

## Introgression of *Lilium rubellum* Baker chromosomes into *L. longiflorum* Thunb.: a genome painting study of the F<sub>1</sub> hybrid, BC<sub>1</sub> and BC<sub>2</sub> progenies

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Received 27 September 1999; received in revised form and accepted for publication by Pat Heslop-Harrison 13 December 1999

**Key words:** genomic *in-situ* hybridization (GISH), interspecific hybrid, *Lilium longiflorum*, *Lilium rubellum*, preferential pairing

### 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<sub>1</sub> hybrids (**LR**, 2n = 2x = 24) and BC<sub>1</sub> individuals (**LLR**, 2n = 3x = 36) were phenotypically uniform for plant height, flowering time, leaf shape and flower colour. The BC<sub>1</sub> 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<sub>2</sub> 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* hybridization (GISH) was applied to establish the parentage of the chromosomes of the F<sub>1</sub> hybrids and the BC<sub>1</sub> and BC<sub>2</sub> progenies. GISH confirmed the **LLRR** constitution of the doubled amphimonoploid (allodiploid), and the **LLR** constitution of all BC<sub>1</sub> plants. The three selected BC<sub>2</sub> 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<sub>1</sub>, BC<sub>1</sub> and BC<sub>2</sub> plants. In spite of the high frequencies of homoeologous recombination in the F<sub>1</sub> hybrids (**LR**) pollen was found to be sterile in all cases. At metaphase I of the pollen mother cells of the BC<sub>1</sub> 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<sub>1</sub> (amphidiploid) and BC<sub>1</sub> plants. It seems that the preferential pairing in the F<sub>1</sub> and BC<sub>1</sub> hybrids hinder the introgression of the chromosome segments or species-specific genes into the recipient for breeding purposes.

### Introduction

The genus *Lilium* (Liliaceae) comprises over 80

species, which are classified into seven taxonomic sections (Comber 1947, De Jong 1974). All species are endemic to the mountainous areas 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; Martin 1966) and all of them are diploid ( $2n = 2x = 24$ ), except for *L. lancifolium* of which triploids occur as well (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 hybridization is difficult because of pre- and postfertilization 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 & Myodo 1977, Van Tuyl *et al.* 1991) and *in-vitro* culture techniques combined with ovary, ovule and embryo cultures are applied.

The production of interspecific hybrids between *L. longiflorum* and *L. rubellum* is interesting for introduction of the pink flower colour of the latter species. The  $F_1$  hybrids which 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* hybridization (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.* 1997) 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, 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 polyploidization was performed by *in-vitro* treatment of the bulb scales in 0.003% oryzalin (3,5-dinitro- $N^4, N^4$ -dipropylsulphanilamide) solution for 3 h. 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.

### Chromosome preparation

For the study of mitotic metaphase complements, the fast growing root tips were collected in the morning and pretreated in saturated  $\alpha$ -bromonaphthalene solution for 2 h 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 mmol/L citrate buffer (pH 4.5) for about 1–1.5 h 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.

Table 1. Genome composition and average value of several phenotypic characteristics of the parents (*L. longiflorum* and *L. rubellum*), amphimonoploid and amphidiploid hybrids, BC<sub>1</sub> and BC<sub>2</sub> progenies.

	Genome composition <sup>a</sup>	Genotype	Phenotypic characters			
			Flower colour	Leaf shape	Plant height (cm ± SD)	Forcing time (days ± SD)
Parent	<b>LL</b>	<i>L. longiflorum</i> 'Gelria'	White	Narrow	101 ± 3.7	95 ± 3.5
	<b>RR</b>	<i>L. rubellum</i>	Dark pink	Wide	ND	ND
F <sub>1</sub> (2x)	<b>LR</b>	<i>L. longiflorum</i> 'Gelria' × <i>L. rubellum</i>	Pink	Wide	40.6 ± 8.9	46.7 ± 1.2
F <sub>1</sub> (4x)	<b>LLRR</b>	Chromosome doubled <b>LR</b> hybrid	Pink	Wide	48.2 ± 4.5	52.9 ± 2.7
BC <sub>1</sub> (3x)	<b>LLR</b>	<i>L. longiflorum</i> 'Snow Queen' × <b>LLRR</b>	Light pink	Intermediate	82.4 ± 8.2	74.6 ± 2.7

<sup>a</sup>**L** and **R** represent *L. longiflorum* and *L. rubellum*, respectively. ND, not determined.

