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Progenies of allotriploids of Oriental × Asiatic lilies (*Lilium*) examined by GISH analysis

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Abstract With the aim of utilizing allotriploid ($2n = 3x = 36$) lily hybrids (*Lilium*) in introgression breeding, different types of crosses were made. First, using diploid Asiatic lilies ($2n = 2x = 24$), reciprocal crosses ($3x - 2x$ and $2x - 3x$) were made with allotriploid hybrids (AOA) obtained by backcrosses of F_1 Oriental × Asiatic hybrids (OA) to Asiatic cultivars (A). Secondly, the AOA allotriploids were crossed with allotetraploid (OAOA, $2n = 4x = 48$), in $3x - 4x$ combination. Finally, the AOA allotriploids where crossed to $2n$ gamete producer F_1 OA hybrids ($3x - 2x(2n)$). Two types of triploids were used as parents in the different types of crosses, derived from: (a) mitotic polyploidization and (b) sexual polyploidization. Ploidy level of the progeny was determined by estimating the DNA values through flowcytometry as well as chromosome counting. The aneuploid progeny plants from $3x - 2x$ and reciprocal crosses had approximate diploid levels and in $3x - 4x$ crosses and $3x - 2x\,(2n)$ the progeny had approximate tetraploid levels. Balanced euploid gametes (x, $2x$ and $3x$) were formed in the AOA genotypes. Recombinant chromosomes were found in the progenies of all crosses, except in the case of $2x - 3x$ crosses through genomic *in situ* hybridization (GISH) analyses. Recombinant chromosomes occurred in the F_1 OA hybrid when the triploid AOA hybrid was derived through sexual polyploidization, but not through mitotic polyploidization with two exceptions. Those recombinant chromosomes were transmitted to the progenies in variable frequencies.

Keywords $2n$ gametes · Allotriploids · Genomic *in situ* hybridization · *Lilium* · Poliploidization

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Introduction

Oriental and Asiatic lilies *(Lilium)* belong to two different taxonomic sections, viz., Archelirion and Sinomartagon, respectively. The cultivars of each of these two groups consist of diploid ($2n = 2x = 24$) hybrids of species within the taxonomic series. It is an important goal in breeding to produce new cultivars that combine desirable horticultural characters of the cultivars, or species, from the two groups of lilies. Because of

their taxonomic distance it is difficult to hybridize the cultivars of the two different sections. Hybridization can be accomplished through the use of special techniques (Asano 1978, 1980; Van Tuyl et al., 1988, 1991, 2000). The intersectional interspecific hybrids obtained such as Oriental × Asiatic lilies (OA) are totally sterile due to failure of chromosome pairing. The F_1 hybrids can be utilized in breeding only after chromosome doubling through colchicine or oryzalin treatment (Van Tuyl et al., 1992) to produce allotetraploids ($2n = 4x = 48$) or through the use of $2n$ gametes (Van Tuyl & De Jeu, 1997; Karlov et al., 1999; Lim et al., 2001; Barba-Gonzalez et al., 2004). Both of these approaches have been used to produce a number of backcross (BC_1) progenies by crossing with Asiatic lilies (AA). One consequence of this approach is that in both cases allotriploid (AOA) BC_1 progenies are produced. These are generally sterile. In order to utilize these triploids in further introgression breeding, a critical assessment of their crossability and intergenomic recombination was necessary in order to use them in breeding.

