Euploid Endosperm of Triploid $\times$ Diploid/tetraploid Crosses Results in Aneuploid Embryo Survival in *Lilium*

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Abstract. The main objective of the present study was to identify chromosome number variation in the progenies from triploid $\times$ diploid/tetraploid ($3x \times 2x/4x$) crosses in *Lilium* to analyze the survival of aneuploid embryos from $3x \times 2x/4x$ crosses and the potential significance of triploid for lily breeding. Two male-sterile autotriploid Asiatic lily cultivars were crossed with four diploid and/or two autotetraploid Asiatic cultivars. The results showed that the fruits of all triploid $\times$ diploid/tetraploid ($3x \times 2x/4x$) crosses fully developed, and seedlings were obtained from the $3x \times 2x/4x$ crosses, indicating that male-sterile autotriploid lilies can be used as female parents. Chromosome preparation showed that the progenies of the $3x \times 2x/4x$ are predominantly aneuploid. Based on megasporogenesis, we deduce that the endosperm of $3x \times 2x$ is $7x$ and that of $3x \times 4x$ is $8x$ in *Lilium* and conclude that the aneuploid embryo survival of $3x \times 2x/4x$ in *Lilium* is the result of the euploid endosperm. Lily is usually propagated by scaling or tissue culture; therefore, aneuploids obtained from triploid $\times$ diploid/tetraploid lilies would be a good source for cultivar breeding.

Lily is an important ornamental crop worldwide. Modern lily cultivars originated from wild species of the genus *Lilium* L. of the family Liliaceae (McRae, 1990, 1998; Van Tuyl et al., 2000, 2002a, 2002b). Most of them are classified into four groups, Asiatic, Longiflorum, Oriental and Trumpet, each representing one lily genome (A, L, O, and T, respectively). Wild species of *Lilium* are diploid ($2n = 2x = 24$), except *L. lancifolium* Thunb., which has some triploid taxa (Kim et al., 2006). Triploid lilies ($2n = 3x = 36$) are also produced by diploid $\times$ tetraploid crosses or $2n$ gametes (Barba-Gonzalez et al., 2004, 2005a, 2005b; Lim et al., 2000). Based on their genome composition, triploid lilies with three identical genomes are regarded as autotriploid, e.g., AAA, whereas others such as LAA and AOA are allotriploid. Both types have abnormal meiosis during gamete formation, causing sterility, which blocks further introgression breeding. However, some lily allotriploid $\times$ diploid/tetraploid crosses have been successful, and their progenies have been analyzed for their genome com-

position and intergenomic recombination using genomic in situ hybridization (Barba-Gonzalez et al., 2006; Khan et al., 2009; Lim et al., 2003; Xie et al., 2010; Zhou, 2007). Nevertheless, few lily autotriploid $\times$ diploid/tetraploid ($3x \times 2x/4x$) crosses have been reported. In the present study, we made $3x \times 2x/4x$ crosses of Asiatic lilies to identify variation of their chromosome number and discussed why the aneuploid embryos from the $3x \times 2x/4x$ crosses were able to survive and the potential significance of triploid for lily breeding.

Materials and Methods

Plant materials. Eight Asiatic lily cultivars were used (Table 1). Six were bought from the Hongyue Flower Company, Zhejiang Province, China, and two were supplied courtesy of the Yunnan Flower Institute, Yunnan Province, China. In Mar. 2009, the cultivars were planted under natural light with regular watering in a glass greenhouse at Zhejiang University, China.

Chromosome preparation for the cultivars. Chromosome preparation was according to Zhou et al. (2008) with minor modifications. When lily roots were ≈ 1 cm long, they were cut off and pretreated with 0.7 mM cycloheximide (Amresco) at room temperature for 4 h and then fixed in ethanol: acetic acid (Sino-pharm Chemical Reagent Co., Ltd.) (3:1) overnight. The root tips were softened with a 1% (w/v) cellulase RS (Dutcheffa Biochemie) and 1% (w/v) pectinase Y23 (Dutcheffa Biochemie) mix at 37 °C for ≈ 1 h. The meristem was mixed with a drop of 1% acetocarmine (Fluka) and squashed on a glass slide. Each slide was checked with an Olympus microscope (BH-2) and metaphase cells were photographed with 40 $\times$ objective oil lens and a charge-coupled device camera. Usually, more than five cells with good metaphase were used to determine the chromosome numbers of the cultivars.

