

# Use of $2n$ Gametes for Inducing Intergenomic Recombination in Lily Hybrids

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## Abstract

Genetic recombination is an important pre-requisite for transferring specific genetic traits across distantly related plant species. With a view to transfer some of the desirable characters like resistances against viruses, *Fusarium* and *Botrytis*, besides many horticultural traits, we have made interspecific hybrids between different species of lilies (*Lilium*,  $2n=2x=24$ ). The  $F_1$  hybrids in all these cases are totally sterile because of the lack of chromosome pairing. Traditional method of somatic chromosome doubling (mitotic polyploidization) can produce fertile allotetraploids. But, because of strict autosyndetic pairing in allotetraploids, no genetic recombination occurs in the progenies. In order to overcome this difficulty, we have selected  $2n$  gamete producing  $F_1$  hybrids of different *Lilium* species and used them successfully for sexual polyploidization (meiotic polyploidization). An important feature of meiosis in the  $F_1$  hybrids is that a certain amount of homoeologous chromosome pairing does occur in them. When  $2n$  gametes originate from such  $F_1$  hybrids through first division restitution (FDR) they are expected to possess recombinant chromosomes. Cytological analyses, using genomic in situ hybridization (GISH), of the sexual polyploid progenies have proved that considerable amount of intergenomic recombinant chromosomes can be recovered in the chromosome complements. One example of the sexual polyploid progenies from Oriental x Asiatic hybrid lilies possessing intergenomic recombinant chromosomes will be illustrated and discussed.

## INTRODUCTION

Lily (*Lilium* L.,  $2n=2x=24$ ) is one of the most important ornamental bulbs worldwide. The taxonomic classification divides the group into seven sections (Comber, 1949; De Jong, 1974) that comprises over 80 species. Lilies of ornamental importance are the result of crosses within three sections. The Asiatic hybrids (Sinomartagon section) are the most important group. They possess a wide variety of flower colours and some of them carry resistance to some viruses and *Fusarium*; characteristics which are not present in the Oriental hybrids (Archelirion section). Similarly, Oriental hybrids have white and pink flowers and in some cases resistance to *Botrytis* that are not present in the Asiatic hybrids. The third group is the Longiflorum hybrids (Leucolirium section) with white trumpet-shaped flowers.

The generation of new lily cultivars, with a combination of desirable characteristics, is only possible by intersectional species hybridisation, which is the most important feature in the actual *Lilium* breeding. In recent years, it has been possible to make such intersectional hybrids with the development of methods to overcome pre- and post-fertilisation barriers. Some of these methods to overcome pre-fertilisation barriers include intrastylar pollination in cut-style (Myodo et al., 1977), in vitro grafted style pollination and mentor pollen (Van Tuyl and De Jeu, 1997). To overcome post-fertilisation barriers ovule, ovary and embryo culture has been performed (Van Creij et al., 1993). Examples are *L. longiflorum* x Asiatic hybrids (LA-hybrids), *L. longiflorum* x *L. rubellum*,

*L. longiflorum* x Oriental hybrids (LO-hybrids) and Oriental x Asiatic hybrids (OA-hybrids) (Van Tuyl et al., 2002a) among others.

However, as in many other *taxa* the F<sub>1</sub> hybrids are sterile. To restore fertility, oryzalin has been applied as a spindle inhibitor to produce allopolyploid lilies (Van Tuyl et al., 1992). A major drawback of this technique is that due to strict autosyndetic pairing, intergenomic recombination is absent. The use of unreduced ( $2n$ ) gametes that occurs commonly angiosperms (Ramanna and Jacobsen, 2003) is an alternative to overcome the sterility of the F<sub>1</sub> hybrids. The mechanisms of  $2n$  gamete formation are generally classified into two groups because of their genetic consequences, viz., the first division restitution (FDR) gametes which comprise the non-sister chromatids of each homologous chromosome and the second division restitution (SDR) gametes which comprises the sister chromatids of each homologous chromosome. A third mechanism, indeterminate meiotic restitution (IMR) has also been recently described in lily, its genetics consequences does not fit to either of the above two mechanisms. In this case, each parent contributes with a disproportionate number of chromosomes to the daughter cell (Lim et al., 2001).

Genomic in situ hybridisation (GISH) techniques have been applied for the identification of parental chromosomes in interspecific hybrids of *Alstroemeria aurea* x *A. inodora* (Kamstra et al., 1999), *L. longiflorum* x Asiatic hybrids (Karlov et al., 1999), *L. longiflorum* x *L. rubellum* (Lim et al., 2000) among others. The aim of this work is to illustrate and describe one example of the sexual polyploid progenies from Oriental x Asiatic hybrid lilies possessing intergenomic recombinant chromosomes.

