Study on lily introgression breeding using allotriploids as maternal parents in interploid hybridizations

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Based on a recent hypothesis, "Five same genomes of endosperm are essential for its development in *Lilium*", it is expected that allotriploid lily (OTO) can be hybridized with diploid Oriental lily (OO) for introgression breeding in *Lilium* L.. To test the hypothesis, OTO lilies, 'Belladonna', 'Candy Club' and 'Travatore', were used as the maternal parents and crossed with two diploid OO cultivars, 'Siberia' and 'Sorbonne', and the species *L. regale* Wilson (TT). Results showed that capsules of all OTO × OO hybridizations developed well and 0.8~3.3 viable seedlings per ovary were obtained through normal pollination and embryo rescue; however, all OTO × TT crosses failed. Genomic *in situ* hybridization showed that the progenies of the OTO × OO hybridizations were aneuploid and a variable number of T-genome chromosomes were introduced into the progenies through the allotriploid lilies. The present results not only demonstrate that allotriploid OTO lilies, although male sterile, can be used as maternal parents to produce aneuploid progenies, but also strongly support the new hypothesis in lily breeding.

Key Words: aneuploid, endosperm genome composition, five-same-genomes, Fritillaria-type embryo sac, *Lilium*.

Introduction

Lily (*Lilium* L.) is an important bulb flower worldwide. Most lily cultivars originating from intra-sectional hybridizations of the genus *Lilium* are classified into four groups: Asiatic (A), Longiflorum (L), Oriental (O) and Trumpet (T) (Van Tuyl *et al.* 2000). Hybridizations within each group are usually straightforward and their F1 hybrids are fertile, however, those between different groups need cut-style pollination and embryo rescue, and such distant F1 hybrids are highly sterile (McRae 1998, Van Tuyl *et al.* 1988, 1991, 2002a, 2002b, Zhou *et al.* 2008b). Notwithstanding, these distant F1 hybrids can spontaneously or artificially produce 2*n*-gametes and result in sexual polyploidization (Barba-Gonzalez *et al.* 2004, 2005a, 2005b, 2006, Khan *et al.* 2009, Lim *et al.* 2000, Zhou 2007, Zhou *et al.* 2008a). With the polyploidy advantages and the variation caused by inter-genomic recombination of 2*n*-gametes, many new cultivars have been directly selected from such allotriploid BC1 progenies by lily breeders (Zhang *et al.* 2012, Zhou *et al.* 2008a). OTO, which has two sets of O-chromosomes and one set of T-chromosomes, is one of the most promising allotriploid lilies because of the large flowers, strong stems and fragrance.

It is well known that most Polygonum-type triploid plants, such as triploid watermelon and banana, are sterile and seedless, so are usually not the ideal source for further introgression breeding (Brandham 1982). In contrast, the triploid lilies (2*n* = 3*x* = 36), both autotriploid (AAA) and allotriploid (AOA, LAA, LLO), can be used as maternal parents to hybridize with appropriate diploid (2*n* = 2*x* = 24) or tetraploid lilies (2*n* = 4*x* = 48), although they are also male sterile due to abnormal meiosis (Barba-Gonzalez *et al.* 2006, Chung *et al.* 2013, Khan *et al.* 2009, Lim *et al.* 2003, Natenapit *et al.* 2010, Xie *et al.* 2010, Zhou 2007, Zhou *et al.* 2011, 2012). The basis for this is the difference in the embryo sac formation between Polygonum-type and Fritillaria-type plants. From normal megasporogenesis of the Fritillaria-type embryo sac, Zhou (2007) deduced that triploid lilies produce aneuploid eggs and hexaploid central cells (secondary nuclei). Based on the crossability of these 3*x* × 2*x*/4*x* interploid hybridizations, the "Five same genomes of endosperm are essential for its development in *Lilium*" ("five-same-genomes") hypothesis was proposed, to explain the success or failure of 3*x* × 2*x*/4*x* crosses in *Lilium* (Zhou *et al.* 2012) (see detail in discussion). Based on this hypothesis, we expected that OTO × OO crosses could be used in lily introgression breeding. However, very few cases regarding OTO lilies as the maternal parent have been reported (Chung *et al.* 2013, Zhou *et al.* 2012). In order to confirm whether the new theory applies to OTO × OO crosses, which would offer a new source for lily