Table 1. The Asiatic lily cultivars used in the present study, indicating their code, ploidy levels, and source.

Cultivar	Code	Ploidy level	Source
Eleganza	AA ⁰⁰²	$2n = 2x = 24$	Hongyue
Monte Negro	AA ⁰⁰³	$2n = 2x = 24$	Hongyue
Renoir	AA ⁰⁰⁵	$2n = 2x = 24$	Hongyue
Pollyanna	AA ²⁶	$2n = 2x = 24$	Yunnan
Navona	AAA ⁰⁰⁴	$2n = 3x = 36$	Hongyue
A18	AAA ¹⁸	$2n = 3x = 36$	Yunnan
Brunello	AAAA ⁰⁰¹	$2n = 4x = 48$	Hongyue
Val di Sole	AAAA ⁰⁰⁷	$2n = 4x = 48$	Hongyue

Fig. 1. The chromosomes at mitotic metaphase of the lily cultivars. (A) AAAA⁰⁰¹ ($2n = 4x = 48$), (B) AA⁰⁰² ($2n = 2x = 24$), (C) AA⁰⁰³ ($2n = 2x = 24$), (D) AAA⁰⁰⁴ ($2n = 3x = 36$), (E) AA⁰⁰⁵ ($2n = 2x = 24$), (F) AAA¹⁸ ($2n = 3x = 36$), (G) AAAA⁰⁰⁷ ($2n = 4x = 48$), and (H) AA²⁶ ($2n = 2x = 24$). Bar = 10 μ m.

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Table 2. Number of pollinated flowers, rescued fruits, embryo sacs (Em.S.), embryos (Em.), and seedlings of the 3x × 2x/4x lily crosses.

Code	Female	×	Male	Flowers (no.)	Fruits (no.)	Condition of endosperm ^z		Seedlings (no.)
						Em. S. (no.)	Em. (no.)	
	(2n = 3x = 36)		(2n = 2x = 24)					
090088	AAA ⁰⁰⁴		AA ⁰⁰²	11	8	15	8	8
090047	AAA ⁰⁰⁴		AA ⁰⁰³	4	4	12	2	0
090020	AAA ⁰⁰⁴		AA ⁰⁰⁵	5	5	24	7	15
090044	AAA ⁰⁰⁴		AA ²⁶	4	4	18	7	7
090010	AAA ¹⁸		AA ²⁶	4	2	8	13	8
Subtotal				28	23	77	37	38
	(2n = 3x = 36)		(2n = 4x = 48)					
090046	AAA ⁰⁰⁴		AAAA ⁰⁰¹	5	5	15	15	9
090045	AAA ⁰⁰⁴		AAAA ⁰⁰⁷	4	4	22	35	32
090089	AAA ⁰⁰⁴		AAAA ⁰⁰⁷	14	11	17	40	55
Subtotal				23	20	54	90	96

^zEmbryo sacs or embryos could be isolated for culture only from ovules with well-developed endosperm.

Fig. 2. The fruits of 3x × 2x/4x crosses collected for embryo rescue. (1) 3x × 2x crosses and (2) 3x × 4x crosses. All appeared to develop well. Bar = 1 cm.

Pollen germination. Pollen from open anthers was scattered on pollen germination medium containing 100 g·L⁻¹ sucrose (Sinopharm Chemical Reagent Co., Ltd.), 5 g·L⁻¹ bacteriological agar (Sinopharm Chemical Reagent Co., Ltd.), 20 mg·L⁻¹ boric acid (Shanghai Yunling Refinery, China), and 200 mg·L⁻¹ Ca(NO₃)₂ (Shanghai Naihui Pengzheng-Yingfang Refinery, China) in a petri dish and kept at 25 °C overnight. Three to five easily countable scopes, containing ≈150 to 400 pollen grains, were observed under a stereo microscope (OPLINIC-SZM745T) to calculate the percentage of pollen germination.

Pollination. Anthers of seed plants were removed before anthesis. Styles were pollinated by hand and wrapped in aluminum foil to avoid contamination by other pollen.

