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Meiotic Polyploidization with Homoeologous Recombination Induced by Caffeine Treatment in Interspecific Lily Hybrids

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ABSTRACT

Caffeine solution was injected into the flower bud to recover F_1 fertility of the intersectional diploid *Lilium* species hybrid (2n=2x=24). 0.3% of caffeine solution was the most effective concentration to produce fertile 2n-gametes. The male and female gametes had a range of fertility following caffeine injection in relation to the different sizes of flower buds. The gametes from the treated OA-hybrids showed to be fertile. They were crossed both as male and female parents to the Asiatics, and produced 279 BC_1 progeny plants. Flowcytometric and chromosomal observation confirmed that all progenies were triploid (2n=3x=36), indicating that the 2n-gametes from the OA-hybrid were functional following caffeine treatment. There was no homoeologous recombination between parental species in the progeny derived from male gametes of the OA-hybrid. However, three plants originating from the female gametes showed homoeologous recombination between Oriental and Asiatic genomes. The functional female gametes were produced when treated at the range of 20-23mm and 34-37mm of flower bud length. Based on the chromosome constitution of the triploid BC_1 progenies, it was concluded that FDR 2n-gamete was functional and homoeologous recombination had occurred during the origin of 2n gametes.

Key words: chromosome analysis, 2n-gametes, *Lilium*, GISH, cytokinesis.

INTRODUCTION

Lily is a widely grown bulb crop as cut flower, pot and garden plant. There are more than 100 species in the genus *Lilium*. Among them, only limited materials belonging to few species are used for commercial breeding. Section Archelirion (Oriental hybrids), Sinomartagon (Asiatic hybrids) and Leucolirion (Longiflorum hybrids) are most frequently used for interspecific hybridization and commercial breeding. Interspecific hybridization techniques have been applied for the new cultivars breeding not only between intra-sectional but also inter-sectional species. Like in several plant genera, interspecific hybridization of lily has played a key role in the creation of modern cultivars. Integrated techniques such as cut style pollination and embryo rescue methods are developed for the

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introgression breeding to produce new type of flower color, forms and especially disease resistant cultivars (Van Tuyl and Van Holsteijn, 1996).

Unfortunately, the most described feature of interspecific lily hybrids (2n=2x=24) between widely related species is their sterility. This classically includes genic sterility (negative combinations between parental genes), chromosomal sterility (lack of structural homology disturbing meiotic pairing), and cytoplasmic sterility (unfavorable interactions between nuclear genes and foreign cytoplasm). In most cases, the sterility is caused by anomalous chromosome pairing, centromere division, spindle formation or cytokinesis during micro- and mega-sporogenesis that show irregular meiotic division such as unbalanced segregation of homoeologous chromosome (a counterpart chromosome of different species) sets during anaphase I and lagging chromosomes during anaphase I and II on the equatorial plane or irregular cytokinesis resulted in different number of chromosomes in daughter cells. F_1 interspecific hybrids also often show mismatches in spindle attachment to the homoeologous chromosomes, in movement of homoeologous chromosomes into both poles, and between cytokinesis and chromosome movement in anaphase I. It generally assumed that the successive type of cytokinesis is more likely to disturb the division of chromosomes and cell wall formation than in simultaneous type during meiosis.

In general, 70% of angiosperms have undergone polyploidization at least once. It is assumed that 2n-gametes mainly contributed for the origin of natural polyploids (Harlan and de Wit, 1975). Some interspecific lily hybrids produce relatively high frequencies of numerically unreduced (2n) gametes (Asano, 1984; Van Tuyl et al., 1989; Lim et al., 2001). Such hybrids offer the prospect of using the diploid hybrid genotypes directly for introgression breeding without the need of mitotic chromosome doubling. In lily, this possibility has already been demonstrated through chromosome analysis of some progenies derived from the functioning of 2n-gametes (Karlov et al., 1999; Lim et al., 2001, 2003, 2004).