Table 2. Genome constitution and chromosome composition of the F<sub>1</sub> hybrids, BC<sub>1</sub> and BC<sub>2</sub> progenies as determined by GISH.

	Genome composition	CPRO-accession number	No. of individuals examined	Somatic chromosome number (2n)	Chromosome constitution	
					L	R
F <sub>1</sub> (2x)	<b>LR</b>	921250	2	24	12	12
F <sub>1</sub> (4x)	<b>LLRR</b> <sup>a</sup>	940303	1	48	24	24
BC <sub>1</sub>	<b>LLR</b>	961003	9	36	24	12
BC <sub>2</sub>	<b>LLL</b>	982275-1	1	43	36	7
	<b>LLL</b>	982211-20	1	42	36	6
	<b>LLL</b>	982211-27	1	44	36	8

<sup>a</sup>**LLRR** was derived from artificial chromosome doubling of the **LR** hybrid.

### DNA probes preparation

Total genomic DNA of *L. longiflorum* 'Snow Queen' was used as a probe. The probe DNA was labeled 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 hybridization

The *in-situ* hybridization protocol was carried out according to Kuipers *et al.* (1997) with minor modifications. Briefly, slides were pretreated with RNase A (100 µg/ml) for 1 h and pepsin (5 µg/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. Hybridization

followed using a mixture consisting of 2 × SSC, 50% formamide, 10% sodium dextran sulphate, 0.25% SDS, 2.0 ng/µl digoxigenin-11-dUTP-labelled total genomic DNA of *L. longiflorum* 'Snow Queen' and 30–40 ng/µl herring sperm DNA for blocking. The DNA was denatured by heating the hybridization mixture at 70°C for 10 min and then placed on ice for 10 min. For each slide, 40 µl hybridization mixture was used. The preparations were denatured at 80°C for 10 min. After overnight hybridization at 37°C in a humid chamber, slides were washed at room temperature in 2 × SSC for 15 min and 0.1 × SSC at 42°C for 30 min. The digoxigenin-labelled probe DNA was detected with 20 µg/ml anti-dig-FITC (fluorescein isothiocyanate; Boehringer Mannheim) and 20 µg/ml rabbit-anti-sheep-FITC (Vector Laboratory). Slides were counterstained with DAPI (4',6-diamidino-2-phenylindole) or PI (propidium iodide) and examined under a Zeiss Axiophot microscope equipped with epi-fluor-

escence illumination and single band filters for DAPI, FITC and PI. Images were photographed on 400 ISO colour negative film and scanned at 1012 dpi for digital processing in Photoshop (Adobe Inc.).

## Results

### *Phenotypic and cytogenetic observations of the diploid and amphidiploid F<sub>1</sub> hybrids*

Phenotypic characteristics of the F<sub>1</sub> hybrids and their backcross progenies were intermediate between the parents. All the F<sub>1</sub> hybrids showed a pink colour which is not present in *L. longiflorum*.

As a control experiment in the F<sub>1</sub> hybrids, GISH with *L. longiflorum* genomic DNA probed to the somatic metaphase chromosomes of the parental species gave a bright signal on all **L** chromosomes but no signal on the **R** chromosomes. This indicates that the chromosome sets of the two different species in the F<sub>1</sub> hybrids can be unequivocally identified.

In spite of the striking GISH differentiations between the parental genomes in the diploid (**LR**) F<sub>1</sub> hybrids, the occurrence of homoeologous pairing between the **L** and **R** chromosomes was often seen in metaphase I complements (Figure 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<sub>1</sub> hybrid (**LR**) was 3.2<sub>II</sub> + 17.6<sub>I</sub>.

Genome painting of both mitotic chromosomes (Figure 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 per cent while the **LLRR** hybrids ranged from 40 to 50%, indicating the range of pollen fertility. However, this pollen only showed about 20% germination.