In the past, both auto- and allotriploids have been successfully used in crossing (reviews, Kuspira et al., 1986; Brandham, 1982; Ramsey & Schemske, 2002; Ramanna & Jacobsen, 2003) in various plant species. In lilies, allotriploids of Longiflorum × Asiatic hybrids (ALA) have been successfully used for producing BC_2 progenies (Lim et al., 2003). A notable feature is that these triploids can be crossed with both diploid and tetraploid parents so that the aneuploid progenies consisting of near – diploids or near – tetraploids to pentaploid can be obtained. In the present investigation we used allotriploid BC_1 plants derived from two approaches: (1) by crossing somatically doubled allotetraploids (OAOA $= 4x -$ OA) to AA parents; (2) by using $2n$ gametes from OA hybrids for crossing with AA parents. The allotriploids were crossed with diploids, i.e. $3x - 2x$ (or reciprocal) and $3x - 4x$ combinations and with $2n$ gamete producer F_1 OA hybrids in the case of those derived from $2n$ gametes. The progenies from these crosses were analyzed through fluorescent and genomic *in situ* hybridization (FISH and GISH) in order to determine the genome composition of the progenies as well as the extent and transmission of chromosomes with intergenomic recombination. The implications of using allotetraploids for introgression are discussed.

Material and methods

Plant material used for making F_1 hybrids and production of BC_1 progenies has been described earlier by Barba-Gonzalez et al. (2004). The origins of allotriploids have been described by Barba-Gonzalez et al. (2005). The genotypes mentioned in Tables 1 and 2 had originated from allotriploids derived from the use of $2n$ gametes ($2n$) as well as mitotic polyploidization (MP). Because the parents involved in making the initial and subsequent crosses were cultivars, which are interspecific hybrids, the species names are avoided. The plant material utilized for crossing work was grown in greenhouses with standard procedures.

Ovule and embryo rescue

Swollen fruits were collected 40 to 60 days after pollination, surface-sterilized by submerging in 80% ethanol and flamed. The successfully fertilized ovules were recognizable by their increased size. The embryosacs containing embryos or the embryo only were excised from the ovules and placed in enriched media according to Van Tuyl et al. (1991).

Chromosome preparation

Root tips were collected early in the morning, pretreated in saturated α-bromonaphtalene solution in ice-water overnight, fixed in the ethanol – acetic acid solution (3:1) for 12 h and stored at $-20\,^{\circ}$C until use. Before squashing, the root tips were incubated in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase Y23 (Sigma-Aldrich, Germany) 0.2% (w/v) cellulase RS (Yakult pharmaceutical, Japan) and 0.2% (w/v) cytohelicase (Sigma-Aldrich, Germany) in 10 mM citrate buffer (pH 4.5) at 37 °C for about 2 h. Squash preparations were made in a drop of 50% acetic acid and frozen in liquid nitrogen; the cover slips were removed by using a razor blade. Slides were dehydrated in absolute ethanol and air-dried.

DNA probe preparation

For GISH analyses, sonicated genomic DNA (1–10 kb) from Oriental cultivar 'Sorbonne' was used as a probe. Probe was labelled by nick-translation

Table 1 DNA content, ploidy level and chromosome number of progeny plants derived from $3x - 2x$, $2x - 3x$ and $3x - 4x$ crosses

Cross	Genotype	Parents		DNA content	Ploidy C-level	Chromosome number*
		Female	Male			
			$3x(2n) - 2x$			
AOA × AA	042627-1	022605-7	'Pollyana'	103.78	2.1	26[e]/25°
AOA × AA	042627-2	022605-7	'Pollyana'	138.10	2.8	34[e]/32°
AOA × AA	042627-3	022605-7	'Pollyana'	120.35	2.4	30[e]/31°
AOA × AA	042627-4	022605-7	'Pollyana'	129.98	2.6	32[e]/30°
AOA × AA	042627-5	022605-7	'Pollyana'	114.67	2.3	29[e]/29°
AOA × AA	042627-6	022605-7	'Pollyana'	120.56	2.4	30[e]/29°
AOA × AA	042627-7	022605-7	'Pollyana'	127.08	2.5	32[e]/32°
			$2x \times 3x$ (MP)			
AA × AOA	022118-2	'Amarone'	022687-8	99.21	2.1	25[e]/27°
AA × AOA	022118-4	'Amarone'	022687-8	103.68	2.2	26[e]/27°
AA × AOA	022118-7	'Amarone'	022687-8	99.77	2.1	25[e]/24°
AA × AOA	022118-8	'Amarone'	022687-8	102.63	2.2	26/[e] 27°
			$3x$ (MP)× $4x$			
AOA × 4x-OA	022308-2	022188-25	981022	228.01	4.8	57[e]/49°
AOA × 4x-OA	022308-4	022188-25	981022	233.10	4.9	58[e]/62°
AOA × 4x-OA	022308-8	022188-25	981022	167.73	3.4	42[e]/44°
AOA × 4x-OA	022308-9	022188-25	981022	179.24	3.6	45[e]/44°
			$3x(2n) \times 4x$			
AOA × 4x-OA	032971-1	002531-12	014076	178.02	3.6	44[e]/38°
AOA × 4x-OA	032971-2	002531-12	014076	186.29	3.8	46[e]/46°
AOA × 4x-OA	032971-3	002531-12	014076	149.59	3.0	37[e]/44°
AOA × 4x-OA	042119-4	002531-12	981013	168.83	3.4	42[e]/42°
AOA × 4x-OA	042119-5	002531-12	981013	175.79	3.5	44[e]/43°
AOA × 4x-OA	042119-7	002531-12	981013	191.80	3.8	48[e]/48°
AOA × 4x-OA	042119-8	002531-12	981013	187.47	3.7	47
			$3x(2n) \times 2x^{**}$			
AOA × OA	042734-2	002531-12	951502-1	145.89	2.9	36[e]/38°