Each meiotic event leading to 2n-gametes is genetically equivalent to either first-division restitution (FDR) or second-division restitution (SDR) mechanisms (Peloquin, 1983), or recently described indeterminate meiotic restitution (IMR) mechanism (Lim et al., 2001). In lily, most of unreduced gametes are produced by FDR, and very rarely by SDR and IMR (Lim et al., 2001, 2003, 2004). Generally, in monocots the first cytokinesis occurs at the end of first meiotic division and the second cytokinesis takes place after second meiotic division (successive cytokinesis type). However, in the dicots, the first and the second cytokinesis take place simultaneously at the same time at the end of meiosis (simultaneous cytokinesis type). Therefore, in a monocot like lily, if the former or the latter cytokinesis is omitted, unreduced gametes can be produced (Pagliarini et al., 1999).

The recovery of F_1 fertility in distantly related interspecific hybrids by chemical treatment was hitherto achieved through somatic chromosome doubling. However, in this case there are several disadvantages such as time and labor consumption, less or no homoeologous recombination between parental species (Lim et al., 2000, 2001). The necessity of direct chromosome doubling without spending time and labor was required for the practical breeding. Theoretically, spindle fiber inhibitors such as colchicine and cytokinesis inhibitor like caffeine can be used *in situ* to the dividing meiocytes (Park et al., 1990). Those chemicals may show the same behaviour as mitotic chromosome doubling to produce unreduced gametes. Therefore the aim of this research was to restore F_1 fertility directly from reproductive cells for inducing meiotic polyploidization in lily through injection of caffeine into the flower buds.

MATERIALS AND METHODS

Plant material and caffeine treatment

The F_1 interspecific hybrid (OA-hybrid=951301, 2n=2x=24) was obtained by crossing the Oriental and Asiatic cultivars 'Mero Star' and 'Connecticut King', respectively. Bulbs of the F_1 hybrid were planted in a greenhouse and grown in standard condition for lily growth and development. When flower buds emerged, upper part of the flower buds was cut and injected caffeine solution to the flower buds until solution filled up to the top of flower bud and sealed to avoid evaporation.

In a preliminary experiment, Caffeine (1,3,7-Trimethylxanthine, Sigma cat. # C0750) solution was injected at different flower bud stages and concentrations to define the best size and concentration (data not included). After injection the plants were kept at the

Table 1. Parentage of materials used in this experiment.

Accession number	Type of crosses	Parentage	
		Female	Male
951301	**O × A**	Oriental hybrid 'Mero Star'	Asiatic hybrid 'Connecticut King'
002684	**OA × A**	951301	Asiatic hybrid 'Lanzarote'
002687	**A × OA**	Asiatic hybrid 'Lanzarote'	951301

same growing condition.

Pollen germination test and embryo rescue

It has been confirmed that both male and female gametes from **OA**-hybrids are absolutely sterile by back crossing to Asiatics and pollen germination tests of more than 500 flowers per plant (clone) for 4 years. Mature pollen was placed on the artificial agar medium containing 100 g sucrose, 5 g agar and 20 mg boric acid per litre. After 12 hrs culture at 20°C the germinated pollen was counted and classified as large (2n) and small (n).

The fertile pollen was reciprocally pollinated to Orientals and Asiatics parent species, respectively. About 45-50 days after pollination embryos were rescued *in vitro* and cultured on MS (RM-1962) medium. The embryo rescue method has been described earlier (Van Tuyl, 1997).

Chromosome preparation and GISH

Root tips from BC_1 progeny were harvested in a saturated α-bromonaphthalene solution during early morning and kept overnight at 4°C for accumulation of the metaphase cells. The next morning, the material was fixed in ethanol-acetic acid solution (3:1) for at least 2 hrs following washing with distilled water three times and stored in 70% ethanol solution at -20°C until use. Root tips were washed with distilled water three times and treated with a pectolytic enzyme mixture (0.3% Pectolyase Y23, 0.3% Cellulase RS and 0.3% Cytohelicase) in 10 mM citric acid buffer at 37°C for about 1 hr and squashed in a drop of 60% acetic acid solution. Chromosome nomenclature of *Lilium* species was followed as Stewart (1947).