Embryo rescue. Fruits were harvested when they became soft or yellowing after pollination. In a laminar air flow cabinet, each fruit was sterilized using 80% ethanol (Sinopharm Chemical Reagent Co., Ltd.), cut open, and the seeds put in a sterile petri dish. The seedcoats were carefully removed, and the embryo sacs or embryos put on lily embryo rescue medium (pH = 5.8) containing 2.2 g·L⁻¹ Murashige and Skoog (MS) medium (Duthefa Biochemie), 60 g·L⁻¹ sucrose, and 4 g·L⁻¹ gelrite (Duthefa Biochemie). They germinated in a dark chamber at 25 °C for 40 to 60 d and then were transferred to lily propagation medium (pH = 5.8) containing 2.2 g·L⁻¹ MS medium, 50 g·L⁻¹ sucrose, and 4 g·L⁻¹ gelrite at 25 °C and 40 μmol·m⁻²·s⁻¹ light intensity for ≈10 weeks.

Table 3. Chromosome numbers of the progenies obtained from autotriploid × diploid/tetraploid crosses showing the contribution by the male and the female.

Type of crosses	Progeny code	Chromosome numbers	Contributed by male ^z	Contributed by female ^y
AAA ⁰⁰⁴ × AA ⁰⁰⁵	090020-4	39	12	27
	090020-5	29	12	17
	090020-11	33	12	21
	090020-15	27	12	15
	Average	32	12	20.0 a ^x
AAA ¹⁸ × AA ²⁶	090010-1	28	12	16
	090010-4	29	12	17
	090010-5	28	12	16
	090010-6	32	12	20
	Average	29.3	12	17.3 a
AAA ⁰⁰⁴ × AAAA ⁰⁰¹	090046-2	40	24	16
	090046-4	45	24	21
	090046-5	45	24	21
	090046-6	42	24	18
	090046-7	43	24	19
	090046-9	46	24	22
	Average	43.5	24	19.5 a
AAA ⁰⁰⁴ × AAAA ⁰⁰⁷	090045-2	42	24	18
	090045-4	41	24	17
	090045-5	41	24	17
	090045-6	40	24	16
	090045-9	44	24	20
	090045-20	41	24	17
	090045-23	43	24	19
	090045-24	45	24	21
	090045-26	57	24	33
	090045-30	43	24	19
	090045-31	40	24	16
	Average	43	24	19.4 a

(Continued on next page)

Table 3. (Continued) Chromosome numbers of the progenies obtained from autotriploid $\times$ diploid/tetraploid crosses showing the contribution by the male and the female.

Type of crosses	Progeny code	Chromosome numbers	Contributed by male ^z	Contributed by female ^y
AAA ⁰⁰⁴ $\times$ AAAA ⁰⁰⁷	090089-2	42	24	18
	090089-3	40	24	16
	090089-5	41	24	17
	090089-10	44	24	20
	090089-12	37	24	13
	090089-14	42	24	18
	090089-16	46	24	22
	090089-19	43	24	19
	090089-20	43	24	19
	090089-23	44	24	20
	090089-25	45	24	21
	Average	42.5	24	18.5 a

^zNumber of chromosomes contributed by male.

^yNumber of chromosomes contributed by female.

^aNo significant difference between the number of the chromosomes contributed by female in these $3x \times 2x/4x$ crosses at $P \leq 0.05$.

Chromosome preparation for progenies.

The seedlings obtained by embryo rescue were numbered according to the crossing code and the lines of seedlings. For example, the eight lines of seedlings obtained from 090088 were numbered 090088-1, 090088-2, 090088-3, 090088-4, 090088-5, 090088-6, 090088-7, and 090088-8, respectively. Chromosome preparation was as for cultivars, but, because it was not always possible to obtain good root tips, the maximum number of progeny possible was analyzed.

Results

Chromosome preparation showed that four of the eight lily cultivars were diploid

($2n = 2x = 24$), two triploid ($2n = 3x = 36$), and two were tetraploid ($2n = 4x = 48$) (Fig. 1; Table 1). Pollen germination of diploid and tetraploid cultivars was 50% to 80%, indicating that they were male-fertile, whereas pollen germination of triploid cultivars was less than 1%, suggesting that they would not serve as acceptable male parents in a breeding program. Therefore, the two triploid cultivars were used as seed plants and pollinated with diploid and/or tetraploid pollen (Table 2).

