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

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Summary

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

Introduction

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

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

Apart from LA hybrids, we have made a series of hybrids between Oriental × Asiatic groups of lilies (OA hybrids). These are potentially "useful" for combining desirable horticultural traits from the two parents through sexual polyploidisation (Barba-Gonzalez et al., 2004). For this purpose, we have selected a few genotypes of OA hybrids that produce considerable frequencies of *2n* pollen. In order to determine the modes of origin of 2*n* pollen grains in OA hybrids, microsporogenesis was analyzed using genomic *in situ* hybridization (GISH) as well as traditional staining methods. The main objectives were to assess the extent of intergenomic recombination and to test the viability of *2n* pollen. The results are discussed in relation to the progenies that might be obtained in the BC_1 generation.

Material and methods

Plant material

The two groups of cultivars, viz., Oriental and Asiatic hybrids, were all diploids ($2n = 2x = 24$) and were hybridized through cut-style pollination and cultured by either: embryo, embryo-sac or ovule culture methods (Van Tuyl et al., 1991; Van Creij et al., 2000). In all, 10 Oriental and six Asiatic hybrids were used for the production of F_1 OA hybrids, among which the *2n* gamete producing genotypes were selected (Table 1) (Barba-Gonzalez et al., 2004). Because all the cultivars of lilies are intra-sectional hybrids between different taxonomic species, it is not appropriate to mention the botanical names of the species and therefore avoided.

Pollen germination

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

Table 1. Selection of OA hybrids

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

Cytological methods

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

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

In situ hybridization

Sonicated genomic DNA (1–10 kb) from the Oriental cultivar 'Sorbonne' was used as a probe after labeling with Biotin-16-dUTP (Biotin-16–2′-deoxyuridine-5′-triphosphate) by nick translation according to the manufacturer's instructions (Roche). Autoclaved DNA (100–500 bp) from the Asiatic cultivar 'Connecticut King' was utilised for blocking the non-hybridized sequences. The *in situ* hybridization protocol was carried out according to Barba-Gonzalez et al. (2004).

Results

Chromosome pairing

Meiosis was investigated in three F_1 hybrid genotypes that produced 5% or more pollen that would

Table 2. Chromosome pairing in three genotypes of OA hybrids

F_1	No. of cells analysed	12_{II}, 0_I	11_{II}, 2_I	10_{II}, 4_I	9_{II}, 6_I	8_{II}, 8_I	7_{II}, 10_I	6_{II}, 12_I	5_{II}, 14_I	4_{II}, 16_I	3_{II}, 18_I	2_{II}, 20_I	1_{II}, 22_I	0_{II}, 24_I	Mean frequency
951502-1	296	0	0	0	0	0	0	1	3	13	26	63	99	91	$1.2_{II} + 21.4_I$
952400-1	231	0	0	0	0	0	0	0	0	2	11	45	67	106	$0.8_{II} + 22.8_I$
962120-1	292	0	0	0	0	0	0	0	0	0	3	18	55	216	$0.3_{II} + 23.3_I$

II and I represent bivalent and univalent respectively.

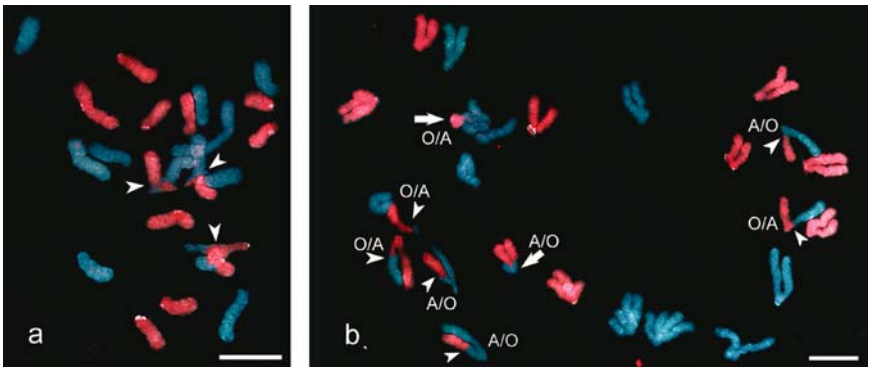

Figure 1. (a–b) Chromosome pairing at Metaphase I and Post-Metaphase I stage in OA hybrid, 951502-1. The Oriental chromosomes were biotin-labeled and detected with Cy3-streptavidin system (pink fluorescence) and the Asiatic chromosomes were counterstained with DAPI (blue fluorescence). (1a) Metaphase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