GISH protocol was basically the same as Lim et al. (2001). Briefly, sonicated genomic DNA (1~10 kb) from *L. Orientals* 'Acapulco' was used as probe after labeling with digoxigenin by nick translation according to manufacturer's instruction (Boehringer Mannheim, Germany). Sheared herring sperm DNA was used for blocking the non-hybridised DNA sequences. After detection steps, slides were counterstained with 5 μg/mL propidium iodide (PI). Images were photographed with a Zeiss Axiophot microscope equipped with epi-fluorescence illumination and single band filters for FITC and Cy3/PI using 400 ISO color negative film. The film was then scanned at 1200 dpi using HP film scanner and the contrast and color balance was adjusted in digital processing software program 'Photoshop version 5.5' (Adobe Inc. USA).

RESULTS

Optimum concentration of caffeine solution

Different concentrations of caffeine were injected into flower buds and the pollen grains were used for crossing following pollen germination test. The shape of most pollen from untreated flowers was shrunken and empty. However, some parts of pollen grains in caffeine treated flowers were aggregated as clusters and were able to germinate(Fig. 1A). Aggregated pollen showed high germination ability (54.6%) mainly from large (2n) pollen. However, most of the normal looking pollen did not germinate.

More than 260 **A OA** progeny plants were obtained by pollinating 45 flowers of Asiatic cultivar with the pollen of caffeine treated **OA**-hybrid. However, only 19 **OA A** plants were produced from 97 flowers crossed with **OA** as female and Asiatic hybrid 'Lanzarote' as male. Flowcytometric measurement of the BC_1 progenies confirmed that they were all triploids (2n=3x=36).

Optimum stage of caffeine injection

There were two peaks in embryo formation, which indicate that the egg cell had recovered its fertility by caffeine treatment otherwise no fertility. The optimum injection stage was confirmed 20-23 mm and 34-37 mm

Table 2. Number of plants derived from the crossing of **OA**-hybrid following 0.3% caffeine treatment to the different size of flower buds.

Accession	Flower bud length (mm)	Number of			
		Flowers pollinated	Ovary survival	Embryos formatted	Progeny
002684	Control	15	7	0	0
	16 - 19	14	7	1	1
	20 - 23	38	17	11	4
	24 - 27	7	2	2	0
	30 - 33	13	3	0	0
	34 - 37	12	4	4	4
	38 - 41	11	5	2	1

of flower bud length (Table 2). The male meiosis stage of the first peak (flower bud length=20-23 mm) and second peak (34-37 mm) were known as before and after meiosis. Hence, meiotic polyploidization of two peaks was considered from pre- and post-meiotic doubling. There are, in general, serious cross incompatibility between Oriental as female and **OA**-hybrid as male or reciprocal combination as well. In this study, any case of backcrosses couldn't overcome cross incompatibility between F_1 **OA**-hybrid '951301' and **O**-genome or reciprocally. Therefore, we made only backcrosses F_1 **OA**-hybrid with Asiatics (**A**-genome) pollen as a counterpart.

GISH analysis

GISH analysis was carried out for 11 **A OA**-hybrids (002687-1~-11) and confirmed that **OA** F_1 hybrid produced functional gametes with unreduced chromosome number (2n=24). However, there was no homoeologous recombination between the parental chromosomes of **O**- and **A**-genomes during microsporogenesis. On the other hand, five plants from **OA A** cross analyzed by GISH confirmed that **OA**-hybrid transmitted unreduced female gamete to subsequent generation. Three out of five analyzed through GISH showed a range of homoeologous recombinations between parental chromosomes (Table 3; Fig. 1B, C). In the case of '002684-4', two Asiatic chromosomes (Chr #2 and #10) had homoeologous recombinations (arrow-heads in Fig. 1B). Due to its genome composition (**A**=24, O=12), '002684-4~-6' showed the same number of chromosomes, i.e. a set of

Table 3. Genome analysis of progenies derived from backcrosses of **OA** following caffeine treatment.