### *GISH analysis of BC<sub>1</sub> progenies*

All BC<sub>1</sub> plants from the cross combination **LLRR** × **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 constitution was 2n = 3x = 36 (**LLR**) in all cases (Table 2). In the triploid (**LLR**) BC<sub>1</sub> plants there was no evidence for the presence of homoeologous chromosomes (Figure 1c,d).

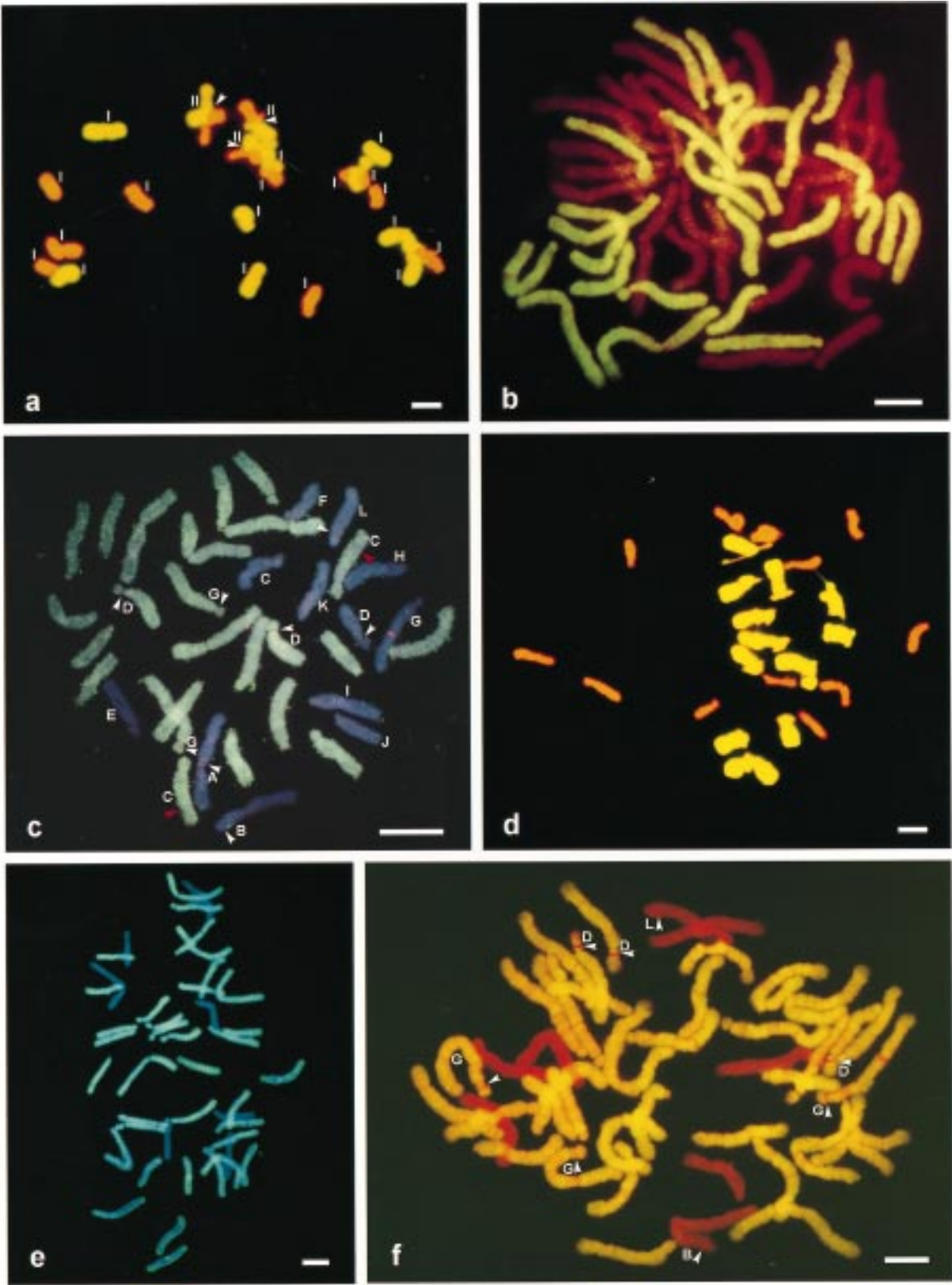
### *BC<sub>2</sub> progenies and their chromosome constitution*

Three best growing BC<sub>2</sub> 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 (Figure 1e,f). None of the three BC<sub>2</sub> plants displayed **L/R** recombinant chromosomes, indicating that, as expected, homoeologous recombination was absent in the BC<sub>1</sub> plants.

