingham 1979; Peloquin 1983; Parrot and Smith 1984). In addition, mechanisms genetically equivalent to SDR known as premature cytokinesis 1 and 2 (Mok and Peloquin 1975) have been reported (Veilleux et al. 1982; Veilleux 1985; Britognolle and Thompson 1997). It is evident from those reviews that most studies were confined to dicotyledonous plants like *Brassica*, *Capsicum*, *Glycine*, *Medicago*, *Solanum* and *Fragaria* (see review Veilleux 1985), and relatively rare in monocots (Parrot and Smith 1984). In this context, the present investigation on *Lilium* hybrids and those on *Alstroemeria* (Kamstra et al. 1999a,b) are pertinent additions to the subject of $2n$ -gamete formation in monocotyledonous species. For *Alstroemeria* and *Lilium*, *in situ* hybridisation techniques (GISH and FISH) have been used for the estimation of the origin of $2n$ -gametes. In these cases the use of GISH and FISH offered a new perspective for the elucidation of restitution mechanisms, the extent of genetic recombination (both crossing-over and chromosome assortment) and the composition of $2n$ -gametes.

An important feature of restitutional meiosis in plants with successive cytokinesis in meiosis, as in *Lilium*, is how the order of chromosomal division and cytokinesis occur. The cytological observations in the present study clearly indicated how meiosis is modified. Instead of chromosome disjunction at anaphase I, all univalents and half-bivalents were aligned in a single equatorial plane. The chromosomes divided simultaneously as a group (Fig. 2a), followed by equational wall formation. This observation explains satisfactorily how FDR gametes can occur. When only univalents are formed in a pollen mother cell, FDR gives rise to $2n$ -gametes without recombinant chromosomes. On the other hand, when both univalents and bivalents are present, FDR with recombinant chromosomes can occur. Both of these possibilities are evident from the meiotic observations in this study and corroborate the results of BC plants derived from $2n$ -pollen of LA-hybrids (Chapter 5).

The occurrence of three types (FDR, SDR and IMR: *indeterminate meiotic restitution*) of $2n$ -gametes can be explained as follows. In the first type FDR $2n$ -gametes without- and with-recombinations were formed. In the case of FDR $2n$ -gametes without recombination, meiocytes at metaphase I form only univalents,

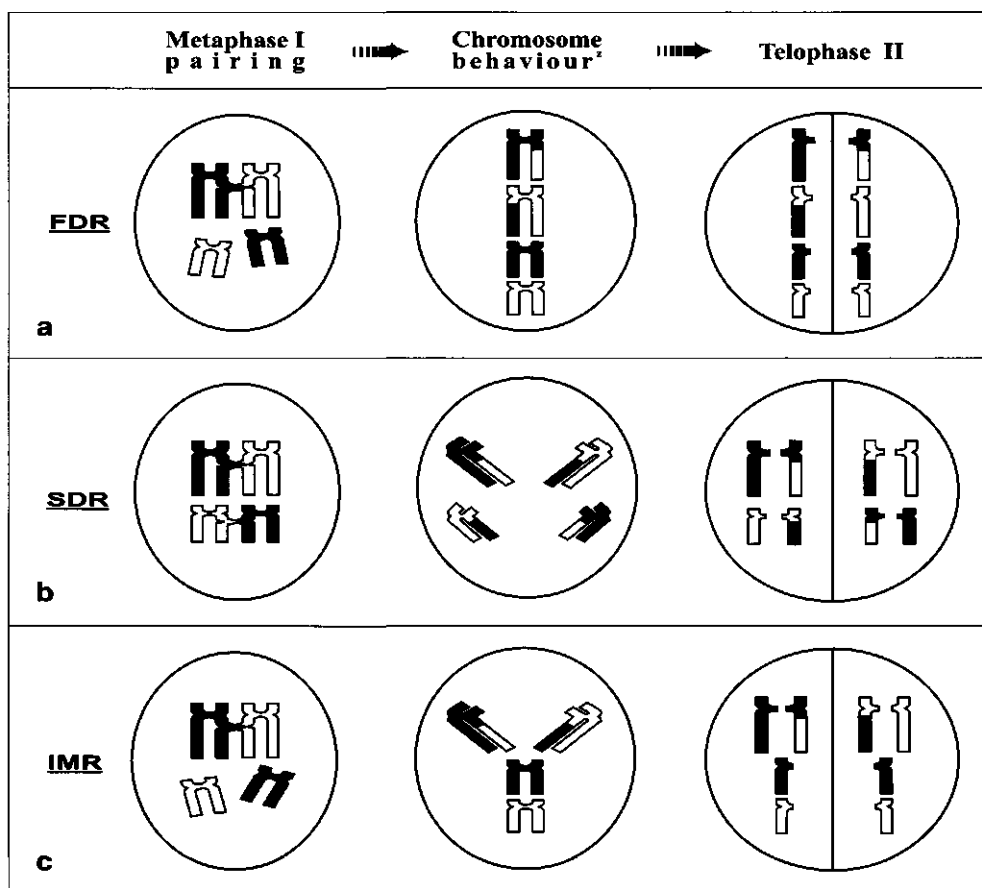
which are oriented at the equatorial plane in the PMC. The equational division of the centromeres results in diploid sets of chromatids moving to the two poles at anaphase I and the complete L- and A-genomes are reconstituted during the first meiotic division. The anaphase in this case is a modified form of anaphase I because the centromeres divide before telophase I. During modified telophase I, cell wall formation gives rise to a dyad with two identical 2n-gametes (figure not shown). In the case of FDR 2n-gametes with recombination(s), it is assumed that homoeologous cross-overs between L- and A-homoeologues occur. Consequently, the meiocytes display both univalents and bivalents at metaphase I. The bivalents always disjoin prematurely at metaphase I resulting in half-bivalents, and remain aligned on the equatorial plane together with all the univalents (Fig 2a). The resulting FDR-gametes now include recombinant chromosomes with the complete genome possessing twelve centromeres of both L- and A-genome chromosomes.

In the second type (SDR), 2n- and normal n-spores are formed as follows: after normal reductional division (anaphase I), a reductional wall is formed during telophase I. The equational wall is formed only in one of the cells of a pollen mother cell during the second division. This leads to a triad with one 2n- (SDR) and two identical (or nearly identical) n-microspore(s) (Fig. 2e). In this case, SDR 2n-gametes contain an even number (12 + 12) of centromeres of the parental genomes, but never an odd number.

In the third type (IMR), numerically imbalanced chromosomes occur due to a restitution mechanism, which cannot be categorised either FDR or SDR. In this case, although the 2n-gamete can have the balanced number of 24 chromosomes (from chromosome 1 to 12 in lily), each set has not contributed the same number. This anomalous situation can be explained as follows: during the first meiotic division all univalent(s) divide equationally whereas bivalents disjoin (reductionally) and then the half-bivalents move to both poles as in normal anaphase I. Following that step, cytokinesis takes place dividing the two groups of chromatids and half-bivalents. Consequently, the sister-centromeres (chromatids) of each of the half-bivalents are included in the same nucleus of the daughter cell (Fig. 2b). The odd number of centromeres of the parental chromosomes cannot be determined in FDR or SDR. We have coined the term as *Indeterminate Meiotic Restitution* (IMR) for this type of anomalous meiotic restitution. The equational division of univalents in the first meiotic division has often been recorded in plants such as *Taraxacum* (Gustafsson 1935), *Lilium* (Richardson 1936; Ribbands 1937; Asano 1982a, 1984), *Chondrilla* (Bergman 1950) and potato (Ramanna 1983; Jongedijk et al. 1991). Interestingly, Asano (1984) observed the same chromosome behaviour of univalents and bivalents in an interspecific hybrid of *Lilium auratum* var *platyphyllum* × *L. henryi*, similar to

the one observed in the present LA-hybrids (Fig. 3c). However, he did not determine the consequences of such a division for the type of restitution that could occur. Asano (1982b, 1984), however, recorded a high frequency of restitution and 2n-pollen. In that case, only a low frequency of 2n-pollen was viable as determined through pollen germination tests (Asano 1984).

Based on traditional cytological and genetical approaches, only two types of meiotic restitution mechanisms, viz., FDR and SDR (Fig. 3a and b), were recognised until now (Mok and Peloquin 1975; Ramanna 1979; Veilleux et al. 1982). From the present approach using GISH, however, a new restitution mechanism, IMR, has been discovered. It has proven that one of the BC₁ plants showed an odd number of parental chromosomes (Chapter 5). In this type, it is evident that genetic recombination can occur during the origin of 2n-gametes, not only through homoeologous crossing-over but also through the assortment of homoeologous chromosomes (univalents and half-bivalents). This means that wide interspecific



hybrids showing disturbed chromosome association such as the one described in the present study have the potential to produce $2n$ -gametes from FDR, SDR and IMR. Although half-tetrad analysis using traditional genetic markers or molecular markers is helpful for establishing the restitution mechanism and the extent of crossing-over, the cytological approach of the present study is more direct.

The frequency from dyad formation (25 %, Table 3) to stainable pollen (4 %, Table 1) and pollen germination (<1 %, Table 1) in the LA_1 hybrid showed a dramatic decrease compared with LA_2 and LA_3 . This is probably due to an imbalanced chromosome composition through IMR. Despite the potential occurrence of various types of $2n$ -gametes in the diploid hybrids, only a few types seem to be recoverable in BC_1 progenies. In case of *Lilium*, both types of FDR gametes (i.e., with and without recombinant chromosomes) (Karlov et al. 1999) and one of an IMR gamete have been discovered in BC_1 progeny (Chapter 5). However, the frequency with which they were discovered in the progenies may not be proportional to the frequency they are produced in the hybrid (Table 1 and 3). This might be caused by gametic, zygotic and post-zygotic selections. Furthermore, they are not recovered in the progenies. Although there is the potential for the formation of SDR gametes, especially in *L. 'Enchantment' × L. pumilum* hybrid where the homoeologous chromosomes paired completely in most of the cells (personal observation), probably the SDR gametes in wide interspecific hybrids are lethal owing to their chromosomal imbalance or they have a selective disadvantage.

Figure 3. A schematic representation of three possible types of meiotic nuclear restitution in a diploid interspecific hybrid in case of $2n=2x=4$. The homoeologous pairs of chromosomes are shown as black and white chromosomes. (a) FDR with recombination. At metaphase I, one bivalent and two univalents are formed. In the subsequent stage two Half-bivalents and two univalents align on the equatorial plate and divide equationally. The result is that the homoeologous chromosomes do not assort independently, and that the centromeres of both genomes are intact in the $2n$ gametes. (b) SDR with recombination shows independent assortment of homoeologous pairs of chromosomes. In this case both pairs of homoeologous chromosomes disjoin at anaphase I but restitute subsequently i.e., without the second division. The notable features of SDR are that the homoeologous pairs assort independently of each other and the number of centromeres of the parental genomes are not preserved intact in the resulting $2n$ gametes. Moreover, each centromere is always represented in pairs. (c) IMR showing unequal distribution of the centromeres of the parental genomes. At metaphase I a bivalent and two univalents are formed. The bivalent disjoins normally as in the anaphase I, whereas the two univalents divide equationally. Consequently, the chromosome constitution of the parental genomes is not preserved in the $2n$ -gametes and, furthermore, the centromeres of each of the parental genomes are present in odd numbers.

^z In all cases, meiosis is incomplete. Because of this, the different stages of meiosis cannot be strictly defined.

Another aspect, which needs to be mentioned, is the usefulness of $2n$ -gametes in breeding. Most of the work so far has been focused on the use of $2n$ -gametes for breeding autopolyploids of alfalfa, potato, clover and many other crops (Bingham and McCoy 1979; Peloquin 1982; Mariani and Tavoletti 1992a,b). In these cases, the value of FDR gametes is transferring heterosis and parental gene combinations intact in sexual polyploids. However, little attention has so far been paid to the systematic use of $2n$ -gametes in breeding of allopolyploids or disomic polyploids. Even in the absence of systematic breeding efforts there have been numerous instances in horticultural crops where sexual polyploids from distant interspecific hybrids have given rise to valuable cultivars (Ramanna 1992; Van Tuyl 1997). In these cases, $2n$ -gametes have been most useful for combining genomes of distant taxa in spite of the diploid hybrids being "sterile", in the sense of not producing normal n -gametes. Thus, in those cases where the hybrids would have been completely sterile, $2n$ -gamete formation would be an alternative for utilizing such hybrids in breeding. Besides these advantages, the occurrence of intergenomic recombination in the $2n$ -gametes of the present LA-hybrids as well as in *Alstroemeria* (Kamstra et al 1999a,b) open the possibilities for transferring desirable chromosome segments more purposefully in breeding.

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5

Indeterminate meiotic restitution (IMR) leads to unusual chromosome constitution in 2n-gametes: a GISH analysis of BC₁ sexual polyploids of lily

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Abstract

Introgression of species specific genes into recipient species or cultivars in lily is often blocked by high sterility of the wide interspecific F_1 -hybrids and by preferential chromosome association in amphidiploid obtained through mitotic chromosome doubling. As an alternative way to circumvent F_1 sterility and lack of homoeologous recombination, $2n$ -gametes of F_1 -hybrids have been considered. Three $2n$ -gametes producing F_1 -hybrids of *Lilium longiflorum* (L) \times Asiatic hybrid (A) ($2n = 2x = 24$) and the genomes of eight BC_1 individuals derived from crossing with Asiatic hybrids were investigated cytogenetically for meiotic recombination. Genomic *in situ* hybridisation (GISH) probed to mitotic chromosome complements, allowed the distinction between L- and A-genome chromosomes, and established the number of parental chromosomes in the F_1 and BC_1 individuals. GISH was also applied to assess if translocations occurred between L- and A-chromosomes in the F_1 hybrid, and homoeologous recombination in the BC_1 individuals. Analysis of the eight BC_1 plants revealed six triploid ($2n = 3x = 36$) and two tetraploid ($2n = 4x = 48$) individuals that had originated through the functioning of the $2n$ -gametes of either the F_1 -hybrid or both parents. Three distinct types of BC_1 individuals were discerned: 1) Three plants (two triploids and one tetraploid) had originated through first division restitution (FDR) $2n$ -gametes without possessing any recombinant chromosomes. 2) Four plants (three triploids and one tetraploid) had originated from FDR $2n$ -gametes with recombinant chromosomes varying from one to nine crossing-overs per individual. 3) One plant (triploid) had originated from a $2n$ -gamete derived from a new type of meiotic nuclear restitution called *indeterminate meiotic restitution* (IMR). A significant feature of IMR is the occurrence of both types of meiotic nuclear restitution, viz., FDR and SDR, simultaneously occurring during the formation of $2n$ -gametes.