Genome	Accession number	Total chromosome numbers (2n=3x)	From female		From male	
			O (**O**/**A**)[z]	**A** (**A**/**O**)[z]	**O** (**O**/**A**)[z]	**A** (**A**/**O**)[z]
A OA	002687-1~-11	36	-	12	12 (0)	12 (0)
OA A	002684-1	36	12 (0)	12 (0)	-	12
	002684-3	36	12 (0)	12 (0)	-	12
	002684-4	36	12 (1)	12 (1)	-	12
	002684-5	36	12 (2)	12 (2)	-	12
	002684-6	36	12 (0)	12 (1)	-	12

[z]**O** and **A** represent Orientals and Asiatics chromosomes. (**O**/**A**) and/or (**A**/**O**) indicate Oriental chromosomes recombinant with Asiatic chromosome segment(s) and/or Asiatic chromosomes recombinant with Oriental chromosome segment(s).

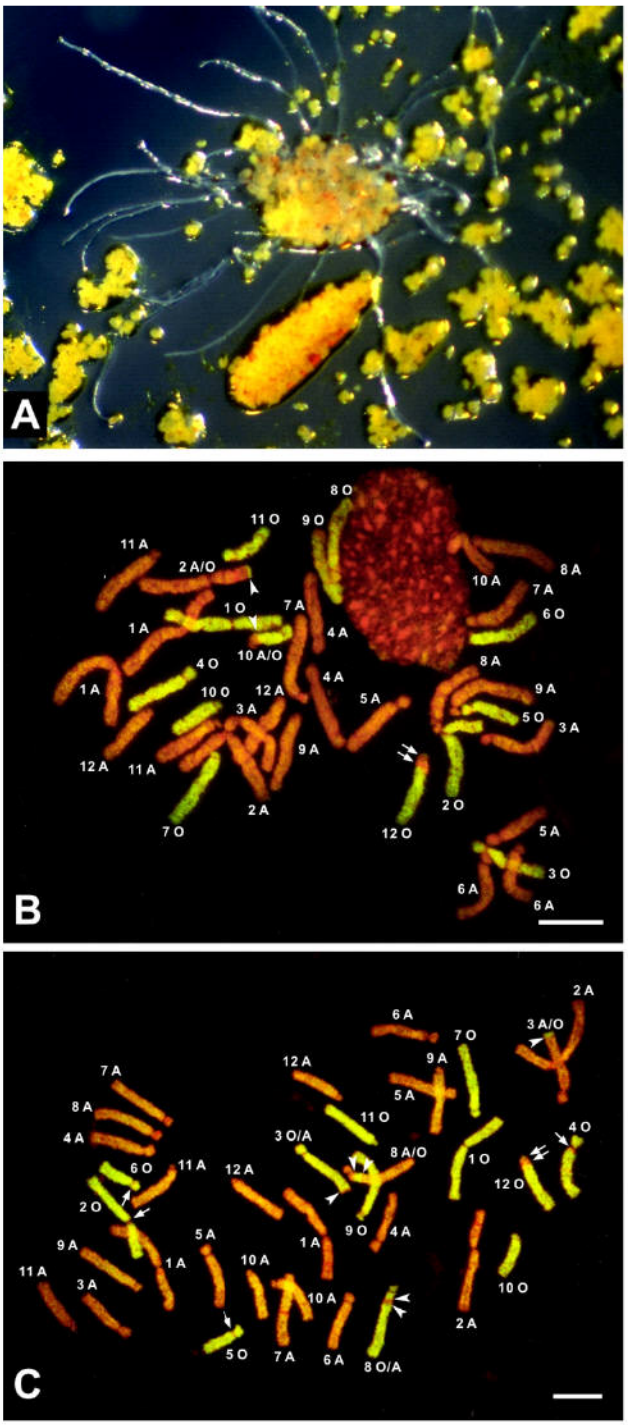

Figure 1. (A) Pollen germination of **OA** intersectional hybrid '951301' following caffeine treatment. Viable pollen was formed in the aggregated pollen, from which germination was high enough for the backcrossing. (B) GISH analysis of triploid (**OA A**, 2n=3x=36) BC_1 plant '002684-4' composted of 24 Asiatics chromosomes (**A**-genome, red fluorescence) and 12 Orientals chromosomes (**O**-genome, green fluorescence). Two Asiatic chromosomes have homoeologous recombination between Oriental (green) and Asiatic (red) chromosomes (arrow-heads). Large domain of rDNA and heterochromatin region is arrowed on 12O (Oriental chromosome #12). (C) Two reciprocal recombinations (arrow-heads) between **O-** and **A-**genomes were occurred during megasporogenesis by FDR mechanism. Arrows indicate ribosomal DNA positions of Oriental chromosomes. Letters in all cases represent putative chromosome number by chromosome nomenclature of *Lilium* (Stewart, 1947). **O/A** and **A/O** represent Oriental chromosome with Asiatics chromosome segment and Asiatics chromosome with Orientals chromosome segment, respectively. Bar=10 μm.