*[e] = Expected chromosome number, ° = Observed chromosome number
** = $2n$ gamete producer
(MP) = Obtained through mitotic polyploidization
($2n$) = Obtained through $2n$ gametes

with biotin-16-dUTP according to manufacturer instructions (Roche, Germany).

In situ hybridization

Slides were incubated at 37 °C in RNase A (100 μg/ml) for 1 h and in pepsin (5 μg/ml) for 10 min, followed by paraformaldehyde (4%) for 10 min at room temperature. Between each step the slides were briefly rinsed in 2 x SSC and finally dehydrated with 70%, 90% and absolute ethanol for 3 min in each and air dried. Hybridization followed using a mixture consisting of 20x SSC, 50% formamide, 10% (*w/v*) sodium dextran sulphate, 0.25% (*w/v*) SDS, 25–50 ng of the probe and 3 μg per slide of autoclaved DNA (100–500 bp) from the Asiatic cultivar 'Connecticut King'. The hybridization mixture was heated at 70 °C for 10 min and placed on ice for at least 10 min. For each slide, 40 μl hybridization mixture was used. The preparations were denatured at 80 °C for 10 min and incubated overnight at 37 °C in a humid chamber. Slides were washed at room temperature in 2x SSC for 15 min and in 0.1x SSC at 42 °C for 30 min. The probe was detected with Cy3 labelled streptavidin (Amersham Biosciences, UK), and amplified with biotinylated goat-antistreptavidin (Vector Laboratories, Burlingame, CA). Chromosomes were counterstained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield

anti-fade (Vector Laboratories. Burlingame, CA) was added for its examination under a Zeiss Axioplan 2 Photomicroscope equipped with epi-fluorescent illumination, filter sets of DAPI and Cy3. Images were captured by a Photometrics Sensys 1,305 × 1,024 pixel CCD camera and processed with Genus Image Analysis Workstation software (Applied Imaging Corporation). The DAPI images were sharpened with a 7 × 7 High Gauss spatial filter and pseudo-coloured in blue. The probe fluorescence was red pseudo-coloured. Optimal brightness and contrast were achieved with Adobe Photoshop image processing.

Results

As a result of extensive crossing of allotriploids with diploid Asiatic cultivars, or diploid or tetraploid genotypes, followed by *in vitro* ovule and embryo culture, a number of progenies were obtained in $3x - 2x$ (and reciprocal to recurrent Asiatic parents), $3x - 4x$ crosses and $3x - 2x$ ($2n$). Whereas the reciprocal crosses were successful in the case of $3x - 2x$ combinations, this was not the case for $3x - 4x$ crosses where reciprocal combinations failed. Because the allotriploid AOA plants were expected to produce a range of aneuploid gametes ($x + 1$ to $3x - 1$) as well as euploid gametes (x, $2x$ and $3x$), the ploidy levels were monitored by measuring DNA values through flowcytometry in different types of progenies (not all the data is included) as well as chromosome counting (Table 1).