## Discussion

This investigation demonstrates that GISH enables unequivocal distinction of the *L. longiflorum* and *L. rubellum* chromosomes in F<sub>1</sub> hybrids, BC<sub>1</sub> and BC<sub>2</sub>

*Figure 1(a–f)* Genomic *in-situ* hybridization of mitotic and meiotic chromosomes of the F<sub>1</sub>, BC<sub>1</sub> and BC<sub>2</sub> plants. **(a)** Metaphase I of the PMC in F<sub>1</sub> 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 *L. rubellum* chromosomes. **(b)** Chromosomes of the F<sub>1</sub> hybrid 940303 (**LLRR**) after artificial chromosome doubling of the **LR** hybrid 921250-2. The probe DNA used and detection were the same as for **(a)**. **(c)** Thirty-six chromosomes of the BC<sub>1</sub> plant 961003-27 without any recombinations. *L. longiflorum* (green fluorescence) and *L. rubellum* (blue fluorescence). Letters indicate the chromosome number according to Stewart (1947). **(d)** The meiotic chromosomes of BC<sub>1</sub> 961003-27 with 12 bivalents (yellow fluorescence) indicating *L. longiflorum* and 12 univalents (red fluorescence) representing *L. rubellum*. **(e)** Aneuploid BC<sub>2</sub> plant 982275-1 from backcrossing of the BC<sub>1</sub> (**LLR**) to 4× (**LLLL**) *L. longiflorum*. Thirty-six chromosomes (three sets) of *L. longiflorum* (green fluorescence) with 7 *L. rubellum* chromosomes (blue fluorescence). **(f)** Aneuploid BC<sub>2</sub> plant 982211-27 from backcrossing of the BC<sub>1</sub> (**LLR**) to 4× *L. longiflorum*. Thirty-six chromosomes (three sets) of *L. longiflorum* (yellow fluorescence) with 8 *L. rubellum* chromosomes (red fluorescence).



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 (Richardson 1936; Asano 1984). As a rare case, the allotriploid  $L. \times$  'Fire King' revealed a maximum of 12<sub>III</sub> (Noda 1971). Because of a high degree of genomic differentiation, the LR hybrid formed 3.2<sub>II</sub> on average with a maximum of five bivalents. As expected, after somatic chromosome doubling, the amphidiploid (LLRR) F<sub>1</sub> 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<sub>1</sub> plants (LLR) showed perfect preferential pairing which resulted in 12<sub>II</sub> (L–L) + 12<sub>I</sub> (R). Similar results have been reported in interspecific triploids between *L. longiflorum* (4 $\times$ ) and *L. cernuum* (2 $\times$ ) (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<sub>1</sub> plants analysed. In other words, the 2 $\times$  gametes contributed by the amphidiploid possessed intact L and R genomes, i.e. without any homoeologous recombination.

If the final aim of the interspecific hybridization programme is to recombine specific horticultural traits, intrachromosomal recombination is essential but 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 addition 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 their alien homoeologue in the cultivar. This is highly laborious and dependent upon the genetic load of the chromosomes involved. Unlike using amphidiploids, the use of numerically unreduced (2n) gametes produced by the diploid F<sub>1</sub> hybrids can greatly enhance the possibility of obtaining homoeologous recombination as has been demonstrated recently in another interspeci-

fic lily hybrid (Karlov *et al.* 1999). Progeny with recombinant chromosomes often have considerable opportunity for substituting either parts of or entire alien chromosomes in a species or a cultivar (Kamstra *et al.* 1999).

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

### Acknowledgements

The authors thank Jannie Wienekers for technical help and Prof. Evert Jacobsen for his critical reading of the manuscript. Ki-Byung Lim is grateful to the Korea Research Foundation (KRF) for financial support.

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