Key Words: *Lilium* • first division restitution (FDR) • homoeologous recombination • interspecific hybrid • sexual polyploidisation • unreduced ($2n$) gametes

Introduction

The genus *Lilium* (lily) comprises about 80 species, which are taxonomically classified into seven sections (Comber 1949; De Jong 1974). For commercial lily breeding, the most important groups of donors are the *L. longiflorum* hybrids including Aurelian hybrids of the section Leucolirion, the Asiatic hybrid group of the

section *Sinomartagon* and the Oriental hybrid group of the section *Archelirion*. All three sections comprise species with distinctive, desirable horticultural characters. *L. longiflorum* is noted for a white, funnel type of flower with a characteristic scent. The Asiatic hybrids have a range of flower colours and are relatively resistant to *Fusarium oxysporum* f.sp. *lilii*, whereas the Oriental hybrids possess large flowers with a distinctive fragrance and resistant to *Botrytis elliptica*. Merging the desirable characters through interspecific hybridisation with different donors forms an important goal in lily breeding (Van Tuyl et al. 1991). Due to a high degree of genome differentiation between sections in the genus *Lilium*, a wide interspecific diploid hybrid possess highly sterile male and female gametes and are not directly appropriate for further breeding steps. One way of restoring fertility is mitotic chromosome doubling of the hybrids through colchicine or oryzalin treatment (Van Tuyl et al. 1992). A drawback of such mitotic polyploidisation is the absence of meiotic recombination between homoeologous chromosomes due to preferential chromosome association (Lim et al. 2000). An alternative way of overcoming hybrid sterility is the use of unreduced (2n) gametes, which occur occasionally in interspecific lily hybrids (Van Tuyl et al. 1989). This natural system of 2n-gametes production has the additional advantage of showing considerable meiotic recombination between homoeologous counterparts in the diploid hybrid (Karlov et al. 1999; Kamstra et al. 1999a,b). When the interspecific hybrids possess desirable characteristics, 2n-gametes can be used effectively to introgress alien chromosome segment(s) through a limited number of backcrosses (Kamstra et al. 1999a,b).

2n-gametes have been used for sexual polyploidisation in *Alstroemeria* (Ramanna 1991, 1992; Kamstra et al. 1999a,b), potato (Den Nijs 1977; Mendiburu and Peloquin 1977; Peloquin 1982; Ramanna 1983; Iwanaga et al. 1989; Hutten et al. 1994; Tai and De Jong 1997), wheat (Alonso and Kimber 1984; Liu et al. 1994) and other crops (Veilleux 1985). Generally, 2n-gametes in plants originate through two different types of nuclear restitution mechanisms. These are, the so-called, *first division restitution* (FDR) and *second division restitution* (SDR) mechanisms (Mok and Peloquin 1975; Ramanna 1979). In the case of FDR, the entire chromosome complement divides equationally during the first meiotic division, i.e., univalent(s) and half-bivalent(s), giving rise to two identical (or nearly identical) 2n-gametes. This process occurs typically in meiotic mutants that show asynaptic or desynaptic behaviour (Ramanna 1979, 1983; Peloquin 1982) and in interspecific hybrids with reduced homoeologous chromosome association and recombination (Asano 1982a; De Jong et al. 1993). A distinctive feature of FDR, because of equational division, is that all centromeres (chromatids) of the parental genomes are preserved in each of the resulting 2n-gametes. In contrast, during SDR normal disjunctional separation of

bivalents occurs at anaphase I. This indicates that the products of disjunction are merely doubled as a result of missing the second meiotic division process. The distinctive features of the gametes from SDR are that: 1) Homo- or homoeologous chromosomes of the parental genomes are randomly assorted during meiosis. 2) Each of the chromosomes (centromere) is doubled and present in the same cell. Consequently, all homologous chromosomes (centromeres) are always present in an even number. However, functional 2n-gametes through SDR in interspecific hybrids could be very rare because only in exceptional cases all chromosomes are paired as bivalents.

Besides FDR and SDR a new mechanism has been reported by present authors, the so-called "*indeterminate meiotic restitution* (IMR)" in a lily hybrid (Chapter 4), which can lead to 2n-gametes with odd number of parental chromosomes (centromeres).

Previous studies on interspecific lily hybrids have demonstrated the power of genomic *in situ* hybridisation (GISH) for the distinction of parental chromosomes (Karlova et al. 1999; Lim et al. 2000). The technique elucidates different aspects of homoeologous recombination and of the modes of 2n-gamete formation. In this paper cytological evidence based on GISH and FISH has been presented that 2n-gametes originating from IMR infrequently can be functional, which will lead to an unusual chromosome constitution of the backcross sexual polyploidised progeny.

Material and methods

Plant material

Three diploid ($2n=2x=24$) 2n-gametes producing interspecific hybrids of *L. longiflorum* (L) 'Gelria' \times Asiatic hybrid (A) 'Whilito' as well as their backcross progenies were used for GISH analysis. The hybrids were produced through integrated pollination and embryo rescue methods (Van Tuyl et al. 1991). The detailed information of these F_1 -hybrids (LA_1 - LA_3) and BC_1 plants (ALA_1 - ALA_8) is given in Table 1. All F_1 -hybrids were known to produce viable 2n-pollen ranging from about 0.1 % up to 15 %. In order to produce backcross progenies, four different Asiatic hybrids (all diploids) were used as female parents and crossed to the three above mentioned LA -hybrids. Among four female parents, three were cultivars ('Montreux', 'Puccini' and 'Meribel') and one genotype was a breeding parent ('78251'). Plants were grown in a greenhouse at 20 - 25 °C during the day and 14 - 18 °C during the night.

Table 1. Origin and parentage of interspecific hybrids and their backcross progeny.

	Genome type ^a	Accession number	Female parent × Male parent
F ₁ -hybrids	LA ₁₋₃	88542-24, 52, 69	<i>L. longiflorum</i> 'Gelria' × Asiatic hybrid 'Whilito'
BC ₁ progeny	ALA ₁	901122-1	Asiatic hybrid '78251' × '88542-24'
	ALA ₂₋₄	921238-1,2,3	Asiatic hybrid 'Montreux' × '88542-52'
	ALA ₅₋₆	985099-1,2	Asiatic hybrid 'Puccini' × '88542-52'
	ALA ₇₋₈	942460-1,2	Asiatic hybrid 'Meribel' × '88542-69'

^a L and A represent *L. longiflorum* and Asiatic hybrid, respectively.

Chromosome preparation

Root tips were collected in the early morning, pre-treated in a saturated α -bromonaphthalin solution and kept at 4 °C until next morning for accumulation of metaphase cells. Subsequently the root tips were fixed in ethanol – acetic acid solution (3:1) for at least 2 hours and stored in –20 °C until use. The material was digested with pectolytic enzyme mixture (0.3 % pectolyase Y23, 0.3 % cellulase RS and 0.3 % cytohelicase in 10 mM citric acid buffer, pH 4.5) at 37 °C for 1 hour and squashed in a drop of 60 % acetic acid solution. Slides were then frozen in liquid nitrogen and their cover slips were removed with a razor blade. The slides were dehydrated in absolute ethanol for a few minutes, air-dried and finally stored at 4 °C for several weeks before further treatment.

Genomic *in situ* hybridisation

GISH protocols were carried out according to Chapter 2 with some modifications. 45 S rDNA sequence from pTa71 (18S-5S-25S rDNA; Gerlach and Bedbrook 1979) was used after probing with biotin-16-dUTP (Boehringer Mannheim) which were labelled by nick translation. The hybridisation mixture consisted of 2x SSC buffer, 50 % formamide, 10 % (W/V) sodium dextran sulfate, 0.25 % SDS, 1.0 ng/μL of digoxigenin-labelled total genomic *L. longiflorum* 'Snow Queen' DNA, 15-20 ng/μL herring sperm DNA as block DNA. The hybridisation mixture was denatured at 70 °C for 10 min and placed on ice for about 5 min. Then 40 μL of hybridisation mixture was applied to each slide, denatured at 80 °C for 5 min and incubated for overnight at 37 °C in a humid chamber. Post-hybridisation washes followed in 2x SSC for 15 min at room temperature and 0.1x SSC for 30 min at 42 °C. Digoxigenin-labelled probe DNA was detected with FITC-antidigoxigenin detection system (Boehringer

Mannheim, Germany). Biotin-labelled rDNA sequences were detected with avidin-streptavidin conjugated to Cy3 (Vecta laboratories). All slides were counterstained with 2 µg/mL DAPI (4',6-diaminido-2-phenylindole) and 2 µg/mL (propidium iodide). Images were photographed on 400 ISO colour negative film, scanned at 1200 dpi and processed digitally with Photoshop 5.0 (Adobe Systems Inc.; www.adobe.com).

Chromosome nomenclature

The chromosomes of lily species were denoted with the letter "L" or "A" for *L. longiflorum* and Asiatic hybrid, respectively, followed by figure "1" to "12" for the short-arm of chromosome in sequence of decreasing length.

Results

Somatic chromosome constitution of the F_1 -hybrids

The control experiments in the chromosome complement of the F_1 -hybrids showed clear GISH signals despite some extent of cross-hybridisation and allowed differentiation between all L- and A-chromosomes.

All three F_1 -hybrids possessed the diploid ($2n = 2x = 24$) chromosome number. GISH with labelled total genomic DNA of *L. longiflorum* as probe revealed 12 out of 24 chromosomes with yellowish-green fluorescence and 12 chromosomes with the red fluorescing propidium iodide (PI) (Fig 1a). Because the centromere showed clearly weak fluorescence, the karyotypes of the parental species could be determined. In both karyotypes, the largest and the second largest chromosomes were metacentric and submetacentric, respectively.

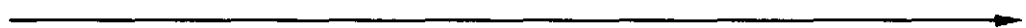
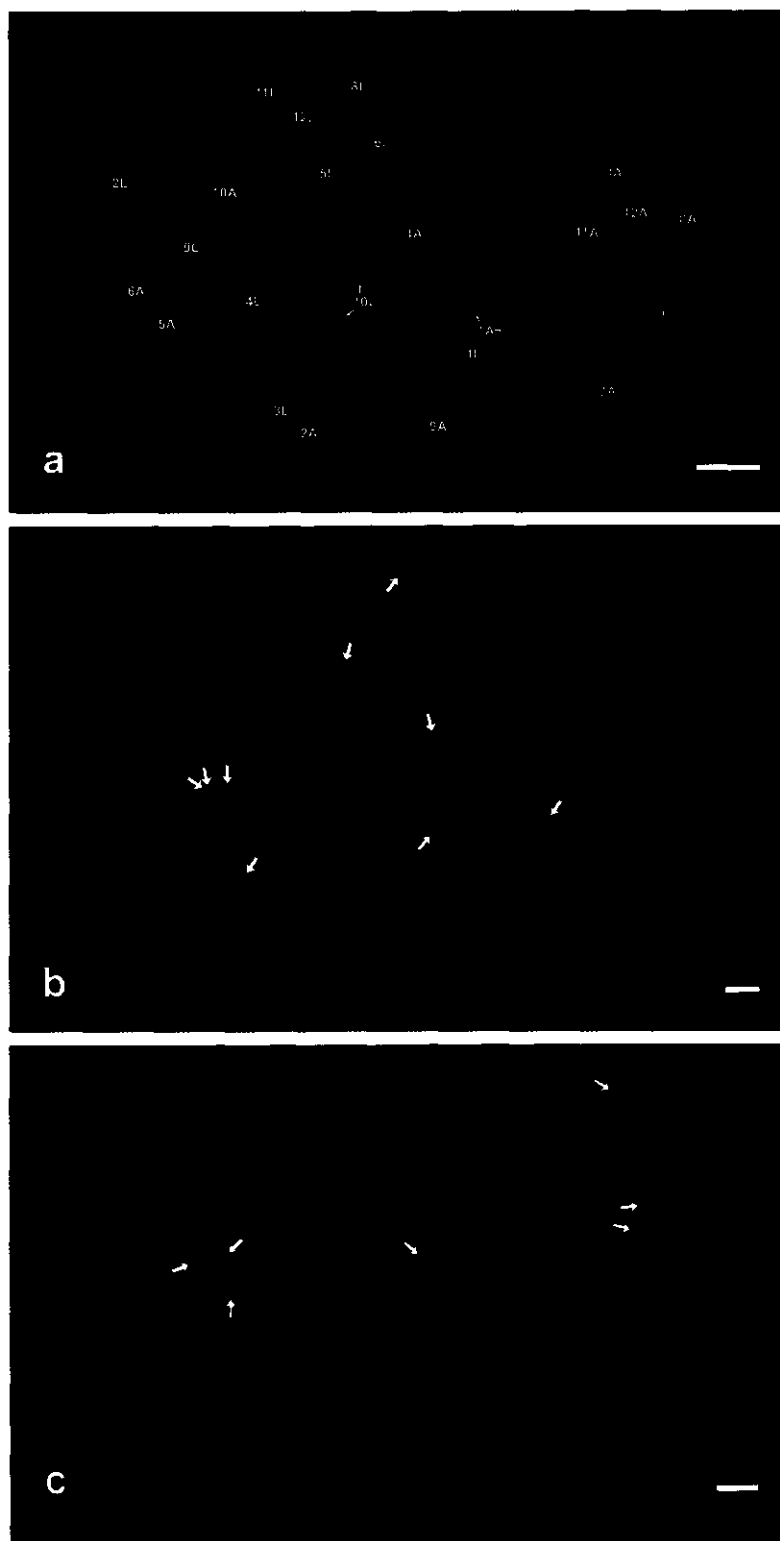


Figure 1. Somatic chromosome constitution of the 2n-gamete producing LA_1 hybrid and its polyploid backcross progenies determined by GISH. The chromosomes of *L. longiflorum* (L-genome) fluoresces yellowish-green (FITC) and those of the Asiatic hybrid (A-genome) are red (propidium iodide). Bar represents in all cases 10 µm. (a) A diploid ($2n = 2x = 24$) chromosome complement of the LA_1 hybrid showing twelve chromosomes each of the L-genome (yellowish-green) and the A-genome (red). Note that there are no recombinant chromosomes in the LA_1 hybrid. Arrows of 10L and 1A indicate the short and long arm of the chromosome. (b) Triploid BC_1 'ALA₅' derived from the functioning of a FDR 2n-gamete with recombinations. There are twelve chromosomes of the L-genome (green) and twenty-four chromosomes of the A-genome (brown). Seven recombinant chromosomes with nine breakpoints (arrows) are visible in this genotype. (c) Tetraploid ($2n = 4x = 48$) BC_1 plant (ALA₆) derived from the functioning of 2n-gamete from both parents. In this case, there are twelve chromosomes of the L-genome (yellow) and thirty-six chromosomes of the A-genomes (red). There are four recombinant chromosomes with five recombination breakpoints (arrows).