The fruits of both $3x \times 2x$ and $3x \times 4x$ crosses developed well (Fig. 2). In all crosses, some ovules developed well, i.e., their endosperm or embryos were visible, so embryo sacs or embryos could be isolated (Table 2). Thirty-eight seedlings were obtained from

23 fruits of $3x \times 2x$ crosses and 96 seedlings from 20 fruits of $3x \times 4x$ crosses (Table 2). Only 090047 was not successful. Similar results were observed with three other triploid Asiatic lily cultivars in 2010 (data not shown). Based on these results, we concluded that male-sterile autotriploid lilies could be used as seed parents to cross with appropriate males.

Chromosome preparation showed that the progenies of these $3x \times 2x/4x$ crosses were predominantly aneuploid with chromosome numbers as follows: AAA⁰⁰⁴ $\times$ AA⁰⁰⁵ progenies ranged from 27 to 39; AAA¹⁸ $\times$ AA²⁶ from 28 to 32; those of AAA⁰⁰⁴ $\times$ AAAA⁰⁰¹ (090046) from 40 to 46; AAA⁰⁰⁴ $\times$ AAAA⁰⁰⁷ (090045) from 40 to 57; and those of AAA⁰⁰⁴ $\times$ AAAA⁰⁰⁷ (090089) from 37 to 46 (Table 3; Fig. 3). Based on the results, it was derived that the functional eggs contributed by triploids AAA⁰⁰⁴ and AAA¹⁸ in both $3x \times 2x$ and $3x \times 4x$ crosses were predominantly aneuploid with an average chromosome number of 18.9. Although the numbers of the chromosomes donated by the triploid lilies were apparently variable, analysis of variance gave no significant difference between the different crosses.

Analysis and Discussion

The present results could be well explained by theoretical analysis. Based on megasporogenesis of *Fritillaria*-type plants (Maheshwari, 1948), it is derived that the endosperm of $3x \times 2x$ crosses is $7x$ and that of $3x \times 4x$ is $8x$. It is clear, as shown in Figure 4, that a secondary nucleus of *Fritillaria*-type embryo sac is equal to the sum of the nuclei of four daughter cells

Fig. 3. The chromosomes at mitotic metaphase from root tips of the representative $3x \times 2x/4x$ progenies. (A) 090020-4 ($2n = 3x+3 = 39$), (B) 090010-5 ($2n = 2x+4 = 28$), (C) 090046-9 ($2n = 4x-2 = 46$), (D) 090045-5 ($2n = 3x+5 = 41$), (E) 090089-12 ($2n = 3x+1 = 37$), and (F) 090089-19 ($2n = 4x-5 = 43$). Bar = 10 μ m.

Fig. 4. Ideogram of megasporogenesis of *Fritillaria*-type embryo sac in *Lilium*. The arrows indicate the origin of the secondary nucleus, showing that the secondary nucleus is just equal to the sum of the nuclei of four daughter cells resulting from meiosis.

produced by a megaspore mother cell. When the megasporogenesis occurs in a triploid lily, its abnormal meiosis usually produces four aneuploid daughter cells. Because the sum of nuclear DNA of the four meiotic aneuploid daughter cells also originates from the duplication of the nuclear DNA of a megasporeocyte, the secondary nucleus of triploid lily must be hexaploid ($6x$) because the secondary nucleus consists of the four meiotic daughter cells. This means that the ploidy level of a secondary nucleus of a *Fritillaria* embryo sac is invariably twice that of its somatic cells, whether this embryo sac forms through normal or abnormal meiosis. Therefore, the endosperm of $3x \times 2x/4x$ is euploid, and it is reasonable to consider that the euploid endosperm of $3x \times 2x/4x$ crosses is responsible for the survival of the aneuploid embryos resulting from these crosses.