Table 1. Results of OTO × OO and OTO × TT hybridizations in *Lilium*

Code	Maternal ♀	Paternal ♂	Flowers No.	Pollinating method	Capsules No.	Embryo sac No.	Embryo No.	Seedlings No.	Seedlings per capsule
100513	OTO[b]	OO[so]	19	Normal	18	115	4	31	1.6
100539	OTO[c]	OO[so]	14	Normal	13	78	4	11	0.8
100534	OTO[t]	OO[so]	8	Normal	7	31	0	7	0.9
100558	OTO[c]	OO[si]	10	Normal	9	69	12	33	3.3
100512	OTO[b]	TT[r]	6	Normal	6	5	0	0	0.0
100531	OTO[c]	TT[r]	5	Normal	1	0	0	0	0.0
100519	OTO[t]	TT[r]	5	Normal	5	7	0	0	0.0

introgression breeding, we carried out controlled hybridizations between OTO and other diploid lilies, and analyzed their progenies using genomic *in situ* hybridization (GISH). We conclude with a discussion of the significance of allotriploids in lily introgression breeding.

Materials and Methods

Plant materials

Three OT cultivars ($2n = 3x = 36$) ('Belladonna', 'Candy Club' and 'Travatore') and two Oriental cultivars ($2n = 2x = 24$) ('Siberia' and 'Sorbonne') were supplied by Hongyue Flower Company, Hangzhou, China. One species, *L. regale* Wilson ($2n = 2x = 24$), was donated by Drs. Jisen Shi and Mengli Xi, Nanjing Forestry University, Nangjing, China. Since 'Belladonna', 'Candy Club' and 'Travatore' are allotriploid, with two sets of O-chromosomes and one set of T-chromosomes (Zhang *et al.* 2012), they were coded as OTO[b], OTO[c] and OTO[t], respectively. Similarly, the diploid Oriental cultivars 'Siberia' and 'Sorbonne' are represented by OO[si] and OO[so], and *L. regale*, the main origin of Trumpet lilies, by TT[r]. The OTO cultivars were used as the maternal donor and other diploid lilies as the paternal donor in the controlled hybridizations, and the crosses numbered 100513, 100539, etc. (Table 1).

Pollination and embryo rescue

At the end of September 2010, the cultivars were grown under natural light in a plastic greenhouse in Zhejiang University. When the temperature inside the greenhouse dropped to 18°C during winter, the automatic heating system was turned on. The flowering period was from the end of December 2010 to the beginning of January 2011. Pollination and embryo rescue were according to Zhou *et al.* (2013). Anthers were removed prior to anthesis. After pollination, styles were wrapped with aluminum foil. The soft or yellow fruits were cut off for in vitro embryo rescue in a laminar air flow cabinet because the seeds did not develop as well as normal seeds. Each was sterilized using 80% ethanol (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) for 3–5 seconds. The seed coats were carefully removed, and the embryo sacs or embryos put on a medium (pH = 5.8) containing 2.2 g·L⁻¹ MS (Duchefa Biochemie, Haarlem, The Netherlands), 60 g·L⁻¹ sucrose and 4 g·L⁻¹ gelrite (Duchefa Biochemie). They were then germinated in

a dark chamber at 25°C for 40–60 d, transferred to a medium (pH = 5.8) containing 2.2 g·L⁻¹ MS, 50 g·L⁻¹ sucrose and 4 g·L⁻¹ gelrite (Duchefa Biochemie) at 25°C, and kept at 2500 lux light intensity for 12 hours per day for about 10 weeks.

Chromosome preparation

The protocol was according to Zhou *et al.* (2013). When lily roots of in vitro plantlets were approximately 1 cm long, they were cut off and pretreated with 0.7 mM cyclohex-imide (Amresco, Solon, OH), at room temperature for 4 h, and then fixed in ethanol: acetic acid (Sinopharm Chemical Reagent Co.) (3 : 1) overnight. Root tips were then softened with a 1% (w/v) cellulase RS (Duchefa Biochemie) and 1% (w/v) pectinase Y23 (Duthefa Biochemie) mix at 37°C for 1 h. The meristem was mixed with a drop of 45% acetic acid on a glass slide, covered with a glass cover slip and squashed. Each slide was examined under a phase contrast microscope (BH-2; Olympus, Tokyo, Japan) for *in situ* hybridization.