The remaining chromosomes were (sub-) acro-centric. Thus, the F_1 plants were identified as true hybrid with full haploid complements of the *L. longiflorum* and the Asiatic hybrid parents. No recombinant or translocation chromosomes, involving L- and A-crossing-overs, were observed in the F_1 -hybrids.

Chromosome constitution of the backcross progenies (BC₁)

Although all genotypes of the parents used for backcrossing were diploid ($2n = 2x = 24$), chromosome counts in the GISH preparations showed that the BC_1 plants were either triploid ($2n = 3x = 36$) or tetraploid ($2n = 4x = 48$) (Table 2, Fig. 1b and 1c). For establishing the genome composition of these polyploids, the number of parental centromeres rather than whole chromosome arms was considered to avoid ambiguity with arms containing L- and A-chromosome fragments. All triploids except one (ALA_1 ; Fig. 2a and Table 2) of BC_1 plants possessed one L-genome and two A-genomes (Fig. 1b). Considering the diploid nature of the parent, it can be concluded that these backcross individuals resulted from $2n$ -gamete formation of the male parent and a full haploid chromosome set of the Asiatic mother (Table 1).

The ALA_1 , a triploid BC_1 plant, possessed twenty-seven A- and only nine L-

Table 2. Number of chromosomes and chromosome constitution of interspecific hybrids and their backcross progeny.

	Genome type ^a	Somatic chromosome number (2n)	Chromosome constitution ^a		No. cross-over breakpoints	No of recombinant chromosomes			Mechanism of the 2n-gamete involved
			L	A		L/A ^b	A/L ^b	Total	
F_1	LA_1	24	12	12	0	0	0	0	-
	LA_2	24	12	12	0	0	0	0	-
	LA_3	24	12	12	0	0	0	0	-
BC_1	ALA_1	36	9	27	10	1	4	5	IMR
	ALA_2	36	12	24	7	2	1	3	FDR
	ALA_3	36	12	24	0	0	0	0	FDR
	ALA_4	48	12	36	0	0	0	0	FDR
	ALA_5	36	12	24	9	3	4	7	FDR
	ALA_6	48	12	36	7	5	0	5	FDR
	ALA_7	36	12	24	1	0	1	1	FDR
	ALA_8	36	12	24	0	0	0	0	FDR

^a L and A indicate *L. longiflorum* and Asiatic hybrid, respectively.

^b L/A and A/L represents recombinant chromosomes of *L. longiflorum* chromosome (centromere) with Asiatic chromosome segment(s) and Asiatic hybrid chromosome (centromere) with *L. longiflorum* chromosome segment(s).

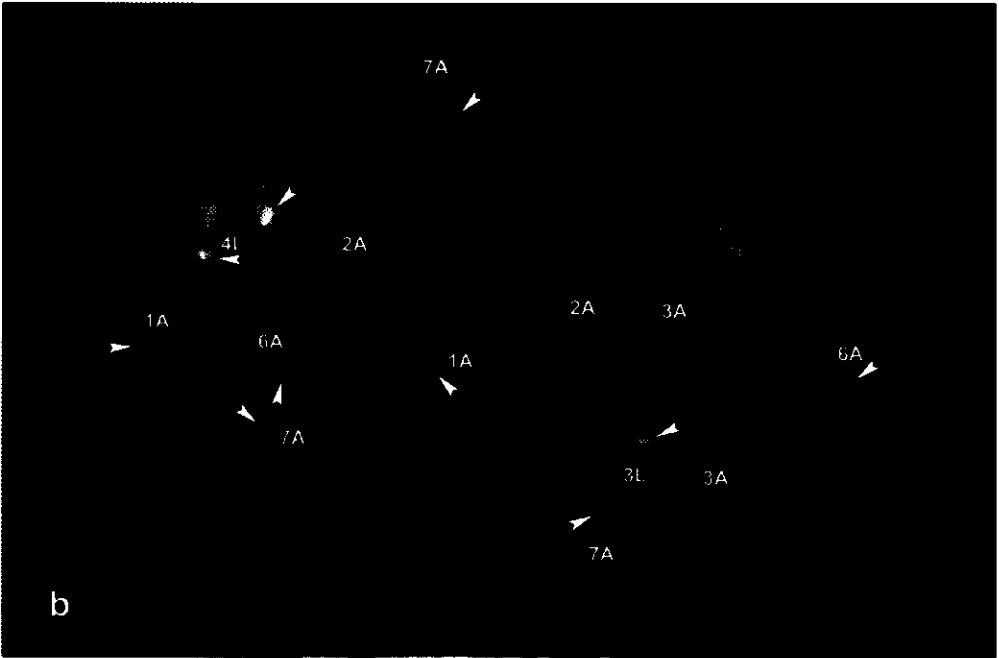
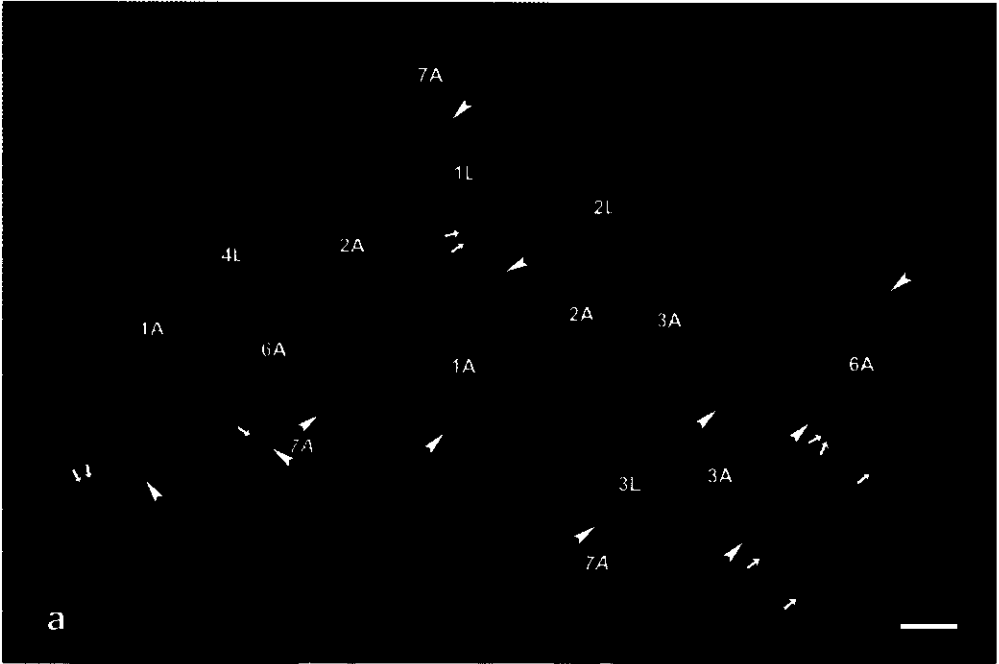
chromosomes (Fig 2a). Despite the imbalanced distribution of centromeres, this plant possessed thirty-six chromosomes as if three complete sets of the chromosome were present. Hybridisation with the 45S rDNA probe and reverse PI/DAPI bands as diagnostic chromosome markers revealed that chromosome 7 of the L-genome was substituted by its homoeologous Asiatic chromosome (indicated by arrowheads in Fig. 2b). By measuring the length of the short-arm, it is assumed that Asiatic chromosome 11 and four *L. longiflorum* chromosomes (chromosome 5, 7, 10 and 12) were substituted with their homoeologous one (Fig. 3). Five substituted chromosomes showed that Asiatic chromosome 5 had no homoeologous recombination, *L. longiflorum* chromosome 11 and Asiatic chromosomes 7 and 10 had recombinations in one chromosome, and Asiatic chromosome 12 had recombinations in both chromosomes. As a consequence of IMR in which bivalents (half-bivalent) always disjoin reductionally as SDR and univalents always divide equationally as FDR simultaneously in the same PMC (Chapter 4), *L. longiflorum* genome has gained one and lost four chromosomes, resulting in 3 missing chromosomes ($+1-4 = -3$). In the Asiatic genome, the opposite situation was obtained, resulting in $+4-1 = 3$ additional chromosomes (Fig. 2a, b and Fig. 3).

An interesting feature of all BC₁ individuals was the presence of up to seven recombinant chromosomes per complement showing L/A (or A/L) crossover sites. Most of these recombinations occurred at proximal sites (fifteen in all) and a few at interstitial and distal regions (Fig 1b, c and 2a). Two types of recombinant chromosomes were distinguished: 1) those with an L-centromere and an A-chromosome segment(s) and, 2) the reciprocal type with an A-centromere and L-segments, which are indicated as L/A and A/L types, respectively (Table 2). Both types of recombinant chromosomes were found in BC₁ plants.

The two tetraploids, 'ALA₄' and 'ALA₆' apparently originated through the functioning of 2n-gametes from both diploid parents. GISH analysis confirmed that these plants contained the expected three A-genomes and a single L-genome (Fig 1c).

Discussion

For the purpose of introgression, recombination following homoeologous cross-over is the most important event in BC plants (Kamstra et al. 1999a,b; Lim et al. 2000). In a previous study a fertile amphidiploid F₁ hybrid (LLRR) derived from chromosome doubling of the F₁-hybrid of *L. longiflorum* × *L. rubellum* (LR) and its BC₁ and BC₂ progenies were analysed.



No intergenomic recombinations between the L- and R-chromosomes were observed in the BC₁ and BC₂ progenies (Lim et al. 2000). By such an approach, the introgression of whole individual chromosomes rather than recombinant segments is obtained after further backcrossing. Thus, mitotic polyploidisation in these hybrids seems not the ideal strategy for introgression of specific desirable characters by recombination of the alien chromosome segments. However, in the present LA-hybrids with the natural potential of producing 2n-gametes, considerable numbers of homoeologous recombinations were achieved. Chromosome analysis of the eight BC₁ plants, Asiatic hybrids \times (*L. longiflorum* \times Asiatic hybrid), revealed 34 homoeologous recombination breakpoints, ranging from zero to ten cross-overs per individual BC₁ plant (Table 2). Under these circumstances at the diploid level, the homoeologous chromosomes are virtually "forced" to pair and to produce cross-overs resulting in intergenomic recombination. The chromosome constitution of all eight BC₁ plants (ALA₁₋₈ in Table 2) clearly showed that the LA₁, LA₂ and LA₃ hybrids in all cases had contributed 2n-gametes to the progeny which contain an euploid chromosome number, i.e., twenty-four. All resulting BC₁ plants were triploid ($2n=3x=36$) or tetraploid (ALA₄ and ALA₆, $2n=4x=48$) originating from both abnormal micro- and macrosporogenesis leading to 2n-gametes (Fig. 1c). A closer look at the genome painted chromosome complements of the eight BC₁ plants clearly demonstrated that three distinct types of 2n-gametes were involved.

In the first group of BC₁ plants (ALA₃, ALA₄ and ALA₈), no recombinant chromosomes could be discerned (Table 2). In this case, the LA₂ and LA₃ hybrids had contributed a set of non-recombinant chromosomes from both parental genomes through the FDR mechanism. In the second group of BC₁ plants (ALA₂, ALA₅, ALA₆ and ALA₇), 2n-gametes that resulted through FDR in the LA₂ and LA₃ hybrids contained homoeologous recombinant chromosomes.

Figure 2. (a) Triploid ($2n=3x=36$) chromosome constitution of BC₁ (ALA₁), derived through the functioning of a 2n-gamete that had originated by IMR. There were twenty-seven centromeres of the A-genome and nine centromeres of the L-genome, both are odd numbers. Five recombinant chromosomes with ten breakpoints are indicated. Arrows mark the recombination breakpoints. White coloured letters indicate the confirmed chromosomes number by pTa71 (45S rDNA sequence) detection as in Fig. 2b and yellow coloured letters represent assumed chromosome number based on the observation of short arm length. Italic letters represent the substituted chromosome numbers indicating that all were paired as bivalents during meiosis I. Arrowheads indicate the centromere position of substituted chromosomes. Bar represents 10 μ m. (b) Detection of 45S rDNA gene sequence (pTa71) on the same chromosome complement as Fig. 2a. Arrowheads indicate the position of rDNA hybridisation on the nucleolar bearing chromosomes. A red arrowhead on the short arm of Asiatic chromosome 2 showed a faint signal of rDNA but can not recognisable in this photo.