## **MATERIAL AND METHODS**

### **Plant Material**

The Asiatic cultivar 'Connecticut King' was used as the male parent in an intersectional cross with the Oriental cultivar 'Pesaro'. Pollination was performed by the cut-style method. Embryo rescue methods were needed to obtain some hybrids (Van Tuyl et al., 2000, 2002b). One of the resulting Oriental-Asiatic (OA) hybrids, which produced  $2n$  gametes, was subsequently used as the male parent in a cross with the Asiatic cultivar 'Gironde' to produce AOA offspring. In both cases, six to eight weeks after pollination, embryo, embryo sac and ovule culture methods were used in order to overcome post-fertilisation barriers.

### **Flow Cytometry and Chromosome Preparation**

Young leaves tips of the AOA offspring were collected and the ploidy level was determined according to Van Tuyl and Boon (1997). Complementary, root were collected early in the morning, pretreated in 0.7 mM cyclohexamide solution at room temperature for 4 h, fixated in Carnoy's solution (alcohol:acetic acid, 3:1) for at least 12 h and stored at -20°C until use. The root tips were incubated at 37°C for about 1-1.5 h in a pectolytic enzyme mixture containing 0.3% (w/v) of each: pectylase Y23, cellulase RS and cytohellicase in 10 mM citrate buffer (pH 4.5). Squash preparations were made in a drop of 45% acetic acid and frozen in liquid nitrogen; the coverslips were removed with a razor blade. Slides were dehydrated in absolute ethanol and air-dried.

### **Genomic In Situ Hybridization**

Sonicated genomic DNA from the Oriental cultivar 'Sorbonne' of a size ranging 1-10 kb was labelled with biotin using the Biotin-Nick translation Mix (Boehringer Mannheim, Germany) and used as probe. Autoclaved genomic DNA of the Asiatic cultivar 'Connecticut King' of a size of 100-500 bp was used as block. The GISH procedure was described previously (Lim et al., 2000). The hybridization mix consisted in 20x SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS, 25-50 ng/ml of probe DNA, 5-10 µg/ml of block DNA and water. It was denatured at 70°C for 10 min and directly placed on ice for at least 10 min. Hybridization of the chromosomal DNA was performed by adding

40  $\mu$ l of the hybridization mix at 80°C for 10 min followed by overnight hybridization at 37°C in a humid chamber. The hybridized probes were detected with Cy3 labelled streptavidin (Amersham Biosciences, UK) and amplified biotinylated goat-antistreptavidin (Vector laboratories, CA). Chromosomes were counterstained with DAPI (4,6-diamino-2-phenylindole). The hybridization signals were visualised and recorded in a chilled CCD camera (Sensys 1400, Photometrics) and pseudocolor images were made using the software Cytovision, Genus (Applied imaging).

## RESULTS AND DISCUSSION

Among a large number of OA hybrids that were screened for the production of  $2n$  pollen grains, one genotype: 951502-1 ('Pesaro' x 'Connecticut King') was found to produce a reasonably high frequency (0-100%). This was used as a male parent in cross with 'Gironde' as the female parent. Following this, the embryos were cultured in vitro. A large number of embryos germinated and the progenies were obtained (Table 1). The OA hybrid 951502-1 produced functional  $2n$  gametes and were expected to have originated through FDR because this was the most likely mechanism in a hybrid that had disturbed chromosome pairing. In order to establish whether the FDR gametes had indeed originated in the OA hybrid, the progenies of the above cross (AOA) were tested for ploidy level through flow cytometry. With the exception of two plants (Table 1), all were triploids ( $2n=3x=36$ ). Obviously, this confirmed that all these plants had originated through the functioning of euploid ( $2n$ ) gametes. The two exceptional plants were tetraploid ( $2n=4x=48$ ) (Fig. 1). The tetraploid plants probably had originated through the functioning of  $2n$  eggs from 'Gironde'. The chromosome composition of the progenies was determined through GISH confirmed that the OA hybrid had contributed 12 + 12 parental chromosomes from Oriental and Asiatic hybrid parents respectively through  $2n$  pollen to AOA as well as to AAOA progeny. A remarkable feature was that both  $3x$  and  $4x$  progenies possessed recombinant chromosomes varying from one to five in different plants. These recombinant events were obviously the result of homoeologous recombination that occurred during microsporogenesis in the OA hybrid (data not shown).

In the traditional method of breeding an allopolyploid crop such as the one described here, one doubles the chromosome number of the sterile  $F_1$  hybrid (e.g., OA) either through treatment with colchicine or oryzalin. This can only give rise to the so-called permanent hybrid that is not suitable for inducing intergenomic recombination. In contrast, however, as this study demonstrates, induction of sexual polyploids through the use of  $2n$  gametes can be undoubtedly successful in inducing intergenomic recombination. Recombination occurs in this case because the homoeologous chromosomes of the alien genomes, viz., O and A, are "forced" to pair in the diploid hybrid in the absence of a homologous partner. The only way of recovering such relatively rare events of homoeologous recombination events is through making use of  $2n$  gametes. In view of this, however rare and difficult it might be to select  $2n$  gamete-producing genotypes, the labour is worth the effort.

