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 2n-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 2n-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 (2n = 3x = 36), both autotriploid (AAA) and allotriploid (AOA, LAA, LLO), can be used as maternal parents to hybridize with appropriate diploid (2n = 2x = 24) or tetraploid lilies (2n = 4x = 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 3x × 2x/4x 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 3x × 2x/4x 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

Communicated by H. Iketani
Received October 17, 2013. Accepted January 25, 2014.
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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, Nanjing, 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°, OTOε and OTOτ, respectively. Similarly, the diploid Oriental cultivars ‘Siberia’ and ‘Sorbonne’ are represented by OO° and OOε, and L. regale, the main origin of Trumpet lilies, by TT°. 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 cycloheximide (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 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, Carlsbad, 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

| Code       | Maternal | Paternal | Flowers No. | Pollinating method | Capsules No. | Embryo sac No. | Embryo No. | Seedlings No. | Seedlings per capsule |
|------------|----------|----------|-------------|--------------------|--------------|----------------|------------|----------------|--------------------|
| 100513     | OTO°     | OO°      | 19          | Normal             | 18           | 115            | 4          | 31             | 1.6                |
| 100539     | OTO°     | OO°      | 14          | Normal             | 13           | 78             | 4          | 11             | 0.8                |
| 100534     | OTO°     | OO°      | 8           | Normal             | 7            | 31             | 0          | 7              | 0.9                |
| 100558     | OTO°     | OO°      | 10          | Normal             | 9            | 69             | 12         | 33             | 3.3                |
| 100512     | OTO°     | TT°      | 6           | Normal             | 6            | 5              | 0          | 0              | 0.0                |
| 100531     | OTO°     | TT°      | 5           | Normal             | 1            | 0              | 0          | 0              | 0.0                |
| 100519     | OTO°     | TT°      | 5           | Normal             | 5            | 7              | 0          | 0              | 0.0                |
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 were pollinated with OO, 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 (Och), seven T-chromosomes (Tch), 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
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).

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 × 4x and 4x × 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 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 (Och), Trumpet chromosomes (Tch), and recombinant chromosomes (O/T ch), and chromosome numbers contributed by pollen (Pollench) and egg (Eggch).

| Code    | Chromosome no. | Och (no.) | Tch (no.) | Pollench (no.) | Eggch (no.) |
|---------|----------------|-----------|-----------|----------------|-------------|
| 100513-3 | 32             | 24        | 7         | 1              | 12          |
| 100539-4 | 32             | 24        | 9         | 0              | 12          |
| 100558-1 | 27             | 22        | 2         | 3              | 12          |
| 100558-5 | 33             | 23        | 8         | 1              | 12          |
| 100558-11 | 25           | 21        | 2         | 2              | 12          |
| 100558-13 | 25            | 21        | 2         | 2              | 12          |
| 100558-14 | 33             | 21        | 9         | 3              | 12          |
| 100558-20 | 29             | 22        | 4         | 3              | 12          |
| 100558-23 | 27             | 21        | 3         | 3              | 12          |

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 × 2x/4x interpollid 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 megsporogenesis, it has been deduced that megsporogenesis 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 × 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).
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

We thank the National Natural Science Foundation of China (31071821) and project 863 (2011AA100208) for financial support, Dr. Shirley Burgess for English correction and Drs. Jisen Shi and Mengli Xi for supplying L. regale.

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