In these two groups, LA-hybrids contributed twelve of full L- and A-chromosome sets based on centromere counting.

The third type of restitution mechanism was observed in one BC₁ plant, ALA₁, where the 2n-gamete had transmitted only nine L-chromosomes (centromeres) instead of the expected twelve. The remaining twenty-seven A-chromosomes came from the Asiatic mother (12 chromosomes) and the LA-father (15 chromosomes). The *L. longiflorum* chromosome 7 was substituted with its Asiatic homoeologous chromosome complement because one rDNA signal indicating the NOR region was visible on the short-arm of the Asiatic chromosome 7 (arrow in Figure 2b). Based on the careful analysis of its karyotype, it was established that five chromosomes (Asiatic chromosome 11 and *L. longiflorum* 5, 7, 10 and 12) were substituted by their homoeologous chromosomes resulting from IMR because they were paired whereas the rest of all chromosomes (univalents) were divided equationally as in pseudohomoeotypic division (Gustafsson 1935). The occurrence of this type of 2n-gametes was described in the previous paper by analysing the behaviour of chromosomes of the F₁ hybrid during meiosis (Chapter 4).

The important features for the transmission of 2n-gametes from the F₁ to the BC₁ progenies are that the gametes must have a balanced chromosome number (with regard to genomes) and they must be viable. In the case of FDR gametes of LA-hybrids, both of the parental genomes are present and, therefore, they are balanced and viable. In the case of ALA₁ only nine chromosomes of *L. longiflorum* and fifteen chromosomes of the Asiatic genome were present in the 2n-gamete through IMR. In spite of this apparent imbalance of the parental chromosomes, the IMR gamete with imbalance chromosome constitution was viable and transmitted through the male parent.

The karyotype of ALA₁ is also unusual in another important aspect (Fig. 3). The two sister chromatids of a half-bivalent are included in the same restitution nucleus. It means that homologous chromosomes showing recombination events can be readily identified in the cell. For example, the homologous pair of chromosome 12 showed differences in the number of recombinant breakpoints of two and three (Fig. 2a) in the two individuals. Such differences in crossover points can be convincingly explained by assuming four strands multiple crossovers (model not presented). This is an important advantage, for theoretical purposes, of recovering the sister chromatids from 2n-gametes obtained by IMR.

Eight BC₁ plants have been investigated by GISH analysis. All plants were the result of FDR 2n-gametes and only one of them originated from an IMR 2n-gamete. This is a clear indication that the frequency of functional IMR 2n-gametes is low as indicated in chapter 4.

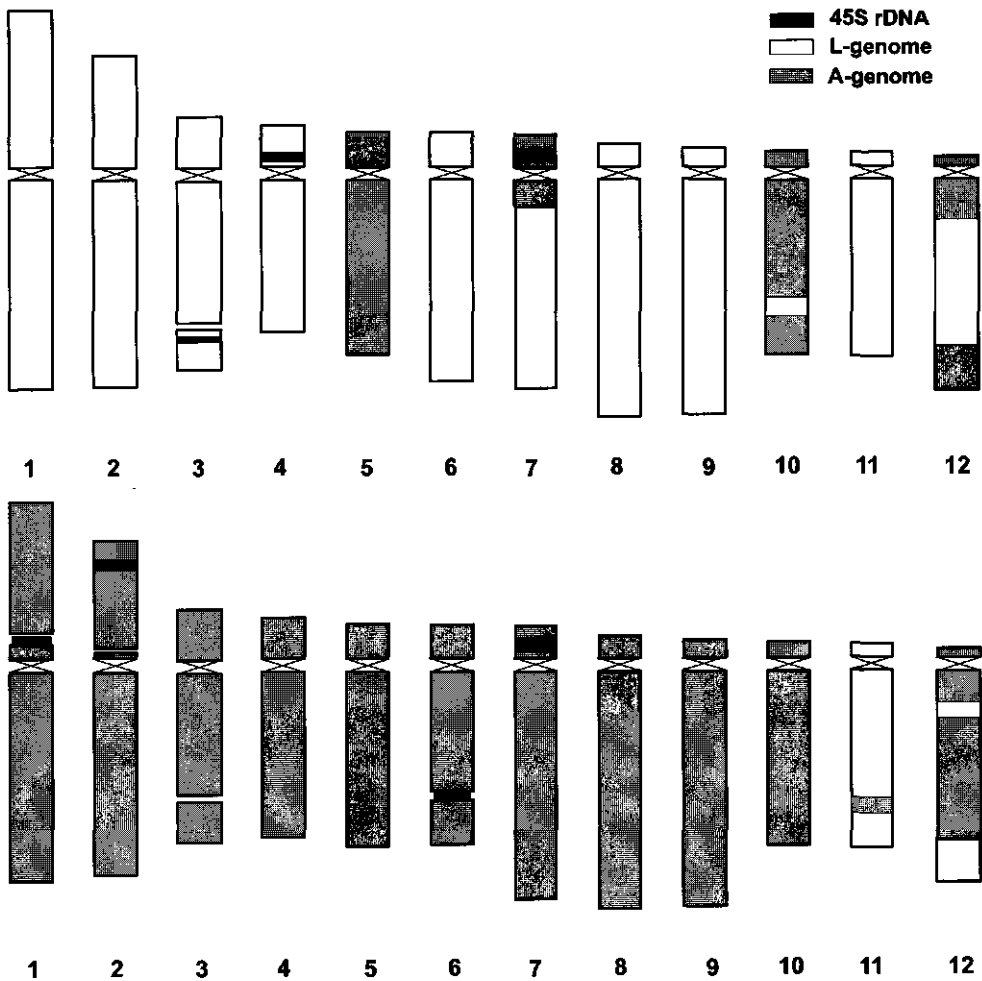


Figure 3. Ideogram of GISH karyotype of triploid ALA_1 '901122-1' represents the chromosome constitution of IMR 2n-gamete including recombination breakpoints. Twelve intact Asiatic chromosomes originating from the female are excluded in this figure. The rest of twenty-four chromosomes are classified into nine L- and fifteen A-genome chromosomes originating from the 2n-gametes producing LA_1 -hybrid. An odd number of parental chromosomes and recombination breakpoints resulting from IMR could be determined in **Figure 2a**. The pTa71 probe was used for the detection of 45S rDNA sites on the NORs bearing chromosomes of both species (see **Figure 2b**).

Figure 4. Flowers of the lily genotypes used in the experiments of this thesis. (a) *L. longiflorum* 'Gelria' (b) *L. rubellum* (c) F_1 -hybrid (940303) of *L. longiflorum* and *L. rubellum* (d) BC_1 -hybrid (LLR; 961003-27), see chapter 3 (e-f) BC_2 -hybrids (LLLR; 982211-26, -27; aneuploids), see chapter 3 (g) Asiatic hybrid 'Whilito' (h) F_1 -hybrid (LA_1), see chapter 4 (i-l) BC_1 -hybrids (ALA_1 -1, 2, 3 and 4), see chapter 5.



6

Evaluation of BC₂ progenies derived from interploidy crosses between triploid BC₁ and several *Lilium* hybrids: a flowcytometric and GISH analysis

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Abstract

A triploid (ALA) BC₁ interspecific hybrid obtained from crosses between a *Lilium* Asiatic hybrid and a 2n-gametes producing interspecific LA-hybrid (*L. longiflorum* × Asiatic hybrid), was used in crosses as a female parent. Different ploidy levels of Asiatic (A) and *L. longiflorum* (L) hybrids together with interspecific hybrid (LLAA) were used as male parent for the production of BC₂ progenies. Fertilisation affinity between cross combinations of different genome composition was analysed by counting the number of embryos rescued and the regeneration ratio. BC₂ progenies were then analysed by flowcytometric DNA measurement and GISH analysis to determine their ploidy levels and parental genome composition. Transmission of recombinant chromosome segments from the BC₁ female parent to the BC₂ generation was analysed by comparing the karyotype of the BC₁. The best fertilisation ratio between the triploid (ALA) BC₁ female parent and genotypes of different genome composition or ploidy level was found in ALA × LLAA (3x × 4x) crosses. A flow cytometric DNA measurement confirmed its relevance for ploidy determination of BC₂ plants. Most of the BC₂ individuals turned out to be aneuploids that originated from megasporogenesis and/or megagametogenesis of the ALA hybrid. These aneuploids are assumed to have originated from random movement of chromosomes during meiosis I and/or from adding whole chromosomes during migration stage of the megagametogenesis. GISH results confirmed that most of the recombinant chromosome segments of the BC₁ female parent were transmitted to the BC₂ progenies. The advantage of transmitted alien chromosome segments in BC₂ plants is that there might be potential for selecting some useful traits already in the BC₂ generation.

Key Words: interspecific hybrid • backcross progeny • polyploid • aneuploid • DNA content

Introduction

Interspecific hybridisation is laborious and time consuming, nevertheless it is highly rewarding. Comparable to many other horticultural crops, interspecific hybridisation has played a considerable role in lily breeding (Van Tuyl et al. 2000). Despite the presence of large genomes in lilies (131.0 – 172.8 pg/4C, Bennett and Smith 1976, 1991; Van Tuyl and Boon 1997) and a fairly large basic chromosome number ($x = 12$), a large number of polyploid cultivars have been produced (Van Tuyl et al. 1989; Schmitzer 1991). Interspecific hybridisation followed by polyploidisation has

contributed to the development of useful breeding material as well as cultivars (Van Tuyl et al. 1989). In many cases, spindle inhibiting or chromosome doubling agents such as colchicine or oryzalin have been successfully used for the induction of polyploids (Blakeslee and Avery 1937; Emsweller and Brierley 1940; Van Tuyl et al. 1992). However, there are also instances in which numerically unreduced (2n) gametes have been shown to be successful for inducing polyploids using interspecific hybrids which are otherwise sterile (Lammerts 1929, 1931; Storey 1956; Stebbins 1970; Van Tuyl et al. 1989, 1990).

The important feature of using 2n-gametes for inducing polyploids is that, in addition to increasing the ploidy level, it is also possible to recover homoeologous recombination in the progenies (Karlova et al. 1999). This aspect of homoeologous recombination can be important in view of introgressing specific characters from a distant species into the cultivars. In our attempts to produce interspecific hybrids of *Lilium* and utilise them for introgression breeding, we have detected hybrid genotypes that produce 2n-gametes in variable frequencies (Van Tuyl et al. 1989, 1990; Chapter 4). Besides elucidating the meiotic mechanism of the origin of 2n-gametes in these genotypes, it was possible to produce backcross progenies, which possessed homoeologous recombinant chromosomes (Chapter 5). In order to gain further insight into the behaviour and chromosome composition of backcross progenies, crosses were made using the hybrids and BC₁ progenies. These crosses involved parents with different ploidy levels. In order to evaluate the ploidy level and the presence or absence of recombinant chromosomes, flow cytometry and genomic *in situ* hybridisation (GISH) were used for estimating the DNA values and recombinant chromosomes. These results form the subject of this chapter and the relevance to introgression is discussed.

Material and methods

Plant material

Interploidy crosses were made between BC₁ triploid (ALA; see Chapter 5) as a female and diploid ($2n = 2x = 24$), tetraploid ($2n = 4x = 48$) of Asiatic hybrids and *L. longiflorum*, and the allotetraploid LLAA ($2n = 4x = 48$) as male parent (Table 1 and 2). Detailed cross combinations and their BC₂ plants are listed in Table 1 and 4.

Table 1. Accession numbers and their parentage of the plant material (BC₂) used in this study.

Accession number	Parentage
997139-1~12	921238-1 (ALA) × 991033 (AA) = [Asiatic hybrid 'Montreux' × (<i>L. longiflorum</i> 'Gelria' × Asiatic hybrid 'Whilito'))] × Asiatic hybrid 'Connecticut King' (2x)
997106-1, 2	921238-1 (ALA) × 85914 (AAAA) = [Asiatic hybrid 'Montreux' × (<i>L. longiflorum</i> 'Gelria' × Asiatic hybrid 'Whilito'))] × Asiatic hybrid 'Connecticut King' (4x)
997147-1~13	921238-1 (ALA) × 87239 (AAAA) = [Asiatic hybrid 'Montreux' × (<i>L. longiflorum</i> 'Gelria' × Asiatic hybrid 'Whilito'))] × tetraploid '78251-1'
997118-1~13	921238-1 (ALA) × 952047-1 (LLAA) = [Asiatic hybrid 'Montreux' × (<i>L. longiflorum</i> 'Gelria' × Asiatic hybrid 'Whilito'))] × (4x <i>L. longiflorum</i> × 4x Asiatic hybrid 'Orlito')

Table 2. Results of crossing the BC₁ triploid (2n=3x=36; ALA) with diploid (2n=2x=24) and autotetraploid, Asiatic hybrid (AAAA), and the allotetraploid LA-hybrid (LLAA).