Ploidy levels of the progenies

A total of 31 progeny plants were examined for their ploidy by chromosome counting. These included 13 plants derived from $3x - 2x$ crosses or $2x - 3x$ crosses and 18 from $3x - 4x$ and $3x - 2x$ ($2n$) crosses (Table 2). From these data, together with flowcytometric measurement of DNA values (not all the data is included) the following conclusions were made: 1. the progenies of $3x - 2x$, and reciprocal crosses, consisted predominantly of plants with circa diploid chromosome numbers (range 24–32); 2. Progenies of $3x - 4x$ and $3x - 2x$ ($2n$) crosses contained largely of circa tetraploid to circa pentaploid chromosome numbers (range, 38–62). Although almost all plants were aneuploids, there were a few cases of euploids ($2n = 2x =$ 24 in 022118-7; 022180-2, $2n = 4x = 48$ in 022302-3; 042119-7 and $2n = 5x = 60$ in 042734-1). Thus, there was an indication that euploid gametes (x, $2x$ and $3x$) are produced by the allotriploid AOA in few cases.

Chromosome constitution and intergenomic recombination

There were two types of AOA plants that were used as parents: (1) those derived from mitotic polyploidization, and (2) those which had originated through sexual polyploidization. In the case of $2x - 3x$ crosses (Table 2), all the triploid genotypes used as male parents had originated by mitotic polyploidization. Out of six plants, none possessed recombinant chromosomes (Table 2). On the contrary, all plants that were analyzed from $3x - 2x$ crosses, in which the female parent was of sexual polyploid origin, possessed at least one recombinant chromosome (Table 2). Although certain amount of intergenomic recombination could occur in the allotriploid, our previous studies had indicated the use of $2n$ gametes give a higher rate of intergenomic recombination (Barba-Gonzalez et al., 2005) in contrast to the use of somatically doubled allotetraploids, where there is few or no recombination. Another difference between the progenies of $2x - 3x$ and $3x - 2x$ crosses was the transmission of extra chromosomes. Whereas only one out of six progeny plants of $2x - 3x$ crosses possessed an extra chromosome of the O genome (Fig. 1a, Table 2), all the progenies of $3x - 2x$ crosses had extra chromosomes originated from this genome.

In the case of the progenies of $3x - 4x$ and $3x - 2x$ ($2n$) crosses, the chromosome numbers varied from $3x + 2$ to $5x + 2$ among which near tetraploids were predominant. Because the male parent in most cases was a typical allotetraploid, mostly a balanced chromosome number of $2x = 24$ (12O + 12A) was contributed and near diploid gametes were functional from the female parent (Table 2). As regards the number of recombinant chromosomes, there were only two progenies with a single recombinant chromosome when allotriploids had originated through mitotic polyploidization (022302-4, and 022304-2) (Fig. 1c). On the other hand, when the AOA was of sexual polyploid origin (032971-1, -2; 042119-5, -7), four out of six plants possessed recombinant chromosomes (Table 2) (Fig. 1b). In two cases, when AOA was crossed with $2n$ gamete producing OA (042734-1, -2) (Figures 1d,e),

Table 2 Genomic composition of progeny plants derived from $3x - 2x$, $2x - 3x$ and $3x - 4x$ crosses