Genomic in situ hybridization (GISH)

The method was according to Zhou *et al.* (2013) with minor modifications. Genomic DNA of Oriental 'Sorbonne' and *L. regale* was isolated using the CTAB method (Rogers and Bendich 1988), and labeled with biotin-16-dUTP as the probe, according to the manufacturer's instructions (Biotin-Nick translation Mix 11745824910; Roche, Mannheim, Germany). The hybridization mix (40 μL) contained 50% deionized formamide, 10% dextran sulfate, 2× SSC (0.3 M NaCl plus 30 mM sodium citrate, pH 7.0), 0.25% sodium dodecyl sulfate (SDS), 25–50 ng probe DNA and 2 μg herring sperm DNA (D3159, Sigma-Aldrich). Signal was detected with Streptavidin-CY3 (Invitrogen, Camarillo, CA) and Biotinylated anti-Streptavidin (Vector Laboratories, Burlingame, CA). After counterstaining with DAPI (Roche), the slides were observed under a fluorescence microscope (BH41; Olympus). Images were taken with an attached CCD (Micropublisher 3.3 RTV; QImaging, Surrey, Canada) driven by Image-Pro® (Media Cybernetics, Rockville, MD).

Results

The main results of the $3x × 2x$ hybridizations are shown in Fig. 1 and Table 1. Capsules of OTO × OO combinations

Fig. 1. Representative fruits of OTO × OO and OTO × TT hybridizations, at harvesting stage for embryo rescue, indicate that the fruits of OTO × OO generally developed better than those of OTO × TT. 100513 = OTO$^{\text{'Belladonna'}}$ × OO$^{\text{'Sorbonne'}}$; 100539 = OTO$^{\text{'Candy Club'}}$ × OO$^{\text{'Sorbonne'}}$; 100534 = OTO$^{\text{'Travatore'}}$ × OO$^{\text{'Sorbonne'}}$; 100558 = OTO$^{\text{'Candy Club'}}$ × OO$^{\text{'Siberia'}}$; 100512 = OTO$^{\text{'Belladonna'}}$ × TT$^{\text{'L. regale'}}$; 100531 = OTO$^{\text{'Candy Club'}}$ × TT$^{\text{'L. regale'}}$; 100519 = OTO$^{\text{'Travatore'}}$ × TT$^{\text{'L. regale'}}$. Bar = 1 cm.

were usually more developed and larger than those of OTO × TT. All the OTO × OO combinations were successful. For example, for 100513 (Table 1), 19 flowers of OTO^b were pollinated with OO^{so}, 18 capsules were harvested for embryo rescue, and 119 well-developed seeds (including 115 embryo sacs and four embryos) were rescued. A final 31 seedlings were obtained. On average, 100513 produced 1.6 seedlings per ovary. Table 1 also shows that 100539, 100534, and 100558 produced 0.8, 0.9, and 3.3 seedlings per capsule respectively. In total, 82 seedlings were obtained from the OTO × OO combinations, while none from any OTO × TT combinations, indicating that OTO × OO hybridizations were more successful than OTO × TT combinations.

Nine seedlings were analyzed using GISH (Fig. 2). Seedling 100513-1 (Table 2) had 32 chromosomes in total: 24 O-chromosomes (O^{ch}), seven T-chromosomes (T^{ch}), and one recombinant chromosome (O/T^{ch}). Since 12 of these 32 chromosomes were contributed by pollen (Pollen^{ch}), the other 20 chromosomes were contributed by egg (Egg^{ch}). It is evident that all of them were aneuploid with 25 to 33 chromosomes, indicating that they are the result of haploid sperm fusing with aneuploid eggs produced by the OTO maternal parents (Table 2). Except for 100539-4, the other eight seedlings contained between one and three recombinant chromosomes, suggesting that allotriploid OTO lilies are a good source for lily introgression breeding.

Discussion

The present results show that, while OTO × OO hybridizations are successful, OTO × TT are not. This is expected from the "five-same-genomes" hypothesis and so strongly supports the hypothesis. As for the variation of different OTO × OO combinations, it is reasonable that genetic differences between cultivars are the main factor causing it.