Interploidy/ Accession	Parentage		Probable genome composition	No of flowers crossed	Successful ovaries (%)	No. of embryos (emb./ov)	No. plant germinated (%) ²
	Female	Male					
<u>3x × 2x</u>	ALA	AA					
997139	921238-1	991033	ALA A	8	8 (100)	39 (4.9)	14 (36)
997147	921238-1	87239	ALA A	7	7 (100)	25 (3.6)	18 (72)
						Avg. = (4.3)	2.1 (50)
	ALA	LL					
997013	921238-1	991031	ALA L	10	0 (0)	0 (0)	0 (0)
<u>3x × 4x</u>	ALA	AAAA					
997106-1	921238-1	85914	ALA AA	14	8 (57)	13 (1.6)	2 (15)
997146	921238-1	85707-1	ALA AA	3	3 (100)	8 (2.7)	4 (50)
997147	921238-1	87239	ALA AA	7	7 (100)	20 (2.8)	18 (90)
						Avg. = (2.4)	0.6 (58.5)
	ALA	LLLL					
997117	921238-1	901015-66	ALA LL	4	0 (0)	0 (0)	0 (0)
997145	921238-1	85842-1	ALA LL	3	0 (0)	0 (0)	0 (0)
997121	921238-1	90239	ALA LL	5	0 (0)	0 (0)	0 (0)
<u>3x × 4x</u>	ALA	LLAA					
997118-1	921238-1	952047-1	ALA LA	3	3 (100)	17 (5.7)	14 (82)
						Avg. = (5.7)	

² All BC₂ plants were derived by embryo rescue techniques.* A, L and LA represent Asiatic hybrid, *L. longiflorum* and their interspecific hybrid, respectively.

Crossing and embryo rescue

The lily bulbs were planted in pots and grown in the greenhouse with the temperature ranging from 14-16 °C during the night to 20 – 22 °C during the day. All crosses were made by the cut-style pollination method (CSM) and encapsulated with aluminium foil on top of the cut-stigma for 7 days. Embryo rescue was carried out at 45 – 60 days after pollination depending on the maturation of the ovary. After the embryo was germinated *in vitro*, the leaf tissue of plantlet was used for DNA measurement by flowcytometry (Van Tuyl and Boon 1997).

Flowcytometric measurement

The protocol of flowcytometric measurement was described by Van Tuyl and Boon (1997). DNA values in the present paper are expressed as 'units' rather than 'picograms' because the DNA values were estimated by using only DAPI staining which is known to be base-biased (Peterson et al. 1999).

Chromosome preparation

Root tips were harvested in a saturated α -bromonaphthalin solution during early morning and kept overnight at 4 °C for accumulation of the metaphase cells. The next morning, the material was fixed in ethanol – acetic acid solution (3:1) for at least 2 hours following washing with mQ water three times and stored at –20 °C until use. Root tips were treated with a pectolytic enzyme mixture (0.3 % pectolyase Y23, 0.3 % cellulase RS and 0.3 % cytohelicase) in 10 mM citric acid buffer at 37 °C for about 1 hour and squashed in a drop of 60 % acetic acid solution. Slides were then frozen by dipping in liquid nitrogen and their cover slips were removed by using a razor blade. Before air-drying the slides were dehydrated in absolute ethanol for a few minutes.

Genomic in situ hybridisation (GISH)

GISH protocols are described in Chapter 2. Briefly, sonicated genomic DNA (1 – 10 kb) from *L. longiflorum* was used as probe after labelling with FITC through nick translation according to manufacture's instruction (Boehringer Mannheim, Germany). Sheared herring sperm DNA was used for blocking the non-hybridised DNA sequences. After detection steps, slides were counter-stained with 5 µg/mL propidium iodide (PI). Images were photographed with a Zeiss Axiophot microscope equipped with epi-fluorescence illumination and single band filters for FITC and Cy3/PI using 400 ISO colour negative film. The film was then scanned at 1200 dpi using HP film scanner and the contrast and colour balance was adjusted in digital processing software program 'Photoshop 5.0' (Adobe Inc. USA).

Results

Embryo rescue

The triploid BC₁ plant '921238-1' was used as female parent successfully for further crossing. This plant was fertile and produced both male and female gametes. Four different genotypes, diploid and autotetraploid of Asiatic hybrids and *L. longiflorum* hybrids, LA-hybrid (allotetraploid; LLAA) were used as male parents for crossing with the triploid BC₁ (ALA) hybrid. Embryo rescue was successful in producing BC₂ individuals (Table 2) in some of the crosses. In case of ALA crossed with diploid and tetraploid *L. longiflorum*, the outgrowth of the ovary failed totally. However, some of the crosses, e.g., ALA × AA, ALA × AAAA and ALA × LLAA, resulted in a number of plants (Table 2). When diploid Asiatic hybrids (AA) were used as male parents, a higher frequency of BC₂ progenies were obtained (2.1 plants per ovary in average) as compared to that of tetraploids (AAAA) (0.6 plant per ovary). However, the allotetraploid LLAA of the LA-hybrid, which consisted of both parental genomes (L and A), gave the best results in producing BC₂ individuals (5.7 plants per ovary).

Ploidy levels of BC₂ progenies

The DNA values of the parents (Table 3) confirmed the expected values of approximately 79.3 units ('Connecticut King'), 112.9 units (ALA female parent), 151.4 units (LLAA) and 157.4 units (AAAA, 4x Connecticut King), respectively. The progeny of 3x × 2x had DNA values ranging from 85.9 to 192.3 units. This indicates that the progenies were expected to consist of a range of aneuploids from near diploids, triploid and pentaploid as well as a pentaploid (192.3 units in 997139-1) based on

Table 3. DNA content from flow cytometric results of parental plants according to their genome composition and ploidy level.

Accession	Genotype	Genome composition	Ploidy level (2n)	DNA content (2C)	
				Value (units)	x value
991033	Asiatic hybrid 'Connecticut King'	AA	2x	79.3	39.6
991031	<i>L. longiflorum</i> 'Gelria'	LL	2x	77.3	38.7
921238-1	BC ₁ plant	ALA	3x	112.9	37.6
85914	4x 'Connecticut King'	AAAA	4x	157.4	39.4
952047-1	4x <i>L. longiflorum</i> × 4x Asiatic	LLAA	4x	151.4	37.9

DNA values (Table 4). If the diploid male parent had contributed haploid ($x=12$) gametes, the triploid BC₁ had also produced near haploid ($x=12+n$) female-gametes giving rise to near diploid progenies (10 out of 12 plants; Table 4) whose DNA values ranged from 85.9 to 99.6 units. Most of the plants from $3x \times 2x$ crosses appeared to have a constitution in between diploid and triploid. Most of the BC₂ plants from $3x \times 4x$ crosses, on the other hand, appeared to be near pentaploid. Expectations based on DNA values regarding ploidy status appeared indeed reliable. The occurrence of near triploid plants in this cross could be explained as due to the formation of near diploid gametes also in the ALA-hybrid, that were fertilised with a normal haploid male gamete from the diploid Asiatic hybrid.

Occurrence of pentaploid plants based on the calculation of DNA values in the progenies of $3x \times 4x$ crosses can be explained through the fact that $2n$ -gametes ($3x$) of ALA-hybrids were functional and united with a $2x$ -gamete from the tetraploid male parent. The result, in this case, was obviously a pentaploid. However, two individuals ('997118-9' and '997118-10') in $3x \times 4x$ crosses were nearly triploid. This was probably the result of the functioning of near $2x$ -gametes ($24 A+? L$), which originated from the ALA-hybrid.

Interestingly, there were some notable differences in the ploidy level of the progenies obtained in the different cross combinations. In case of a diploid male parent, haploid gametes (x) were predominantly fertilised with a haploid female gamete from the ALA female parent, resulting in diploids or near diploids. In the case of the tetraploid male parent ($4x$ Asiatic- and $4x$ LA-hybrid, $2x$ -gametes were predominantly fertilised with a unreduced ($3x$) or a near unreduced ($3x\pm$) female gamete, resulting in pentaploids or near pentaploids (Table 4).

Genome identification through GISH

In order to verify whether the $2C$ DNA values were in conformity with the cytological constitution of the progenies, some of the plants were analysed through GISH. In case of $3x \times 2x$ crosses of ALA \times AA (Table 5), the plants with DNA values ranging from 85.9 to 99.6 units were assumed to be in between diploid and triploid. One example of this is shown for '997139-2' which possessed $2n = 2x = 24+6 = 30$ chromosomes (Fig. 1e). Assuming that the diploid Asiatic (AA) male parent had contributed a normal n -gamete ($x = 12$), the triploid ALA parent had contributed 18 chromosomes (i.e., $13A + 5L$) giving rise to the in between diploid and triploid constitution of '997139-2' with 30 chromosomes.

One genotype '997118-12' was a near pentaploid ($5x+1$) based on DNA value. The chromosome counting of this plant indicated this expectation in having 63

Table 4. DNA values from flowcytometric measurement and probable ploidy levels of the progenies (BC₂) derived from the crosses of allotriploid '921238-1' (ALA) to different ploidy levels.

Accession	Parentage		Expected genome composition	2C DNA value	Probable ploidy level
	Female	Male			
3x × 2x	ALA	AA			
997139-1	921238-1	991033	ALA A	192.3	5x
997139-2	921238-1	991033	ALA A	98.0	2x+6
997139-3	921238-1	991033	ALA A	107.8	2x+10
997139-4	921238-1	991033	ALA A	116.7	3x+1
997139-5	921238-1	991033	ALA A	100.7	2x+8
997139-6	921238-1	991033	ALA A	98.6	2x+7
997139-7	921238-1	991033	ALA A	85.9	2x+3
997139-8	921238-1	991033	ALA A	97.4	2x+7
997139-9	921238-1	991033	ALA A	90.6	2x+4
997139-10	921238-1	991033	ALA A	99.5	2x+7
997139-11	921238-1	991033	ALA A	90.5	2x+4
997139-12	921238-1	991033	ALA A	93.7	2x+5
3x × 4x	ALA	AAAA			
997106-1	921238-1	85914	ALA AA	197.8	5x+2
997106-2	921238-1	85914	ALA AA	196.4	5x+2
997147-1	921238-1	87239	ALA AA	196.4	5x+2
997147-2	921238-1	87239	ALA AA	198.3	5x+2
997147-3	921238-1	87239	ALA AA	196.9	5x+2
997147-4	921238-1	87239	ALA AA	196.0	5x+1
997147-5	921238-1	87239	ALA AA	197.8	5x+2
997147-6	921238-1	87239	ALA AA	197.4	5x+2
997147-7	921238-1	87239	ALA AA	196.4	5x+2
997147-8	921238-1	87239	ALA AA	196.0	5x+1
997147-9	921238-1	87239	ALA AA	197.8	5x+2
997147-10	921238-1	87239	ALA AA	197.4	5x+2
997147-11	921238-1	87239	ALA AA	199.6	5x+3
997147-12	921238-1	87239	ALA AA	197.8	5x+2
997147-13	921238-1	87239	ALA AA	194.6	5x+1
3x × 4x	ALA	LLAA			
997118-1	921238-1	952047-1	ALA LA	195.2	5x+2
997118-2	921238-1	952047-1	ALA LA	194.3	5x+1
997118-3	921238-1	952047-1	ALA LA	192.0	5x+1
997118-4	921238-1	952047-1	ALA LA	192.9	5x+1
997118-5	921238-1	952047-1	ALA LA	196.5	5x+2
997118-6	921238-1	952047-1	ALA LA	190.7	5x
997118-7	921238-1	952047-1	ALA LA	194.7	5x+1

Continued from Table 4.

Accession	Parentage		Expected genome composition	2C DNA value	Probable ploidy level
	Female	Male			
997118-8	921238-1	952047-1	ALA LA	193.8	5x+1
997118-9	921238-1	952047-1	ALA LA	142.7	3x+9
997118-10	921238-1	952047-1	ALA LA	131.2	3x+5
997118-11	921238-1	952047-1	ALA LA	192.0	5x+1
997118-12	921238-1	952047-1	ALA LA	193.8	5x+1
997118-13	921238-1	952047-1	ALA LA	188.3	4x+11

Table 5. Genome composition through GISH analysis of two different crosses.

Accession	Crosses	Flowcytometric results		Actual chr. number	From female parent		From male parent	
		DNA content	Expected chr. number		L(L/A) ²	A(A/L) ²	L(L/A) ²	A(A/L) ²
921238-1	A × LA	112.9	-	36	-	12	12 (2)	12 (1)
997118-12	921238-1 × LLAA	193.8	61	63	12 (2)	27 (1)	12	12
997139-2	921238-1 × AA	98.0	30	30	5 (2)	13 (0)	-	12

² L/A and A/L indicate the recombinant chromosomes with Asiatic and *longiflorum* chromosome segment(s), respectively.

chromosomes (Table 5). Because the male parent was an allotetraploid (LLAA) it was expected to have produced a 2x-gamete with each one L- and A-genome, i.e., contributing 24 chromosomes to the progeny. GISH result confirmed that the triploid had contributed 39 (3x+3) chromosomes of which 12 were from the L-genome and 27 (instead of 24) from the A-genome (Fig 1b; Table 5). Three of the homoeologous recombinant chromosomes (arrowheads) were transmitted from the triploid female (Fig. 1a). The occurrence of aneuploid gametes was expected because of the triploid constitution of the female parent.

GISH results confirmed that the same or less numbers of recombinant chromosomes were transmitted from the triploid female (arrowheads in Fig. 1a, b, and e). It appears that a low frequency of homoeologous recombinations occurred in triploid female parent (ALA) because of the preferential pairing between A- and A- rather than A- and L-chromosomes during meiosis. Comparing the chromosome composition of the female parent (ALA; '921238-1' Fig 1a) and '997139-2' (BC₂ plant), two homoeologous recombinant chromosomes among the five chromosomes of the alien *L. longiflorum* in BC₂ were transmitted from the

triploid female parent (Fig. 1e; compare Fig. 1f and g). Fig. 1f and g shows clearly that recombinant *L. longiflorum* chromosome 9 and 12 were transmitted from the female parent (compare Fig. 1a, f and Fig. 1e, g). Although genome composition of the BC₁ female parent consists of two sets of the A-genome and one set of the L-genome which tend to have preferential chromosome association between two sets of the A-genome, some frequencies of homoeologous chromosome association between A- and L-genomes were observed at metaphase I (unpublished data). Especially *L. longiflorum* chromosome 9 had a new homoeologous recombination during the meiosis of the triploid parent (ALA) (Fig. 1e and g, indicated by red arrowhead). Furthermore, the GISH results demonstrate that the triploid (ALA) female parent contributed one more Asiatic chromosome 4 to the next generation during megasporogenesis or megagametogenesis (Fig. 1e).