## CONCLUSIONS

There are two reasons why intergenomic recombination is important in breeding allopolyploids. A) When the breeding objective is to transfer only one or a few genes into a cultivar, but not the entire alien genome, obviously, transfer of only a recombinant segment is required. B) In a typical allopolyploid, genetic segregation for any of the parental characteristics is almost impossible. This is because of the autosyndetic pairing. However, the presence of recombinant chromosomes in an allopolyploid can lead to multivalent formation and chromosome segregation (Ramanna et al., 2003). This type of segregation can be most useful to create genetic variation that can facilitate selection.

The occurrence of  $2n$  gametes in plants has been recognised for a very long time (Harlan and De Wet, 1975) and it is wide spread. However, the use of  $2n$  gametes in crop breeding has been very limited and mostly confined to autopolyploids (Ramanna and Jacobsen, 2003). Some examples are: potato (Mendiburu et al., 1974), alfalfa (Bingham,

1980; Veronesi et al., 1986), among others. Only recently, the potential of using  $2n$  gametes in breeding allopolyploids has received attention in the case of *Alstroemeria* (Ramanna, 1992; Ramanna et al., 2003). As is evident in the case of lilies, there is a considerable potential for using  $2n$  gametes for breeding cultivars as has been demonstrated in the case of *L. longiflorum* x Asiatic hybrids (Karlov et al., 1999; Lim et al., 2003), *L. longiflorum* x *L. rubellum* (Lim et al., 2000) *L. auratum* x *L. henryi* (Van Tuyl et al., 2002). In all these cases, besides making use of  $2n$  gametes, the application of GISH for the determination of restitution mechanisms in the  $F_1$  hybrids, genomic composition of the progenies and the extent of intergenomic recombination have greatly increased the knowledge on sexual polyploidisation in lilies. This might lead to the development of more systematic approaches for breeding lilies.

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## Tables

Table 1. Progeny obtained and their ploidy level.

Parents		Embryos cultured	Successful germinations	Progeny plants analysed by flow cytometry	Ploidy level of progeny plants	
Female (A hybrid)	Male (OA hybrid)				3x	4x
'Gironde'	'Pesaro' x 'Connecticut King'	65	52	27	25	2

## Figures

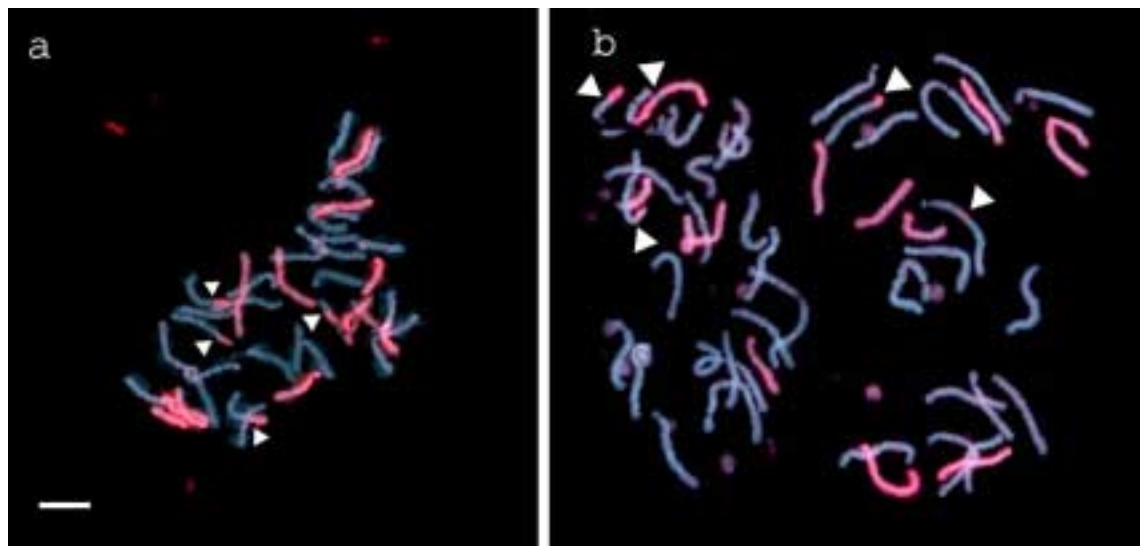


Fig. 1. Chromosome complements showing a triploid and a tetraploid chromosome number. **(a)** 36 chromosomes of 022604-7, plant with four recombinant chromosomes (arrows). The biotin-labeled Oriental DNA was detected with the Cy3-streptavidin system (pink fluorescence) and Asiatic chromosomes were counter stained with DAPI (blue fluorescence) **(b)** A tetraploid chromosome complement of 022604-4, showing five recombinant chromosomes. The probe DNA used and detection were the same as for **(a)**. Bar represents 10  $\mu$ m.