germinate. The main objectives were the assessment of chromosome pairing and the cytological mechanism(s) of nuclear restitution. Without exception there was reduced chromosome pairing at Metaphase I in all cases (Figure 1a). Very low frequencies of bivalents, from 0.3 to 1.3 bivalents per cell, were observed between genotypes (Table 2). Within a plant, bivalent averaged from 0 to 6 per cell. As a result of high frequencies of univalent formation, meiotic division was chaotic as expected in a majority of pmcs. But in 5–45% of the cases, the stages following Metaphase I the chromosomes did not separate into two groups (as in Anaphase I). A notable feature was that the sister chromatids of each chromosome became clearly visible and it was the most ideal stage for detection of intergenomic recombination. For the sake of convenience, this stage will be mentioned hereafter as Post-Metaphase I or PMI. In one of the PMCs four different recombination events were clearly visible (Figure 1b), they are single recombination events. However, there was one case where it could be explained as a four strand double crossover event (Figure 1b arrows).

Nuclear restitution

We analyzed six F_1 hybrid genotypes in which meiosis was fairly asynchronous in each anther and as a result of that, it was possible to find meiotic stages ranging from Metaphase I to Anaphase II and from Telophase I to sporad stages in one and the same preparation (Table 3). In some of the PMCs the first division (Anaphase I)

Table 3. Frequencies of deviating meiotic stages (PMI and PMII) in six different genotypes of OA hybrids and the frequencies (%) of sporads

Cross code	No. of cells analysed	Anaphase separation		Sporads	
		PMI	PMII	Dyads	Others
951502-1	293	37 (12.63)	8 (2.73)	117 (39.93)	131 (44.71)
952400-1	372	1 (0.27)	10 (2.69)	26 (6.99)	335 (90.05)
951462-1	317	6 (1.89)	21 (6.62)	26 (8.20)	264 (83.28)
951584-1	382	5 (1.31)	1 (0.26)	24 (6.28)	352 (92.15)
969023-2	553	10 (1.81)	20 (3.62)	88 (15.91)	435 (78.66)
952059-9	425	2 (0.47)	24 (5.65)	141 (33.18)	258 (60.71)