The present results agree with previous reports (Barba-Gonzalez et al., 2006; Khan et al., 2009; Lim et al., 2003) that progenies of $3x \times 2x/4x$ crosses of *Lilium* are predominantly aneuploid as a result of aneuploid eggs (from x to $3x+3$) surviving (Fig. 5). In contrast, $3x \times 2x/4x$ crosses of most other plants usually produce euploid, i.e., diploid, tetraploid, and pentaploid progenies (Brandham, 1982; Carputo and Barone, 2005; Ramsey and Schemske, 1998). This could be explained by the difference of the secondary nucleus between *Polygonum*-type and *Fritillaria*-type embryo sacs, because endosperm is the primary reason for seed development or abortion (Birchler, 1993; Haig and Westoby, 1991; Johnston et al., 1980; Johnston and Hanneman, 1982; Scott et al., 1998). According to megasporogenesis (Maheshwari, 1948), a triploid *Polygonum*-type plant usually produces an aneuploid egg ($1.5x^*$) and aneuploid secondary nucleus ($3.0x^*$), whereas a triploid *Fritillaria*-type plant produces an aneuploid egg cell ($1.5x^*$) but hexaploid secondary nucleus ($6x$) (Fig. 4). After double fertilization, as shown in Figure 6, both embryo and endosperm of $3x \times 2x/4x$ crosses in *Polygonum*-type plants are aneuploid. However, in *Lilium* $3x \times 2x/4x$ crosses, the embryo is aneuploid but the endosperm is euploid ($7x/8x$), and it is likely that euploid endosperm would develop better than aneuploid endosperm. Therefore, in $3x \times 2x/4x$ crosses of *Polygonum*-type plants, only when eggs are euploid will both euploid endosperm and euploid embryo develop, and the surviving

Fig. 5. Overall distribution of the frequency of eggs with different chromosome numbers produced by auto- and allotriploid lilies. Data on autotriploids from the present study and those on allotriploids from previous research (Barba-Gonzalez et al., 2006; Khan et al., 2009; Lim et al., 2003).

Fig. 6. Schematic comparison of ploidy level of sperm, egg, secondary nucleus, embryo, and endosperm in $3x \times 2x/4x$ crosses of *Polygonum*-type and *Fritillaria*-type plants. The dotted margin and gray of the aneuploid gamete and aneuploid secondary nucleus indicate that they are usually nonfunctional, and the solid margin and black of the euploid gamete and euploid secondary nucleus mean that they are usually functional.

plants will usually be euploid. However, in *Fritillaria*-type plants, whether eggs are euploid or aneuploid, their secondary nuclei are invariably euploid and their endosperm will likely develop and result in embryo survival. Further support comes from the fact that the chromosome numbers of most surviving eggs produced by triploid lilies are $\approx 1.5x^*$ (Fig. 5), which is the same as the expected peak of aneuploid gametes produced by triploid lilies.

Endosperm balance number (EBN), proposed by Johnston et al. (1980) based on potato hybridization, has been used to explain the success or failure of interspecific and interploidy crosses of many plants (Carputo and Barone, 2005; Johnston and Hanneman, 1982). In $2x \times 4x$, $4x \times 2x$, $4x \times 4x$, $2x \times 5x$,

and $5x \times 2x$ crosses, Zhou (2007) noticed the difficulty in selfing the oryzalin-induced amphydiploid lilies, e.g., LALA $\times$ LALA. In contrast, whereas AA $\times$ LALA crosses are usually successful, their reciprocal crosses are not. This cannot be explained with EBN. It is possible that the DNA amount in primary endosperm of LALA $\times$ LALA and LALA $\times$ AA is so high that endosperm development is substantially hindered (Zhou, 2007).

In the present research, the AAA $\times$ AAAA crosses were more successful than AAA $\times$ AA. This is similar to our recent results of LAA $\times$ AAAA and LAA $\times$ AA crosses (unpublished data). The phenomenon can be explained by genomic imprinting, i.e., an excess dosage of paternal chromosomes promotes

endosperm development (Köhler and Weinhofer-Molisch, 2010; Scott et al., 1998).

Based on our results, we suggest that autotriploid lilies, regardless of their male sterility, could be used as a female to cross with appropriate diploid or tetraploid male. We also conclude that the progenies of $3x \times 2x/4x$ are aneuploid in *Lilium* rather than diploid or tetraploid like in *Polygonum*-type plants.

It is well known that a change in chromosome number can bring about large variation in morphological, physiological, and ecological characteristics. Because lily is usually propagated by scaling or tissue culture, the triploid lily may be a good potential source for breeding aneuploid lily cultivars.

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