Cross (Triploid origin)	Genotype	Ploidy level	Genome composition		Total number of recombinant chromosomes	Chromosome contribution of the gametes ♀		♂	
			O (O/A)	A (A/O)		O	A	O	A
			$2x - 3x$ (MP)						
AA × AOA	022118-2	$2x + 3$	0	27	0		12	0	15
AA × AOA	022118-4	$2x + 3$	0	27	0		12	0	15
AA × AOA	022118-7	$2x$	0	24	0		12	0	12
AA × AOA	022118-8	$2x + 3$	0	27	0		12	0	15
AA × AOA	022171-1	$2x + 1$	1	24	0		12	1	12
AA × AOA	022180-2	$2x$	0	24	0		12	0	12
			$3x(2n) - 2x$						
AOA × AA	042627-1	$2x + 1$	1	24(1)	1	1	12		12
AOA × AA	042627-2	$2x + 8$	8(1)	24(1)	2	8	12		12
AOA × AA	042627-3	$2x + 7$	7(1)	24(1)	2	7	12		12
AOA × AA	042627-4	$2x + 6$	6(1)	24(1)	2	6	12		12
AOA × AA	042627-5	$2x + 5$	5(1)	24(2)	3	5	12		12
AOA × AA	042627-6	$2x + 5$	5(1)	24(2)	3	5	12		12
AOA × AA	042627-7	$2x + 8$	8(2)	24(2)	4	8	12		12
			$3x$ (MP) − 4x-OA						
AOA × 4x-OA	022302-3	$4x$	22	26	0	10	14	12	12
AOA × 4x-OA	022302-4	$3x + 7$	18	25(1)	1	6	13	12	12
AOA × 4x-OA	022302-5	$3x + 4$	16	24	0	4	12	12	12
AOA × 4x-OA	022304-1	$3x + 3$	14	25	0	2	13	12	12
AOA × 4x-OA	022304-2	$3x + 7$	19	24(1)	1	7	12	12	12
AOA × 4x-OA	022304-3	$3x + 7$	18	25	0	6	13	12	12
AOA × 4x-OA	022304-4	$3x + 3$	15	24	0	3	12	12	12
AOA × 4x-OA	022308-2	$4x + 1$	20	29	0	8	17	12	12
AOA × 4x-OA	022308-4	$5x + 2$	20	42	0	8	30	12	12
AOA × 4x-OA	022308-9	$3x + 8$	19	25	0	7	13	12	12
			3x (2n) − 4x-OA						
AOA × 4x-OA	032971-1	$3x + 2$	14(1)	24	1	2	12	12	12
AOA × 4x-OA	032971-2	$3x + 10$	22(1)	24	1	10	12	12	12
AOA × 4x-OA	032971-3	$3x + 8$	20	24	0	8	12	12	12
AOA × 4x-OA	042119-4	$3x + 6$	18	24	0	6	12	12	12
AOA × 4x-OA	042119-5	$3x + 7$	19(1)	24(1)	2	7	12	12	12
AOA × 4x-OA	042119-7	$4x$	23(1)	25	1	11	13	12	12
			3x (2n) − 2x*						
AOA × OA	042734-1	$5x$	12(1)	48(1)	2	0	36	12	12
AOA × OA	042734-2	$3x + 2$	14(4)	24(3)	7	2	12	12	12

The number of recombinant chromosomes and the possible chromosome contribution of the parental gametes analyzed through GISH.

*$2n$ gamete producer

(MP) = Obtained through mitotic polyploidization

($2n$) = Obtained through $2n$ gametes

both progenies possessed recombinant chromosomes. The highest number of recombinant chromosomes (7) was found in the genotype 042734-2 (Fig. 1d), which was the product of sexual polyploidization and, probably, both parents had contributed recombinant chromosomes.