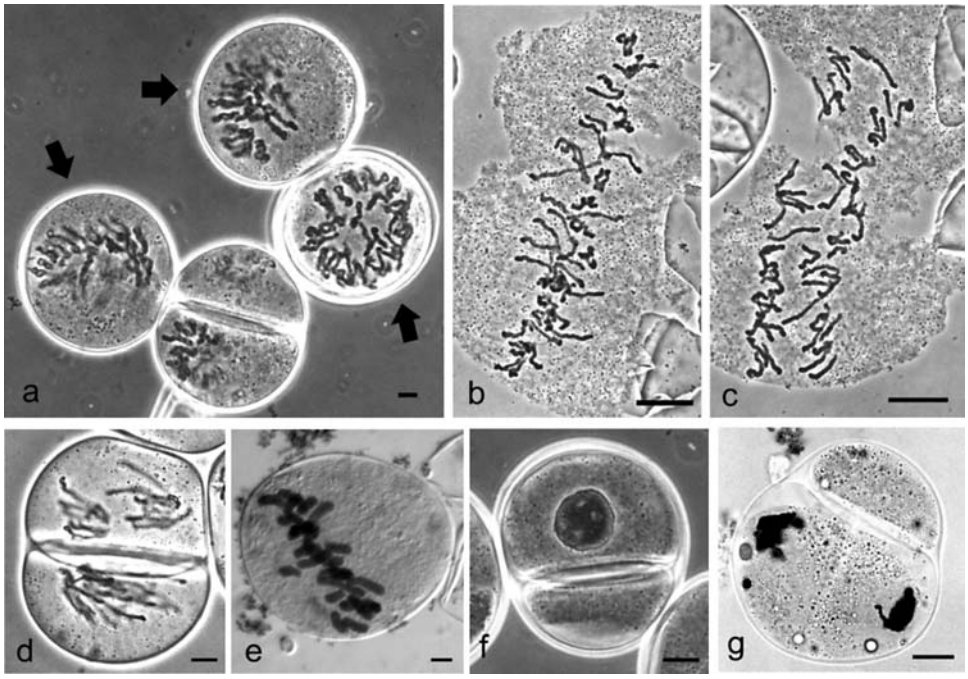

Figure 2. (a–f) Stages of microsporogenesis in OA hybrid 951502-1, showing meiotic nuclear restitution. (2a) Restitution nucleus formation in pmcs without cytokinesis (arrows). (2b) Equivalent of Metaphase II stage showing the orientation of all chromosomes in a single group. (2c) Anaphase separation of chromatids in a stage subsequent to that shown in (2b). (2d) Cytokinesis and cell wall formation at the end of first meiotic division, so-called "successive type" of meiosis in lilies. (2e) Post-Metaphase I orientation that can lead to the equational division of chromosomes (FDR). (2f) Asymmetric cytokinesis in pmc in which the entire nucleus is included in one of the cells. (2g) Nuclear division following asymmetric cytokinesis. *Bar*: 10 μm.

was generally followed by cytokinesis and a cell wall was formed at Telophase I (Figure 2d). This was according to expectation in *Lilium*, which has the so-called successive type of cytokinesis. However, in a considerable number of PMCs, the expected cytokinesis and cell wall formation were absent. In such PMCs, either the entire chromosome complement was aligned at the equatorial position (Figure 2e) and divided equationally or formed restitution nucleus (Figure 2a). The restitution nuclei gave rise to metaphase stage, (equivalent to Metaphase II) and divided equationally (Figures 2b and 2c). This type of division will be indicated as Post-Metaphase II, or PMII division. These two different restitution mechanisms (PMI and PMII) occurred in different frequencies among the F_1 hybrids (Table 3) and obviously led to the formation of a dyad. In addition to PMI and PMII, there were also pmcs in which asymmetrical cytokinesis occurred so that the entire nucleus was included in a single cell (Figures 2f and 2g). Regardless of the occurrence of PMI, PMII or the division of a nucleus in an asymmetrically divided PMC, they all led to an equational division of the whole complements. They obviously conformed to first division restitution (FDR) mechanism.

Viability of 2n pollen

Two criteria were used for the assessment of the viability of 2*n* pollen. (a) *In vitro* germination of pollen and (b) fruit set and embryo or embryo sac germination after using 2*n* pollen in crossing. For *in vitro* germination, two diploid cultivars, one Oriental and one Asiatic, as well as two genotypes of OA hybrids were used (Table 4). Pollen from the two diploid cultivars (controls) showed 95–100% germination when fresh pollen from just opened flowers or flowers one day after opening were used. Also 2*n* pollen from both genotypes of OA showed fairly good germination when fresh pollen was used (0–40% in 952400-1). But 2*n* pollen almost completely failed to germinate from one day old flowers (with the exception of a few flowers of 951502-1 which presented less than 5% pollen germination). This loss of viability of 2*n* pollen in an OA hybrid was strikingly constant. Besides germination *in vitro*, fresh pollen

Table 4. Time effect on the pollen germination of diploid Asiatic (AA) and Oriental (OO) cultivars and diploid OA hybrids 2*n* gametes producers

		Pollen germination (Range %)	
Code (Genome)	Flower no.	Fresh	1-day old
Pollyanna (AA)	12	95–100	95–100
Sorbonne (OO)	15	95–100	95–100
951502-1 (OA)	17	85–100	0–<5
952400-1 (OA)	20	0–40	0

grains could be successfully used in order to produce backcross progenies (results not included). A fairly good number of BC_1 plants were obtained when diploid Oriental and Asiatic hybrids were used as female parents in crosses with OA males. In two combinations, by using OA as female parent and a diploid Asiatic hybrid as male, BC_1 progeny was obtained. This was an indication for the occurrence of 2*n* eggs in OA hybrids.