Oriental and Asiatic chromosomes were transmitted from parental species (**O** and **A**) indicating FDR 2n-gamete was functional when fertilized. Two pairs of reciprocal homoeologous recombinations were observed in four chromosomes (arrow-heads in Fig. 1C). Interestingly, there were two reciprocal homoeologous recombinations; e.g. one had a cross over at the end of long arm of chromosome #3 and the other had two breakpoints in proximal part of the long arm of chromosome #8 (arrow-heads in Fig. 1C). GISH technique successfully distinguished the breakpoints of homoeologous recombination between parental species, which occurred during megasporogenesis.

DISCUSSION

Allopolyploids are important for ornamental plant breeding such as lily. In lily, numerous **OA**-hybrids were made by intersectional hybridization (Barba-Gonzales et al., 2004). As was reported in a previous paper (Lim et al., 2000), mitotic chromosome doubling is a method to recover their fertility in intersectional hybrids. However, in this case there are hardly any homoeologous recombinations between the parental chromosomes (Lim et al., 2000). Homoeologous recombination is a prerequisite for the introgression of alien genes and/or chromosome segment(s) into a recipient species through interspecific hybridization. The BC_1 plants derived from crosses with amphidiploid of intersectional hybrid are normally triploid without any homoeologous recombinations. Such triploid BC_1 plants would be sterile and further crossing will be either very difficult or impossible (Lim et al., 2000). However, triploid BC_1 plant derived from 2n-gametes producing diploid of intersectional hybrid often have some amount of fertility of both male and female gametes (Lim et al., 2003).

Generally, several events of homoeologous recombination occur during the process of meiotic polyploidizations of **LA**-hybrids (Lim et al., 2001, 2003). However, we have seen that there is very low meiotic polyploidization in **OA**-hybrids among several thousand of plants for many years. The meiotic configuration of the **OA**-hybrids showed very low allosyndetic pairing, which indicates that the genetic distance of parental species is wide (unpublished data). It seems that low allosyndetic pairing decrease the formation of unreduced gametes in distantly related interspecific hybrids. Therefore fertility may be reduced by meiotic abnormalities.