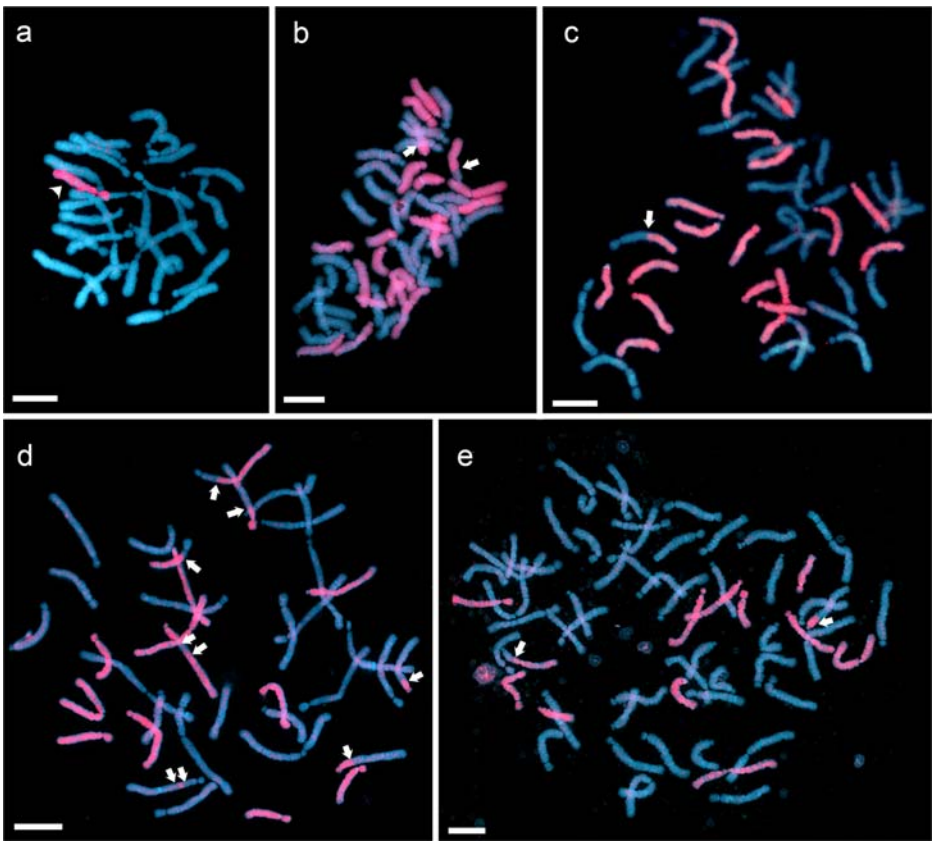

Fig. 1 Detection of intergenomic recombination and chromosome constitution in five BC_2 progenies. In all cases, the biotin-labeled Oriental (O) DNA was detected with the Cy3-streptavidin system (pink fluorescence) and the Asiatic (A) DNA was counterstained with DAPI (blue fluorescence). (**a**) The near-diploid ($2x + 1$) complement of 022171-1, showing 24 A + 1 O (arrowhead) chromosomes. (**b**) The near-tetraploid ($3x + 7$) complement of 042119-5, showing 24 A + 19 O and two recombinant chromosomes (arrows). (**c**) The near-tetraploid ($3x + 9$) complement of 022302-4, showing 27 A + 18 O and a recombinant chromosome (arrow). (**d**) The near-triploid ($3x + 2$) complement of 042734-2, showing 24 A + 14 O with seven recombinant chromosomes (arrows). (**e**) The pentaploid ($5x$) complement of 042734-1, showing 48 A + 12 O with two recombinant chromosomes (arrows). Bar represents 10 μm.

Transmission of recombinant chromosomes

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

Discussion

Generally, allotriploids cannot be used easily as parents in lilies. This investigation shows that allotriploid AOA genotypes can be used as parents to produce a