The present results coincide to a large extent with those reported for other types of triploid lilies, which usually demonstrate limited female fertility and produce aneuploid
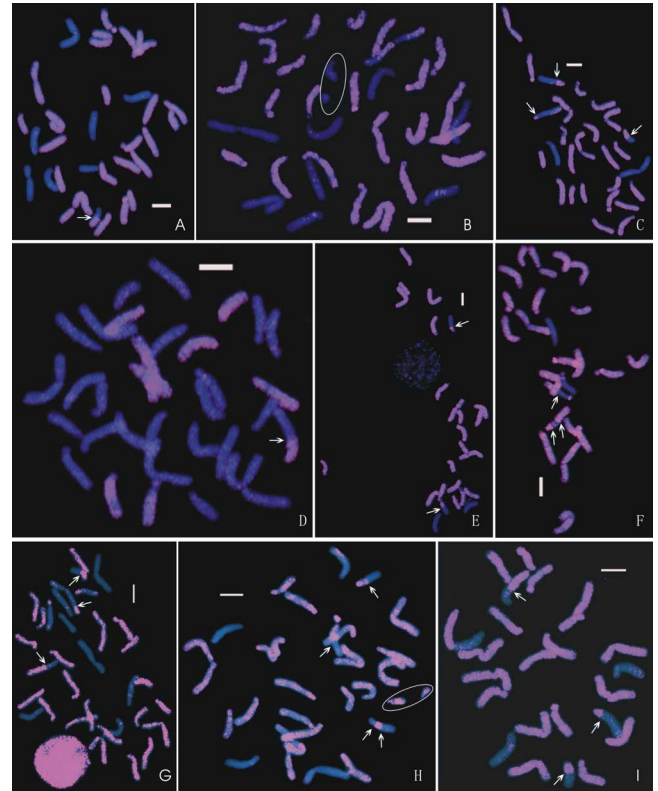
Fig. 2. Genomic *in situ* hybridization (GISH) on the metaphase chromosomes of the nine progenies of OTO × OO hybridizations, demonstrating the chromosomal variation caused by the triploid OTO lilies. In D, T-genome DNA was labeled with biotin and used as probe so the T-chromosomes are pink and O-chromosomes are blue. In all other images, the O-genome DNA was labeled with biotin and used as probe, so the O-chromosomes are pink and the T-chromosomes blue. Arrows indicate the recombination point. The chromosomes marked with an ellipse in images B and H were damaged and part of them is lacking. Bar = 10 μm. (A) 100513-3 ($2n = 24^O + 7^T + 1^{O/T}$); (B) 100539-4 ($2n = 24^O + 9^T$); (C) 100558-1 ($2n = 22^O + 2^T + 3^{O/T}$); (D) 100558-5 ($2n = 23^O + 8^T + 1^{O/T}$); (E) 100558-11 ($2n = 21^O + 2^T + 2^{O/T}$); (F) 100558-13 ($2n = 21^O + 2^T + 2^{O/T}$); (G) 100558-14 ($2n = 21^O + 9^T + 3^{O/T}$); (H) 100558-20 ($2n = 22^O + 4^T + 3^{O/T}$); (I) 100558-23 ($2n = 21^O + 3^T + 3^{O/T}$).

Table 2. The chromosome number of one seedling of 100513, one of 100539, and seven of 100558, illustrating variation in their total chromosomes (Chromosome no.), Oriental chromosomes (O^{ch}), Trumpet chromosomes (T^{ch}) and recombinant chromosomes (O/T^{ch}), and chromosome numbers contributed by pollen ($Pollen^{ch}$) and egg (Egg^{ch})

Code	Chromosome no.	O^{ch} (no.)	T^{ch} (no.)	O/T^{ch} (no.)	$Pollen^{ch}$ (no.)	Egg^{ch} (no.)
100513-3	32	24	7	1	12	20
100539-4	33	24	9	0	12	21
100558-1	27	22	2	3	12	15
100558-5	33	23	8	1	12	21
100558-11	25	21	2	2	12	13
100558-13	25	21	2	2	12	13
100558-14	33	21	9	3	12	21
100558-20	29	22	4	3	12	17
100558-23	27	21	3	3	12	15

progenies (Barba-Gonzalez *et al.* 2006, Chung *et al.* 2013, Khan *et al.* 2009, Lim *et al.* 2003, Xie *et al.* 2010, Zhou *et al.* 2011, 2012). They differ from Polygonum-type triploid plants which are sterile and seedless, or produce a small number of euploid progenies through $3x \times 2x/4x$ interploid hybridizations (Brandham 1982, Carputo and Barone 2005, Ramsey and Schemske 1998, 2002). The reason for the difference has been well explained by analyzing the difference between Fritillaria-type embryo sacs of *Lilium* and the Polygonum-type embryo sacs of most other plants (Zhou *et al.* 2011). From diploid normal megasporogenesis, it has been deduced that megasporogenesis in triploid Polygonum-type plants results in embryo sacs that contain aneuploid eggs and aneuploid central cells, while embryo sacs in Fritillaria-type triploid plants contain aneuploid eggs and euploid ($6x$) central cells (Zhou 2007). After double fertilization in $3x \times 2x/4x$ crosses, survival of *Lilium* aneuploid embryos is due to the euploid endosperm, while only a small number of euploid progenies are obtained in triploid Polygonum-type plants (Zhou *et al.* 2011).