There are three possible explanations of how caffeine might affect the chromosome numbers in the spores and gametes. Firstly, caffeine reacted before prophase I chromosomes. In this case the consequence of meiocyte may have no homoeologous recombination between parental chromosomes like in the premeiotic doubling or mitotic chromosome doubling of distantly related hybrid. Secondly, caffeine reacted at the metaphase I to anaphase I. Forced allosyndetic chromosome association between parental chromosomes is expected as has been reported by Lim et al. (2001). Because caffeine inhibits cytokinesis formation at the stage of cytokinesis I, viable 2n-pollen can be produced at the end of meiosis (Samuels and Staehelin, 1996; Staehelin and Hepler, 1996). The result of this case is FDR unreduced gametes with homoeologous recombination. Thirdly, caffeine may affect at the end stage of micro- or mega-sporogenesis. The consequence of this case is the same result as post meiotic doubling (Bastiaanssen et al., 1998). Terasaka and Niitsu (1987) reported the formation of bi-nucleate cell through *in vitro* culture of pollen grain in the caffeine added medium. Such effect can be used for the mitotic chromosome doubling instead of colchicine or oryzalin treatment (Thomas et al., 1997; Chung et al., 2003).

In this experiment caffeine solution was injected into flower buds comprising male and female organs. Because intersectional **OA**-hybrid possesses low chromosomal homology and therefore disturbed chromosome movement during meiosis (Barba-Gonzalez et al., 2004), more than 300 flowers produced all sterile pollen for four years' test. **OA**-hybrid flowers injected by caffeine produced a range of viable pollen tested by lactophenol fuchsin staining. Otherwise **OA**-hybrid produces absolutely sterile pollen. In such cases, interspecific and/or intergeneric hybrids are highly advantageous to screening the fertile hybrid individual by simply checking pollen viability (Shamina et al., 1999; Lim et al., 2001, 2003). Female gametes were fertilized with Asiatic haploid male gametes and produced three BC plants (**OA A**) with homoeologous recombinations between parental species chromosomes (Table 3).

There are two types of cytokinesis, simultaneous and successive types. Cytokinesis in most dicotyledons does not occur until after the four nuclei are formed (simultaneous cytokinensis). However, in monocotyledons the cytokinesis occurs two times separately after the first and the second meiotic divisions (successive cytokinesis). Caffeine is known to inhibit the cytokinesis of the dividing cells (Valets and Hepler, 1997). *In vitro* caffeine treatment to the pollen grain of monocotyledons '*Pinus densiflora*' increased the formation of binucleate chromatin (Pagliarini et al., 1999). Only few studies for the somatic chromosome doubling using caffeine are hitherto reported in wheat (Thomas et al., 1997) and lily (Chung et al., 2004). We have here attempted for the first time to produce 2n gametes from sterile interspecific hybrid through caffeine injection to immature flower bud. Assuming the effect of caffeine, as Shamina et al. expected (1999), the

homoeologous recombinations shown in our GISH results had occurred during meiosis I and omitted cytokinesis I giving rise to equal number of parental chromosomes (12 Asiatic lily chromosomes and 12 Oriental lily chromosomes) after normal cytokinesis II (Fig. 1B, C). There are several advantages for the induced meiotic polyploidization; Firstly, sterile F_1 interspecific or intergeneric hybrids can be used directly for the subsequent crossing without mitotic chromosome doubling of F_1 hybrids to overcome F_1 sterility which is time consuming work. Secondly, higher chances of homoeologous recombination between parental species are expected as shown in spontaneously produced unreduced gametes (Lim et al., 2003) rather than in somatically chromosome doubled F_1 hybrids (Lim et al., 2000). Thirdly, more wide range of F_1 hybrid materials can be used to produce 2n gametes. Spontaneously 2n gametes producing individuals are limited in F_1 interspecific hybrids, only few plants out of thousands may produce unreduced gametes in some plant taxa. The use of 2n gametes allows breeding at the diploid level, which makes it possible to achieve highly heterotic combinations of genes - whether they are due to multiple allelic interactions or to accumulate of favorable linkages more rapidly than would the breeding at the tetraploid level (McCoy and Walker, 1984; Kim and Seo, 1991). This experiment successfully demonstrates the possibility of direct recovery of fertility of the sterile intersectional lily hybrid by caffeine treatment.

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