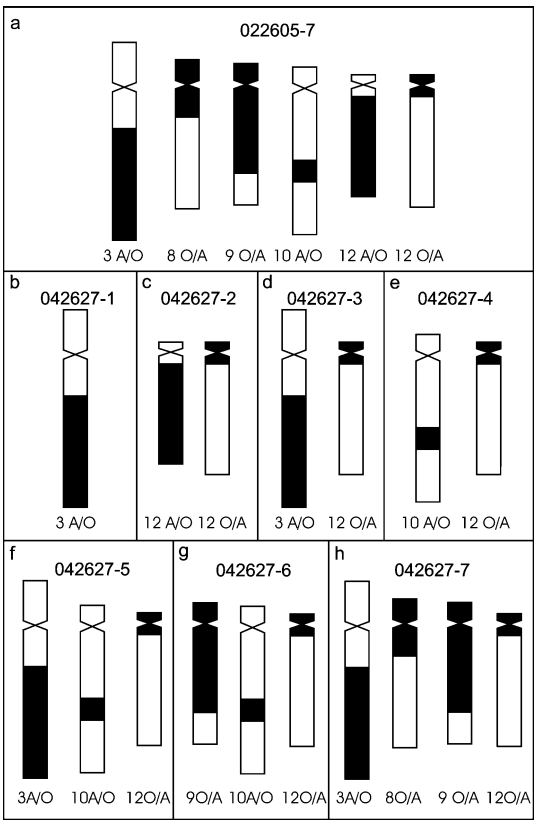

Fig. 2 Diagrammatic representation of the six recombinant chromosomes in the BC_1 allotriploid AOA (a) and the same recombinant chromosomes segregated to seven progeny plants (b–h). The chromosomes are represented as O/A or A/O, where O represents the centromere of the O genome and A the recombinant segment of Asiatic chromosome, and vice versa; the solid (black) parts of recombinant chromosomes represent the O genome chromatin, while the empty (white) ones – the A genome chromatin.

considerable number of progenies. This confirms the results of an earlier investigation, using allotriploids of Longiflorum × Asiatic lilies (ALA), in which a number of BC_2 progenies were produced (Lim et al. 2003). Furthermore, it also confirms the earlier observation that there is a difference in the ploidy levels of the progenies of $3x - 2x$, and reciprocals, as compared with $3x - 4x$ crosses (see above). Such differences in ploidy levels in the progenies of $2x - 3x$ and $3x - 4x$ crosses have been reported in certain autopolyploid crops (Brandham, 1982). Different types of progenies obtained from AOA hybrids might be useful in lily breeding for the following reasons. Although diploid cultivars of lilies have been cultivated for more than 50 years, the polyploid cultivars have appeared recently. There appears to be no assessment of the optimum threshold for growth and vigour and selection has been made without considering the optimum ploidy levels for cultivars. For example, in *Narcissus* the tetraploid level has proven to be the ideal one. This has been achieved during the last century through unconscious selection (Brandham, 1986; Brandham & West, 1993; Brandham et al., 1995). By comparing the DNA values of some of the horticultural plants such as *Narcissus*, *Tulipa*, *Hyacinthus*, among others, these authors have argued that DNA values from 100 to 120 pg are the most ideal values in these cases. If this was to be true, for *Lilium* species in which 1C have 35–36 pg of DNA (Bennett & Smith, 1976, 1991), triploid levels appear to be the optimal because the approximate threshold of 120 pg is attained in triploids. Some of the cultivars involving Longiflorum – Asiatic lilies that we have investigated have shown the triploid form in most of the cases and in a few cases the tetraploid form (unpublished results). It remains to be tested if triploid cultivars can be better than the tetraploid forms.

In most of the aneuploids (Table 2) there were variable numbers of O genome chromosomes. This was expected in view of univalent formation in AOA parents where A genomes paired regularly and univalents from the O genome were distributed irregularly and segregated randomly into the gametes. There was one case in the $3x - 2x$ $(2n)$ cross 042734-1, where the female parent contributed an unreduced gamete and probably the diploid Asiatic cultivar contributed with an unreduced gamete as well (Table 2, Fig. 2e).

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

Identification of different recombinant chromosomes transmitted to the progenies (Fig. 2) demonstrates the usefulness of *in situ* hybridization technique in introgression breeding. In the absence of these techniques, accurate detection of introgressed

chromosomes or alien segments would be impossible. It should be noted, however, that the introgression has been detected cytologically in lilies (Lim et al., 2000; Van Tuyl et al., 2002; Karlov et al., 1999) but the phenotypic expression has not been studied critically. However, with the use of appropriate analyses, and paying attention to well defined characters it might be possible to detect introgression phenotypically as has been done in the case of *Lycompersicon esculentum* × *Solanum lycopersicoides* and *L. esculentum* × *S. sitiens* (Pertuzé et al., 2003).

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