Consequences of nuclear restitution

An important feature of meiotic nuclear restitution in OA hybrids with a high frequency of univalents (Table 2) is that it leads almost exclusively to FDR. In the absence of any recombination, FDR leads to 2*n* gametes with identical genotypes (i.e., the same as parental sporophyte). The occurrence of chiasma formation and crossing-over in the OA hybrid (Figures 1a and 1b) gives rise to 2*n* gametes with different genotypes. An example of the number of genotypes that can result from a random segregation of chromatids after a crossover between a pair of homoelogous chromosomes is illustrated in Figure 3. Thus, four different genotypes of 2*n* gametes were expected to occur with: (a) no recombinant chromosomes, (b) two recombinant (O/A + A/O) (reciprocal products), (c) only O/A and (d) only A/O. From an analysis of BC_1 progenies of OA hybrids all the expected types of 2*n* gametes have been found to be functional (results not included).

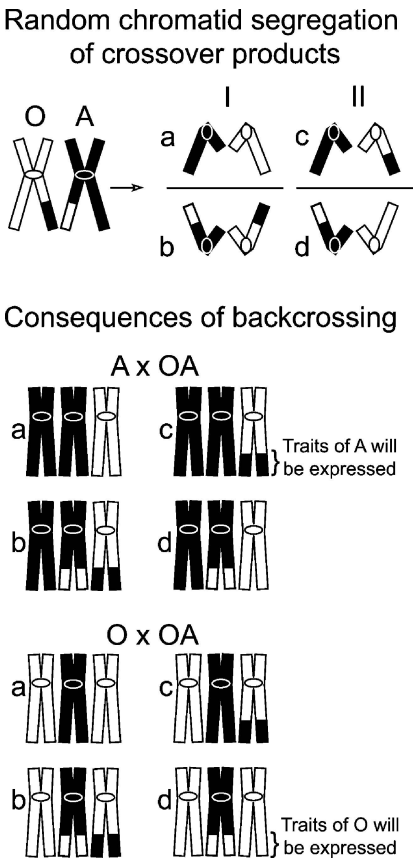

Figure 3. Random chromatid segregation scheme and its implication for the expression of either Asiatic or Oriental traits in subsequent backcrosses. It is noted that homozygosity can occur for the distal recombinant segment in the BC_1 progeny.

Discussion

In the past, we have critically investigated the occurrences of 2*n* gametes in LA hybrids and used them for producing backcross progenies (Karlov et al., 1999; Lim et al., 2001; Van Tuyl & Lim, 2003; Van Tuyl et al., 2000, 2002). Besides these basic investigations, lily breeders have extensively used 2*n* gametes in breeding numerous cultivars (data not shown). In view of this successful story of LA hybrids, the OA hybrids of the present investigation are of practical interest as well as scientific importance. It is of scientific importance because, when using distantly related species in crop improvement, the traditional approach was to produce an allopolyploid from a F_1 hybrid through somatic chromosome doubling. Such allopolyploids were appropriately called "permanent hybrids" because the parental characters almost never segregated in their progenies. In the absence of any genetic variation in the progenies of allopolyploids they were not useful for the selection of cultivars. Therefore the interest of breeders for using somatically doubled allopolyploids diminished, if not completely vanished. However, as is evident from the present

investigation, considerable genetic variation can be generated if 2*n* gametes from F_1 hybrids can be used for backcrossing. Most importantly, parental traits may be expressed in the BC_1 progenies depending on the recurrent parent used. Thus, considering the use of 2*n* pollen where only the O/A recombinant chromatid of a single chromosome is included and if an Asiatic cultivar is used as recurrent parent, the Asiatic traits for those homoleologous chromosomes will be expressed. The opposite case when Oriental traits may be expressed will be when the 2*n* pollen includes only the A/O recombinant chromatid and an Orientla cultivar is used as a recurrent parent (Figure 3).

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

A clear difference was observed with regard to the viability of 2*n* pollen grains based on *in vitro* germination (Table 4). It is not clear whether the difference is physiological. Nevertheless, from the point of view of making crosses, it is important to note that the use of fresh 2*n* pollen grains can ensure success. This is illustrated from the successful crosses that were obtained in both O × OA as well as A × OA combinations (results not included). Viability of 2*n* pollen is one of the considerations that one has to take into account while making crosses. Besides, factors such as the occurrence of very low frequencies of 2*n* gametes and environmental influence can also be limitations in some cases. Nevertheless, from the success we have achieved in producing a large number of backcrosses through sexual polyploidisation proves this method is quite practical in breeding lilies.

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