Discussion

If we recognise the fact that whenever interspecific hybridisation is practised in horticultural crop breeding, there is; a) a likelihood of increasing the ploidy level and b) there is a possibility of introgression of alien chromosome(s) or homoeologous recombinant segments. This investigation, together with the results in chapter 5 on the production of LA-hybrids and BC₁ progeny (ALA), highlights changes in ploidy level as well as introgression.

Considering the backcross procedure of hybridisation using embryo rescue methods, the process of introgression is by no means an easy task. When the obtained hybrid material and backcross progenies are properly analysed, more meaningful approaches can be planned as well as the outcome may become more predictable. In this context, the use of techniques such as flowcytometric DNA measurement for ploidy determination and GISH for the identification of genomes can elucidate chromosome and intergenomic recombination.

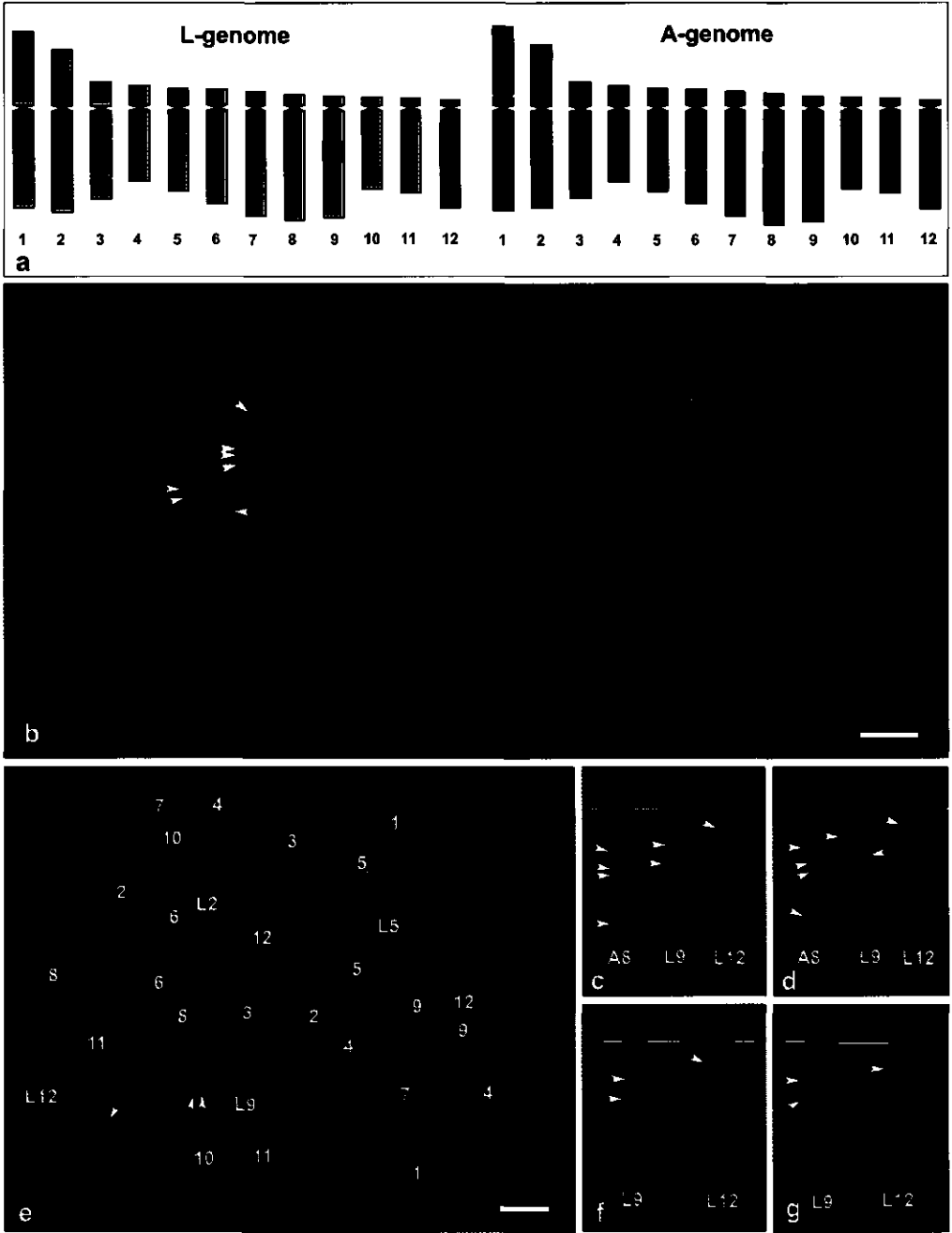
The triploid BC₁ used as a female parent was fertilised preferentially with male gametes according to its genome composition. Table 2 shows some examples of selective fertilisation. Firstly, in case of $3x \times 2x$ crosses (ALA \times AA), since the genome constitution of the female parent (ALA) is two sets of A-genome and one set of L-genome, the frequency of successful fertilisation with Asiatic pollen (haploid gametes) showed better results than with *L. longiflorum*. Similar results were obtained in the $3x \times 4x$ crosses, where tetraploid Asiatic pollen fertilised higher frequencies than the *L. longiflorum* pollen. The results are probably due to genetic affinity in genome composition between male and female gametes. However, $3x \times 4x$ crosses showed a lower number of embryos is formed compared to $3x \times 2x$

crosses. This may be due to the increased genome complexity. Secondly, in case of LA-hybrids (allotetraploid) as male parent, both L- and A-genomes are expected to coincide well with the ALA female parent on their affinity of genome composition. In this case, ALA \times AA, the number of embryos per ovary was higher than in case of the ALA \times AAAA cross.

From the data presented in the Table 4 and 5, it is evident that DNA values fairly reflect the ploidy levels and are helpful for selecting aneuploids (between diploids and triploids). This aspect is certainly most helpful for large scale screening of the progeny. In addition, selecting the more appropriate genotypes for further analysis or use them as parents. Besides, in other plants with large chromosomes, such as *Alstroemeria* (Buitendijk et al. 1997), even aneuploids with single chromosome differences can be identified through flowcytometric measurement.

The type of progeny obtained in the case of $3x \times 2x$ and $3x \times 4x$ crosses shows a clear difference (Table 4). In the case of $3x \times 2x$ crosses the progeny have a near diploid chromosome number. On the other hand, in the case of $3x \times 4x$ cross the ploidy level of the progeny is nearly pentaploid. This obviously means that the $3x$ female parent is capable of producing viable gametes whose chromosome numbers can vary from x ($x=12$) to $3x$ (i.e., $2n$) gametes. When the diploid is used as the male parent, the progenies are nearly diploid, whereas when a tetraploid is the male parent the progenies are nearly pentaploid. This difference in the ploidy levels of the progenies is in a way, comparable to the situation in several other plant species where $3x \times 2x$ crosses produce mostly diploid ($2x$) progenies and $3x \times 4x$ crosses give rise to mostly tetraploid progenies (see Brandham 1982). The occurrence of such differences has been attributed to the differences in the ratios of the ploidy levels of the embryo and the endosperm depending on the type of cross.

The occurrence of diploid plus 6 chromosomes ('997139-2' Table 5) in $3x \times 2x$ crosses is the most attractive because they offer the prospects for creating monosomic alien addition lines, in which one Asiatic chromosome was added from female gamete formation. One of the most convenient features of using GISH is that the genome, chromosomes and recombinations can be accurately identified. Two BC₂ individuals analysed by GISH showed aneuploids (Fig 1b and e). One plant '997118-12' possessed 63 chromosomes, among them 39 chromosomes originated from the female gamete, which composed of 12 chromosomes of the L- and 27 of the A-genome. After telophase II as a typical division of embryo-sac mother cell in lily, a quarter of the nuclei (x) migrates from the micropylar to the chalazal site and reunified into triplex nuclei ($3x$) (Brock 1954). It can be assumed that chromosome number of ALA egg mother cells were unreduced giving rise to 36 chromosomes with the complete L-genome (12 chromosomes) and two sets of the A-genome (24



chromosomes) at tetrad stage. Later, during migration stage, three of the Asiatic chromosomes were left at the same place, but the nine other Asiatic chromosomes were moved from the micropylar to the chalazal site as a rule. Finally, as result of abnormal migration process, the chromosome composition of the female gamete will be 27 A- and 12 L-chromosomes, which are transmitted to the next generation. Another case of a BC₂ plant (997139-2; 2n=30) can be explained as follows;

1. Table 5 shows the probable origin of chromosomes, in which 12 intact chromosomes originated from the male gamete as expected, 5 L- and 13 A-chromosomes originated from the ALA female gamete.
2. At meiosis I, almost perfect preferential chromosome association had occurred giving rise to one trivalent between the A- and L-chromosomes (with *L. longiflorum* chromosome 9 because chromosome 9 had recombinant in the BC₂), 11 A-bivalents, 11 L-univalents.
3. At anaphase I, 11 bivalents disjoined normally and 11 univalents and 1 trivalent moved randomly (5 chromosomes of the *L. longiflorum* moved to one pole and 7 chromosomes to the other pole).
4. Because of the Asiatic chromosome 4 was added (Fig. 1e), it can be postulated that during migration stage, Asiatic chromosome 4 stayed at the same place while the rest of the chromosomes moved to the chalazal site.

An interesting result through GISH analysis of the two BC₂ plants is that there is a much lower frequency of homoeologous recombination occurring than in the BC₁. Once homoeologous recombinants were obtained in BC₁ generation, these can be transmitted to the next generation without any more homoeologous recombination (in case of 997118-12). If this is true, then it will be easy to select the useful genes, which were introduced from the donor species at the BC₂ generation. More

Figure 1. (a) Ideogram of the triploid (ALA) '921238-1' (BC₁, 2n = 3x = 36) used as female showing some homoeologous recombinations between L- and A-genomes. Two of *L. longiflorum* and one of *L. Asiatic* chromosomes were recombined during meiosis of F₁ interspecific LA-hybrid and were transmitted to BC₁. (b) Genome painting of metaphase chromosome complement of BC₂ plant '997118-12' obtained from a 3x × 4x cross. Three recombinant chromosomes were recognised as the same chromosomes assuming that they transmitted from the triploid female to the BC₂ generation without any further homoeologous recombination during meiosis of the BC₁ (compare Fig. c and d). (c – d) Comparison on the recombinant chromosomes of BC₁ (Fig. c) and BC₂ (Fig. d). (e) Genome painting of mitotic metaphase complement of BC₂ individual '997139-2' (2n = 30) derived from cross of a 3x × 2x, indicating that two homoeologous recombinant chromosomes (arrowheads) transmitted from triploid female '921238-1'. Red arrowhead indicates the fact of homoeologous recombination during meiosis of BC₁ '921238-1' (compare Fig. f and g). (f – g) Comparison of recombinant chromosomes of BC₁ '921238-1' (Fig. f) and BC₂ '997139-2' (Fig. g).

important is the fact that it saves time to select out appropriate parents. The use of proper parents and the possibilities for monitoring the types of progenies that are produced are undoubtedly essential ingredients for making rational predictions. The alien chromosome additions at the diploid level open the possibilities for producing alien chromosome substitution lines in lilies in the near future.

7 General Discussion

This thesis has described a cytogenetic study of species and hybrids of the genus *Lilium*. The different chapters, which focus on karyotype analysis of two lily species and chromosome behaviour in meiosis of the interspecific lily hybrids, can have a high impact for lily breeders and taxonomists. In the following paragraphs, the relevance of the present investigations is highlighted and attention is drawn to practical and theoretical aspects of polyploidisation and introgression in *Lilium*.

In our vision on cytogenetics for lily, the aim was to acquire essential background information for the introgressive hybridisation and breeding programs: a) the use of banding techniques for the identification of individual chromosomes in two lily species (*L. longiflorum* and *L. rubellum*) which were used as parents in this study and b) the use of fluorescence *in situ* hybridisation for the distinction of parental chromosomes in mitotic and meiotic cells of interspecific lily hybrids.

Individual chromosome identification and karyotype analysis

The recognition of all chromosomes in a mitotic metaphase complement is one of the major goals in plant cytogenetics. The thus acquired chromosome portrait allows the assignment of linkage groups and economically important genes to individual chromosomes and so can effectively trace their behaviour in consecutive crossing generations. In the absence of species-specific DNA probes, however, the highly conserved rDNA sequences have been effectively used for chromosome identification in lily and in other plant species (Chapter 2). C-banding, in combination with *in situ* hybridisation, can be applied as shown in wheat and related species (Jiang and Gill 1993). For unknown reasons, a relatively small amount of constitutive heterochromatin in *Lilium* species genomes are present (Chapter 2). In view of this, it would be desirable to generate species-specific DNA markers in lily as has been done in *Alstroemeria* (De Jeu et al. 1995). The introduction of fluorescence *in situ* hybridisation (FISH) methods to plant chromosomes using repetitive and single copy DNA sequences as probes has been highly useful. Most common examples are the use of highly conserved rDNAs. These components of the nucleolar organiser regions (NORs) have been used most effectively for the identification of the satellite bearing chromosomes in metaphase complements in several plants (Gerlach and Dyer 1980; Kamstra et al. 1997; Takeda

et al. 1999; Mukai et al. 1991a,b; Leitch and Heslop-Harrison 1992; Jiang and Gill 1994; Fukui et al 1998). In addition, for the two lily species, *L. longiflorum* and *L. rubellum*, the technique showed clear signals on NOR-bearing chromosomes. Together with other banding techniques such as C-banding, DAPI and PI staining most of the individual chromosomes can be identified (Chapter 2). Furthermore, this banding study newly demonstrated two NOR-bearing chromosomes (chromosome 3 and 6) of *L. rubellum*. So far, those chromosomes were neither known as NOR-bearing ones. Chapter 2 in this thesis represents the most detailed karyotype analysis performed until now. However, chromosomes 5 and 6, and 10 and 11 in *L. longiflorum*, and chromosomes 7 and 9, and 10 and 11 of *L. rubellum* are still very difficult to discriminate from each other.