The key factor in determining success or failure of hybridization is the compatibility of the parents. In the present

research, OTO × OO crosses were often successful while all OTO × TT combinations failed, indicating that OTO and OO are compatible. The result can be well explained by the recent "five-same-genomes" hypothesis (Zhou *et al.* 2012). Because the amount of nuclear DNA in *Lilium* central cells is invariably twice that of somatic cells (Zhou 2007), the endosperm genome composition of any lily hybridization can be identified (Table 3). For example, an allotriploid lily (OTO) produces central cells containing four Oriental (O) genomes and two Trumpet (T) genomes, i.e., 4O + 2T, while a diploid OO lily generates pollen grains containing one Oriental genome, so the endosperm genome composition of OTO × OO hybridizations is 5O + 2T. From Table 3, it can be concluded that the endosperm of hybridizations develop well, and the hybridizations are relatively successful, when they contain 5 or more same genomes (+); otherwise, the hybridizations are not likely to be successful (−) (Zhou *et al.* 2012). In the present research, the endosperm of OTO × OO progeny contains 5 O-genomes and 2 T-genomes, hence the endosperm develops well and hybridizations are more successful. In contrast, the endosperm of OTO × TT crosses contains 4 O-genomes and 3 T-genomes, and hybridizations fail. These results give major support to the recent hypothesis.

Other mechanisms for explaining success or failure of plant hybridizations have been described. A 2 : 1 ratio of maternal : paternal genomes of the endosperm itself was proposed by Nishiyama and Inomata (1966). Unfortunately, it cannot explain the success of $2x \times 4x$ and $4x \times 2x$ hybridizations in many plants. EBN (endosperm balance number) has been proposed as a basis for explaining hybridizations between *Solanum* species (Johnston *et al.* 1980, Johnston and Hanneman 1982) and other species (Carputo *et al.* 1999, Carputo and Barone 2005). The difficulty of the EBN hypothesis is that the value of any parent EBN has to be assigned by hybridizations with a standard species. These hypotheses do not conflict with that of the 'five-same-genomes' because the 2 : 1 ratio and the EBN are applied to

Table 3. Relationship between the endosperm genome composition and crossability in lily hybridizations

Hybridizaitons		Central cell	Sperm	Endosperm	Crossability	References
Maternal (♀)	Paternal (♂)					
AA	AA	4A	A	5A	+++	McRae 1998
OTO	OO	4O + 2T	O	5O + 2T	+	This research
LAA	AA	4A + 2L	A	5A + 2L	+	Lim *et al.* 2003
LAA	AAAA	4A + 2L	2A	6A + 2L	++	Zhou *et al.* 2012
LAA	LALA	4A + 2L	L + A	5A + 3L	+	Lim *et al.* 2003
AOA	AA	4A + 2O	A	5A + 2O	+	Barba-Gonzalez *et al.* 2006
AOA	OAOA	4A + 2O	O + A	5A + 3O	+	Barba-Gonzalez *et al.* 2006
LLO	LLTT	4L + 2O	L + T	5L + 2O + T	+	Xie *et al.* 2010
AAA	AA	6A	A	7A	+	Zhou *et al.* 2011
AAA	AAAA	6A	2A	8A	++	Zhou *et al.* 2011
OTO	TT	4O + 2T	T	4O + 3T	−	This research
LAA	LL	4A + 2L	L	4A + 3L	−	Zhou *et al.* 2012
LAA	OO	4A + 2L	O	4A + 2L + O	−	Zhou *et al.* 2012

The successful (+) hybridizations produce endosperms with 5 or more same genomes while the unsuccessful (−) hybridizations produce endosperm with less than 5 same genomes. The more plus (+) symbols, the more successful the hybridization.

Polygonum-type plants while 'five-same-genomes' is applicable to Fritillaria-type plants. All the mechanisms demonstrate that endosperm genomic constitution is essential for success or failure of hybridizations in angiosperms.

Modern lily intra-sectional cultivars are usually diploid and most inter-sectional cultivars are triploid (Li *et al.* 2011, Zhang *et al.* 2012). Few commercial cultivars are aneuploid (Zhou *et al.* 2008a), although triploid lilies can be used as the maternal line to hybridize with appropriate males to produce aneuploid progenies. The reason for this is unclear, but it is known that aneuploidy is quite common in *Hyacinth* cultivars (Rees 1972). Aneuploidy causes considerable variation in morphological and biological traits, thus increasing the diversity for cultivar development. Lily is also easily propagated by micropropagation and bulb scaling, making it possible to multiply promising aneuploid lines. We do not consider that allotriploidy is a bottleneck in lily introgression breeding, and believe that aneuploids will become an important part of new lily cultivar development in the near future.

Acknowledgements

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