Polyploidisation and their cytogenetic analysis in lily

In some important horticultural bulbous crops such as *Narcissus* and *Tulipa*, several thousands of cultivars have been produced by breeding during the last centuries. Lily belongs also to the aforementioned group of horticultural crops and about 7000 cultivars have been registered since 1960 (Leslie 1982-1998). Beside this, *Lilium* species have also been model plants for cytogenetic research because of their large genome and chromosome size. Despite these attractive features, useful cytogenetic information for lily breeders is still scarce as in the case of many other important horticultural crops.

Undoubtedly, a lot of cytogenetic research has been conducted during the last century on polyploid crops - both natural and induced - using traditional cytogenetic techniques. Obviously, there were serious limitations connected to these techniques because it was very difficult, or even impossible, to identify all individual chromosomes of many economically important crops. This situation changed dramatically during the recent decade when molecular cytogenetics became available. It is possible to identify not only individual chromosomes accurately but also homoeologous recombinant segments through GISH (Schwarzacher et al. 1992; Kuipers et al. 1997; Kamstra et al. 1999a,b; Khrustaleva and Kik 1998, 2000; Lim et al. 2000). Moreover, species-specific DNA probes serve as efficient chromosome markers when used in FISH for the identification of individual chromosomes as has been demonstrated in *Alstroemeria* (Kamstra et al. 1997).

The availability of molecular chromosome markers together with banding techniques and GISH have opened new opportunities to investigate polyploids more critically as never before. As it is evident in the case of the analysis of the BC₂ population of LLLR derivatives, homoeologous crossing-over is completely lacking

in the progenies of somatically doubled hybrids (Chapter 3). This means that introgression from donor species into cultivars, using mitotic polyploidisation, is difficult. However, it is certainly possible to generate aneuploid plants that possess the whole alien chromosome additions. Somatic polyploidisation gave important information for producing aneuploid or alien chromosome additions in the BC₂ generation (Chapter 3).

Analysis of flower colour in BC₂ progenies (aneuploid; LLLR), derived from somatic polyploidisation, showed that two individuals were pink and one was white. Because the pink flower colour is derived from the *L. rubellum*, those three plants could be useful for investigating the chromosomes that determine the gene(s) for pink flower colour (Chapter 3).

One of the most important phenomena found in several horticultural crops is the occurrence of spontaneous polyploidisation during breeding for domestication. They mostly originated from interspecific hybrids, which are often sterile due to chromosomal differentiation of the parental species. In such hybrids, the few viable gametes that occur at low frequencies are often unreduced (2n) and capable for producing spontaneous polyploids. This method of spontaneous polyploid formation has been demonstrated in numerous horticultural crops (cf. Ramanna 1992). Therefore, the aspects of the modes of origin of 2n-gametes and, eventually their sexual polyploids deserve more attention through cytogenetics (Chapters 4 and 5).

Unlike in somatic allopolyploids and their backcross derivatives where homoeologous chromosome associations and recombinations are totally absent (Chapter 3), intergenomic recombination can occur in meiotic polyploids (Chapter 4 and 5). This is a fundamental difference between the genetic consequences of mitotic and meiotic polyploidisation in lilies. In fact, even in intersectional hybrids such as *L. longiflorum* (Leucolirion section) × Asiatic hybrids (Sinomartagon section) there is evidence for the occurrence of multiple cross-overs between homoeologous chromosomes (Fig. 1 and 2 in Chapter 4) and they are transmitted to the progeny (Fig. 1 and 2 in Chapter 5).

An intergenomic recombination demonstrated in the present study is the occurrence of two types of recombinations (Fig. 3c, Chapter 4) due to:

- 1) Crossing-over between homoeologous chromosomes and
- 2) The interchromosomal recombination by random orientation and segregation of heteromorphic bivalents of two homoeologous chromosomes.

Intergenomic recombinations can readily be detected through *in situ* hybridisation (Fig. 1 and 2 in Chapter 4 and 5) (Yan et al. 1999; D'Hont et al. 2000; Linares et al. 2000). This aspect has opened the possibilities for shortening the time requirement

for introgression of desirable genes. Furthermore, as was demonstrated in this study, it should be possible to create cytogenetically defined breeding material. If such material becomes available, efficient breeding strategies can be developed for lilies and other crops.

Meiotic nuclear restitution mechanisms

Meiotic nuclear restitution mechanisms in plants are mainly divided into FDR (First division restitution) and SDR (Second division restitution). In FDR during abnormal micro- or mega-sporogenesis (i.e., lack of chromosome association), all centromeres of the univalents and the half-bivalents divide equationally (simultaneously) and the chromatids move to two poles. Because of the equational division of chromosomes, the parental genomes are preserved nearly intact in the resulting FDR $2n$ -gametes (Fig. 3a in Chapter 4). Because of rare occurrence of SDR gametes in wide interspecific hybrids, there was no occurrence in the BC_1 progenies investigated in Chapter 5. Unlike in FDR, in the new mechanism of nuclear restitution 'IMR', demonstrated in this thesis, univalent(s) divide(s) equationally as in FDR but also bivalent(s) divide "reductionally" as in normal anaphase I in the same spore mother cell. The consequence of this type of division is that the homoeologous chromosomes of a bivalent assort by moving to the two opposite poles, whereas all the univalents divide equationally as in FDR. This obviously results in producing $2n$ -gametes in which the two parental genomes are not preserved intact but the euploid number is preserved (Fig. 3 in Chapter 4; Fig. 2 and 3 in Chapter 5). The odd number of parental chromosomes at telophase II of the restituted euploid IMR spores ($2n$) was actually transmitted in one of our genotypes (Fig. 2 in Chapter 5). After the discovery of this mechanism in lily, a recent report on the genome composition of banana ($2n = 3x = 33$) (D'Hont et al. 2000) also showed the unequal chromosome constitution of the triploid cultivar 'Pelipita' ($ABB = 8 A + 25 B$). A logical explanation of this abnormality appears to be IMR.

Besides $2n$ -gametes, aneuploid gametes do occur in lilies as is evident from the chromosome composition of 997139-2 resulting from an ALA (triploid) \times AA (diploid) cross (Chapter 6). The ploidy level of this genotype ($2n = 30$) is between diploid and triploid. 18 chromosomes were originated from a triploid female parent (ALA) transmitting 13 chromosomes from Asiatic genomes and the remaining 5 chromosomes from *L. longiflorum*. From flowcytometric analysis, it is evident that many of such aneuploids are available in these progenies (Table 4 in Chapter 6). These BC_2 plants are potentially useful for developing alien chromosome addition lines of Asiatic hybrids with individual *L. longiflorum* chromosomes at the diploid

level. In several crop plants, alien chromosome addition lines have been shown useful for determining the chromosomal positions of genes.

An important question to answer is what is the best strategy to produce polyploids. Is it mitotic or meiotic doubling? During the last century the polyploidy inducing chemical "colchicine" was hailed as a "wonder drug" for crop improvement but led to disappointment for introgression breeding. The observation that sexual polyploidisation through the use of $2n$ -gametes could be advantageous, stimulated research in the so-called, polysomic polyploids (autopolyploids) in crops such as potato, alfalfa, clover and blue berries among others. The main emphasis in these crops has been paid to increase the heterozygosity of the sexual polyploid progeny using FDR gametes (Ramanna 1979; Ortiz and Peloquin 1991a,b; Vorsa and Rowland 1997; Douches and Maas 1998; Buso et al. 1999). Unfortunately, the allopolyploids received no attention implicating as if sexual polyploidisation is not relevant to this group. Moreover, attention was directed only to dicotyledonous plants and instances of the occurrence of $2n$ -gametes in monocotyledonous plants have been virtually ignored. In this context, the sexual polyploids produced by Kamstra et al. (1999a,b) in *Alstroemeria*, another monocot, and the present investigation on lily are filling the gap.

Summary

Lily has been grown as the most important bulbous crop during the last decade. The genus *Lilium* consists of seven different sections with about 80 species. Among them, three sections are the most commonly used for breeding cultivars, e.g. Asiatic hybrids of the Sinomartagon section, *L. longiflorum* of the Leucolirion section and Oriental hybrids of the Archelirion section. The species and cultivars are mainly diploid ($2n = 2x = 24$) but polyploid forms exist including triploids and tetraploids, which are either induced for breeding purposes or spontaneously found during interspecific breeding programs. Therefore, cultivars include both diploids as well as polyploids of which the latter became more important in recent years.

As in all other crops, there is a need for combining desirable characteristics in new cultivars. In lily, such characters are distributed in several species that belong to different sections in the genus *Lilium*. It is, therefore, essential to hybridise species from different sections and to backcross the F_1 -hybrids to the cultivars in order to select new cultivars. Generally, it is difficult to hybridise species and to produce backcross progenies, but these crossing barriers can be overcome through integrated pollination and embryo rescue methods. The intersectional species hybrids, however, are highly sterile because of a high degree of genome differentiation of the parents. The main aim of the present research was to develop techniques for using sterile interspecific hybrids in lily breeding more effectively. For this purpose, the F_1 -hybrids between *Lilium longiflorum* and *L. rubellum* (LR), the amphidiploids of LR-hybrid, *L. longiflorum* and Asiatic hybrids (LA) and backcross progenies (BC_1 and BC_2) of these hybrids were analysed through molecular cytogenetic techniques, GISH (genomic *in situ* hybridisation) and FISH (fluorescence *in situ* hybridisation).

Because it was essential in this study to identify the genomes and chromosomes of the parental species in F_1 -hybrids and their backcross derivatives, criteria were developed for the identification of individual chromosomes of some of the *Lilium* species (Chapter 2). For this purpose, the karyotype characteristics based on chromosome length, arm length ratios, C-banding patterns and FISH detection of rDNA sequences (5S and 45S rDNA) was performed. Some of these criteria, together with GISH and FISH were used for the analysis of both mitotic and meiotic chromosomes of the F_1 -hybrids and their backcross derivatives (Chapter 3, 4 and 5).

The diploid ($2n = 2x = 24$) sterile F_1 -hybrids were utilised for backcrossing in two different ways, viz., 1) mitotic (somatic) polyploidisation in case of *L. longiflorum* × *L. rubellum* hybrids and 2) meiotic (sexual) polyploidisation in case of *L. longiflorum*

× Asiatic hybrids, where 2n-gametes in certain genotypes were formed. A remarkable difference was observed between the two polyploidisation methods. In case of mitotic polyploidisation there was no evidence of any intergenomic recombination between the chromosomes of *L. longiflorum* and *L. rubellum*. This was because of the complete preferential pairing between the homologous chromosomes of each species in the amphidiploid (Chapter 3). In the triploid BC₁ progenies (LLR) and the aneuploid BC₂ (LLLR) progenies there was a total absence of homoeologous recombinant chromosomes (Chapter 3).

In case of the 2n-gametes producing F₁ LA-hybrids there was clear evidence, through GISH and FISH, for the occurrence of homoeologous chromosome association and recombination as was visible at metaphase I and anaphase I of the microsporogenesis. Most remarkably, these LA-hybrids produced three types of 2n-gametes: FDR (first division restitution) without recombination, FDR with recombinant chromosomes and IMR (indeterminate meiotic restitution) with recombinant chromosomes as well as with assortment of homoeologous chromosomes (Chapter 4 and 5).

The occurrence of such genetic recombination in these LA-hybrids was convincingly proven by analysis of the chromosome composition of the BC₁ (ALA) and BC₂ progenies (Chapter 5 and 6). Thus, it was evident that by using 2n-gametes, as in LA-hybrids, sexually polyploidised offsprings can be produced with the addition of complete alien chromosomes as well as with a variable size of recombinant segments. This is advantageous for the introgression of specific characters from alien species into the cultivars. Some triploid BC₁ progenies (ALA) derived from crossing with 2n-gametes producing LA-hybrids showed a range of fertility (5 – 30 %) and one BC₁ plant (921238-1) was used as female parent for further crossing, in which many BC₂ individuals were produced. The strategy of interspecific hybridisation for introgression breeding of lily is now operational by using 2n-gametes producing interspecific hybrids (F₁) combined with backcrossing (Chapter 6).

Besides, the practical implications of sexual polyploidisation in lily, the results of the investigations, presented in this thesis, are scientifically significant for many reasons. In the first place, a comparison has been made of the consequences of mitotic and meiotic polyploidisation in this crop. Secondly, in the previous cytogenetic studies the meiotic nuclear restitution mechanisms were rigidly categorised into FDR and SDR. There is now convincing evidence that IMR is a mechanism that combines both FDR and SDR in individual PMC. Thirdly, the molecular cytogenetic study using *in situ* hybridisation techniques is a powerful tool which enables us not only to create useful breeding material but also to enhance

our basic knowledge regarding the modes of origin and composition of polyploids in horticultural crops and species.

Samenvatting

Lelie is de laatste decennia uitgegroeid tot het belangrijkste bolgewas. Het geslacht *Lilium* omvat zeven secties met circa 80 soorten. Drie secties zijn tot nu toe het meest gebruikt bij de lelieveredeling, te weten Leucolirion, Sinomartagon en Archelirion, waaruit respectievelijk *L. longiflorum*-cultivars, de Aziatische hybriden en de Oriental hybriden zijn ontwikkeld. De soorten zijn diploïd ($2n=2x=24$), maar polyploïden (vnl. triploïden en tetraploïden) komen ook voor. Deze polyploïden zijn gericht ontwikkeld voor de veredeling of spontaan ontstaan in interspecifieke veredelingsprogramma's. Hierdoor bestaat het sortiment uit diploïden en polyploïden, waarbij de laatsten de afgelopen jaren sterk in belang zijn toegenomen.

Zoals in alle andere gewassen, is het gewenst om gunstige eigenschappen in nieuwe cultivars te introduceren. Bij lelie komen deze eigenschappen voor bij soorten die tot verschillende secties van het geslacht *Lilium* behoren. Het is daarom essentieel om soorten van verschillende secties met elkaar te hybridiseren en de F_1 -hybriden terug te kruisen om zodoende nieuwe cultivars te kunnen selecteren. In het algemeen is het moeilijk om soorten te kruisen en terugkruisingspopulaties te produceren, maar het is mogelijk om kruisingsbarrières te omzeilen door toepassing van geïntegreerde bestuivings- en embryo-reddingsmethoden. De intersectionele soortshybriden, zijn echter in het algemeen zeer steriel door een hoge graad van genoomdifferentiatie van de ouders. Het belangrijkste doel van het huidige onderzoek was om technieken te ontwikkelen waarmee steriele interspecifieke hybriden effectiever gebruikt kunnen worden in de lelieveredeling. Hiertoe zijn de F_1 -hybriden van *Lilium longiflorum* en *L. rubellum* (LR), *L. longiflorum* en Aziatische hybriden (LA), de amphidiploïden van deze hybriden en terugkruisingspopulaties (BC_1 en BC_2) geanalyseerd met moleculair cytogenetische technieken, GISH (genomische *in situ* hybridisatie) and FISH (fluorescentie *in situ* hybridisatie).

Omdat het in deze studie essentieel was om genomen en chromosomen van oudersoorten te identificeren in de F_1 -hybriden en hun terugkruisingsnakomelingschappen, zijn criteria ontwikkeld voor de identificatie van individuele chromosomen van enkele *Lilium*-soorten (Hoofdstuk 2). Hiervoor zijn karyotypische eigenschappen gebruikt zoals chromosoomlengte, armlengte-ratios, C-banderingspatronen en FISH detectie van rDNA sequenties (5S en 45S rDNA). Enkele van deze criteria zijn tezamen met GISH en FISH gebruikt voor de

analyse van mitotische en meiotische chromosoompreparaten van de F₁-hybriden en hun terugkruisingsnakomelingschappen (Hoofdstuk 3, 4 en 5).

De diploïde ($2n = 2x = 24$), steriele F₁-hybriden zijn op twee manieren gebruikt bij het terugkruisen, namelijk 1) door mitotische (somatische) polyploïdisatie in het geval van *Lilium longiflorum* × *L. rubellum* hybriden en 2) door meiotische (generatieve) polyploïdisatie in het geval van *L. longiflorum* × Aziatische hybriden (in enkele genotypen die 2n-gameten vormden). Er is een opmerkelijk verschil tussen de twee polyploïdisatietechnieken waargenomen. In het geval van mitotische polyploïdisatie kan geen enkel bewijs van enige intergenomische recombinatie tussen de chromosomen van *L. longiflorum* en *L. rubellum* gevonden worden. De oorzaak is complete preferentiële paring van de homologe chromosomen van elke soort in de amphidiploïd (Hoofdstuk 3). In de triploïde BC₁ (LLR) nakomelingschappen en de aneuploïde BC₂ (LLLR) nakomelingschappen waren homoeologe recombinante chromosomen dan ook geheel afwezig (Hoofdstuk 3).

Bij F₁-hybriden van *L. longiflorum* × Aziatische hybriden (LA) kon door middel van GISH en FISH duidelijk aangetoond worden dat homoeologe chromosoom-associatie tijdens metafase I en anafase I van microsporogenese plaatsvond. Het meest opmerkelijk was dat de LA-hybriden drie soorten 2n-gameten produceerden: FDR ("first division restitution") zonder recombinatie, FDR met recombinante chromosomen en IMR ("indeterminate meiotic restitution") met recombinante chromosomen én homoeologe chromosomen (hoofdstukken 4 en 5).

Het optreden van dergelijke genetische recombinatie in LA-hybriden werd overtuigend aangetoond door middel van analyse van de chromosoomcompositie van de BC₁ (ALA) en BC₂ nakomelingschappen (ALA) (Hoofdstukken 5 en 6). Zodoende is het duidelijk dat het mogelijk is om generatief gepolyploïdiseerde nakomelingschappen te produceren met een additie van complete, soortsvreemde chromosomen in combinatie met in grootte variërende recombinante segmenten door het gebruik van 2n-gameten. Dit heeft voordelen voor de introgressie van specifieke eigenschappen van verwante soorten naar cultivars. Veel triploïde BC₁ nakomelingschappen (ALA), verkregen na kruising met 2n-gameten producerende LA-hybriden, lieten een variatie in fertiliteit zien (5 – 30 %). Een BC₁-plant (921238-1) werd gebruikt om BC₂-populaties te produceren. De strategie van interspecifieke hybridisatie voor introgressie-veredeling van lelie is nu mogelijk via het gebruik van 2n-gameten producerende hybriden, gevolgd door terugkruisen (Hoofdstuk 6).

Naast de praktische consequenties van generatieve polyploïdisatie bij lelie, zijn de resultaten van dit onderzoek, gepresenteerd in dit proefschrift, om meerdere redenen van wetenschappelijke relevantie. In de eerste plaats is er een vergelijking gemaakt van de consequenties van mitotische en meiotische polyploïdisatie in dit

gewas. Op de tweede plaats zijn tot nu toe in cytogenetische studies de meiotische nucleaire restitutie mechanismen strak ingedeeld in FDR en SDR. Er is nu overtuigend bewijs dat IMR een mechanisme is dat FDR en SDR combineert in een individuele PMC. Op de derde plaats is de moleculaire cytogenetica met *in situ* hybridisatie een krachtig instrument dat ons niet slechts in staat stelt om bruikbaar veredelingsmateriaal te creëren, maar ook om onze fundamentele kennis over de mechanismen achter het ontstaan en de genetische compositie in tuinbouwkundige gewassen te vergroten.

적 요 (摘要)

지난 수 십 년간 百合은 가장 중요한 구근작물로 재배되어 왔다. 百合 屬은 7 亞屬 약 80餘 種으로 구성되어 있다. 그 중에서 3개 亞屬 즉, 아시아틱 (툼나리) 계통의 Sinomartagon, 철포백합 계통의 Leucolirion 그리고 동양계 교잡종 계통의 Archelirion이 상업품종 육성에 있어 가장 중요한 위치를 차지하고 있다. 백합 種의 기본 염색체 수는 2배체 ($2n=2x=24$) 이지만, 상업적 품종의 경우 倍數體도 존재한다. 이들은 대부분 3倍體 혹은 4倍體 이며, 育種過程이나 種間雜種 육종을 통하여 간헐적으로 발생되기도 한다. 따라서, 상업적 품종은 2 배체 와 배수체가 混在 하지만 최근에는 후자가 더욱 중요한 위치를 차지하고 있다.

다른 작물에서와 같이 백합 新品種을 育成하기 위해서는 종간의 有用 遺傳形質들을 조합해야 할 필요성이 있다. 백합에 있어서 이러한 유용 형질들은 百合屬內의 각각 다른 種이나 심지어 다른 亞屬에 분포해 있다. 따라서, 각각 다른 아속간의 종간 잡종이 필수적으로 이루어 져야 하고 신품종 육성을 위해서는 기존의 품종 또는 系統에 兪교잡 하여야 한다. 일반적으로, 종간교잡이 쉽지 않을 뿐 아니라 兪교잡 後代를 생산하는 것 또한 어렵지만, 이러한 문제들의 상당부분은 종합적인 受粉 및 胚培養 기술로 어느정도 극복 가능하다. 그러나, 종간 잡종 특히, 아속간 잡종은 兩親 間의 게놈구성에 있어 차이가 크기 때문에 不稔인 경우가 대부분이다. 본 연구의 주요 목적은 백합 육종에 있어 불임인 종간잡종을 보다 효율적으로 이용하는 기술을 개발하기 위함 이었다. 이러한 목적을 달성하기 위하여 染色體 패인팅 (GISH) 기술을 도입하였으며, *L. longiflorum* × *L. rubellum* (LR), LR-hybrids의 amphidiploid, *L. longiflorum* × Asiatic hybrid (LA) 그리고 이들의 兪교잡 후대들 (BC_1 과 BC_2)을 분석하였다.

F_1 과 兪교잡 후대들의 양친 염색체나 게놈을 구분하는 것이 본 실험의 필수사항이기 때문에 몇몇 백합 종들의 核型分析을 실시하였다 (제 2장). 이를 위하여 염색체의 길이, arm-ratio, C-banding 패턴, rDNA 의 FISH detection을 통한 핵형 분석을 실시하였다. *L. longiflorum* 과 *L. rubellum* 종의 핵형 분석에 근거하여, F_1 종간 잡종과 兪교잡 후대들의 體細胞 및 減數分裂 세포 염색체를 분석하였다 (제 3, 4, 5장).

불임인 F_1 잡종 개체들은 兪교잡 후대를 얻기 위하여 다음과 같은 방법을 통하여 사용되었는데, 첫째, *L. longiflorum* × *L. rubellum* (LR) 교잡의 경우 체세포 배가를 통하여 둘째, $2n$ 花粉을 생산하는 *L. longiflorum* × Asiatic hybrid (LA)의 경우 생식세포 배가법을 이용하였다. 괄목 할 만한 결과가 두 가지 방법을 통하여 얻어 졌는데 체세포 배가법의 경우 *L. longiflorum* 과 *L. rubellum* 사이에 어떠한 異種 染色體 間 組換 (recombination) 도 확인되지 않았다. 왜냐하면 amphidiploid인 LLRR에 있어 두 종간 게놈의 preferential pairing이 일어나기 때문이었다 (제 3장).

따라서 여교잡 1세대 와 2세대인 LLR 과 LLLR 에서는 어떠한 이종간 組換도 일어나지 않았다 (제 3장).

그러나 F_1 LA 중간 잡종에 있어서는 감수분열 第 1中期와 第 1後期の 염색체를 염색체 패인팅 기술을 이용하여 관찰한 결과, 분명한 이종간 組換을 확인할 수 있었으며, 그 후대의 체세포 염색체를 조사해 본 결과, 역시 이를 확인할 수 있었다. 더욱 중요한 결과는 LA 중간 잡종에 있어서 3가지 종류의 $2n$ 화분을 관찰할 수 있었는데 첫째, 組換이 있는 FDR 화분 둘째, 組換이 없는 FDR 화분 셋째, 組換이 있는 IMR 화분 (제 4, 5장). 또한, 이러한 상호 종간 組換은 여교잡 후대를 염색체 패인팅 해 봄으로써 확인되었다 (제 5, 6장). 따라서, LA 중간 잡종에서와 같이 $2n$ 화분을 이용하여 組換에 의한 염색체 단편들이 이입된 후대들이 생산될 수 있음이 입증되었다. 이러한 사실은 다른 종으로부터 特定形質을 원하는品種에 移入시키는데 있어 매우 유용한 방법이다. $2n$ 화분을 생산하는 F_1 중간 잡종으로부터 많은 여교잡 후대들을 얻을 수 있었으며, 백합의 이입 육종을 위한 종간 교잡법 에 있어 $2n$ 화분을 이용한 여교잡법을 통하여 이입정도의 조절이 가능하게 되었다 (제 6장).

그 외에도 백합의 生殖細胞 染色體 倍加法의 실제적인 이용에 있어, 본 연구 결과는 몇 가지 매우 중요한 의미를 지닌다. 첫째, 體細胞 染色體 倍加法 과 生殖細胞 倍加法 을 비교함으로써 그 결과에 대한 원인 및 대책을 提示하였으며, 둘째, 종전의 세포 遺傳學的 研究에 의해 밝혀진 Nuclear restitution mechanism들은 오직 FDR과 SDR 만 알려져 왔으나 본 연구에 의해 추가로 밝혀진 바에 의하면 FDR 과 SDR이 동시에 단일 화분 모세포에서 일어나는 IMR 이 존재한다는 것이 證明되었다. 셋째, 分子細胞 遺傳學的 방법인 염색체 패인팅 기술이 유용 육종 재료의 염색체 구성을 확인하거나 원예작물에 있어 倍數體의 염색체 構成 및 起源에 대해서 가장 강력한 도구로 사용될 수 있다.

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Curriculum Vitae

Ki-Byung Lim was born in YeCheon, KyungBuk, Korea on 23 May 1962. He finished his elementary school and middle school in the small countryside village. In 1977, he studied at the department of Horticulture of Andong Agricultural High School, Andong City, KyungBuk, Korea. He studied BSc at the department of horticulture, Kyungpook National University (KNU) from 1980 to 1984. After graduation from the university, he worked at a seed company (Shin-Nong Co. Ltd, Korea) for two years starting from 1984 and one and half years at the Yeongnam Crop Experiment Station (Milyang city), RDA starting from 1988. He obtained his MSc degree at the KNU in 1988. He worked for 6 years at Hungnong Seed Co during 1990 - 1995. In the mean time at the Hungnong Seed Co., he studied for one year on the physiology of lily at the Florist and Nursery Crops Laboratory, USDA, Beltsville, MD, U.S.A. in 1990 and for three months on seedling production at the Ball Tagawa Greenhouses Inc., CA, U.S.A. He obtained his PhD degree at the KNU in 1996 and a post-doctorate scholarship in 1997 from the Korea Research Foundation (KRF) for two years. In September 1997, he started working at the Centre for Plant Breeding and Reproduction Research (CPRO-DLO; now, Plant Research International), Wageningen with Dr. Jaap Van Tuyl. One year later in 1998, he decided to study for a second PhD at Wageningen University. The results included in this thesis are based the work carried out from September 1997 up to July 2000. At present, he is employed at Plant Research International as a research scientist from September 2000.

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답 설 야 중 거 볼 수 호 난 행
눈덮힌 벌판을 걸어갈 때, 함부로 어지럽게 걷지 말라.

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