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Cytogenetic studies on meiotic chromosome behaviors in sterile Oriental x Trumpet lily

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ABSTRACT. In order to determine the reasons for pollen sterility in lily hybrids, four diploid sterile Oriental x Trumpet (OT) lily cultivars ('Nymph', 'Gluhwein', 'Yelloween', and 'Shocking') were used to investigate the meiotic chromosome behaviors in pollen mother cells (PMCs), using genomic *in situ* hybridization and conventional cytological methods. At metaphase I, chromosome associations were quite variable, not only among different genotypes but also in different PMCs of the same genotype. In addition to bivalents, a certain amount of univalent, trivalents, and quadrivalents were observed in all of the investigated genotypes. In addition, ring octavalents and ring hexavalents were observed in 'Nymph'. Even dodecavalents were observed in 'Nymph'. These abnormal chromosome associations at metaphase I implied the occurrence of chromosome interchanges (translocation) in these intersectional hybrids. At anaphase-telophase, a large number of laggard chromosomes and different kinds of chromosome bridge configurations were observed. At the tetrad stage, micronuclei and polyads were also found in many PMCs. All of these

abnormal chromosome behaviors in PMCs were responsible for the pollen sterility in lily hybrids.

Key words: Pollen sterility; Meiosis; Chromosome behaviors; *Lilium*

INTRODUCTION

The genus *Lilium* comprises about 100 species, and all of them are dispersed in the Northern Hemisphere (McRae, 1998). The diversity of flower color, shape, fragrance, and other phenotypic and physiological characteristics are found in the wild species. Based on 15 morphological characteristics, they are classified into seven taxonomic sections: *Lilium*, Martagon, Pseudolirium, Archelirion, Sinomartagon, Leucolirion, and Oxypetalum (Comber, 1949; De Jong, 1974). Each section has its own unique set of horticultural traits. It is desirable to combine or introgress valuable horticultural traits from different lily genomes (sections) into cultivars through intersectional hybridization. However, hybridization between the cultivars or species from different taxonomic sections in the genus *Lilium* is generally difficult because of pre- and post-fertilization barriers (Van Tuyl et al., 2002). In the past few years, owing to the development of pollination and embryo rescue techniques (Asano and Myodo, 1977a,b; Asano, 1978, 1980; Van Tuyl et al., 1991), a range of intersectional hybrids such as LA (Longiflorum x Asiatic), OT (Oriental x Trumpet), LO (Longiflorum x Oriental), and OA (Oriental x Asiatic) involving distantly related cultivars/species have been cultivated. These intersectional hybrids possess many unique horticultural characteristics that their parents do not have. However, the pollen of these intersectional hybrids is usually sterile and cannot be used for further cross-breeding. Therefore, it is valuable to investigate the meiotic development in pollen mother cells (PMCs) to determine the reasons for the pollen sterility in lily hybrids.

Genomic *in situ* hybridization (GISH) is a powerful and effective molecular cytogenetic tool, which has been successfully used in *Lilium* to discriminate parental genomes of the F_1 interspecific hybrids and to detect intergenomic recombinant chromosomes in the BC_1 and BC_2 progenies (Karlov et al., 1999; Lim, 2000; Barba-Gonzalez, 2005). Therefore, it can be an ideal technique to investigate homologous chromosome pairing and other meiotic chromosome behaviors in distant *Lilium* hybrids.

In the present study, four sterile OT lily cultivars were investigated for their meiotic chromosome behaviors during microsporogenesis with conventional cytological methods and the GISH technique. A number of aberrant meiotic chromosome behaviors were detected and analyzed.

MATERIAL AND METHODS

Plant material

Four diploid sterile F_1 OT lily cultivars ('Nymph', 'Gluhwein', 'Yelloween', and 'Shocking') were used to investigate the homologous chromosome behavior during microsporogenesis.

All the cultivars were supplied by a Dutch lily breeding company. They were grown in a greenhouse following standard growing conditions applicable for lily cultivation.

Chromosome preparation with anthers

Young anthers at the prophase I to telophase II stages were collected from different sized flower buds and fixed in Carnoy's solution (1:3 acetic acid-ethanol) for 24 h at room temperature. After fixing, the anthers were cut in the middle, and PMCs were spread on the slide; then, 16 µL 2% aceto-carmine was swiftly added and gently mixed with the PMCs. The slide was covered with a square plastic cover, pressed gently with the thumb, and finally examined under a light microscope (Carl Zeiss, West Germany). Anthers at the proper meiotic stage were incubated in 10 mM citrate buffer, pH 4.5, containing 1% cellulase RS (w/v), 1% pectolyase Y23 (w/v), and 1% cytohelicase (w/v), for 60 to 70 min at 37°C. A drop of 60% acetic acid was immediately added to the meristem and mixed gently with a needle, and then smeared for several seconds on a slide heater. The slides were then washed with 3:1 ethanol-acetic acid (v/v) and air-dried. The best slides that had good chromosome spreads were selected under a light microscope for GISH analysis.

GISH

Total genomic DNA was extracted from leaves. Before labeling, the total genomic DNA was sonicated to 0.5 to 5 kb and used as a probe. Oriental DNA was labeled with digoxigenin-11-dUTP and Trumpet DNA was labeled with biotin-16-dUTP by nick translation according to the manufacturer protocols (Roche Diagnostics GmbH, Mannheim, Germany).

In situ hybridization was carried out according to Lim (2000). In short, the hybridization mixture contained 2 x SSC, 50% formamide, 10% sodium dextran sulfate (w/v), 0.25% sodium dodecyl sulfate (w/v), and 3.0 ng/µL labeled probe DNA. The hybridization mixture was denatured in water bath at 72°C for 10 min and cooled on ice for 10 min. For each slide, 40 µL hybridization mixture was added, followed by denaturing at 80°C for 5 min and incubating overnight at 37°C in a humid chamber. After hybridization, the slides were washed twice in 0.1 x SSC [including 10% formamide (v/v)] at 42°C for 5 min each time. Biotin was detected using the Cy-3-conjugated streptavidin-antistreptavidin system (Vector Laboratories, UK). Digoxigenin was detected by using the FITC-antidigoxigenin system (Boehringer, Mannheim, Germany). The chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma, US) in Vectashield (Vector Laboratories, UK). Preparations were analyzed using an epifluorescence microscope (Carl Zeiss) and photographed with a digital camera (Canon, Japan) attached to the microscope.

RESULTS

Chromosome associations at diakinesis-metaphase I

Using the GISH technique and traditional cytogenetic method, the frequencies and configuration of the chromosome associations in the four sterile intersectional hybrids were observed, as shown in Table 1. Some pictures of the chromosome associations are showed in Figure 1. The chromosome associations were quite variable, not only among different genotypes but also in different PMCs within the same genotype. The mean frequency of bivalents per cell was highest (8.9) in 'Nymph.' The lowest frequency of bivalents per cell (3.0) was observed in 'Shocking'. Usually, bivalents were formed involving the homologous chromosomes (Figure 1A, B, and C). There were also instances of bivalents resulting from non-homologous association of two

chromosomes in the same genome (Figure 1D). In addition to univalents and bivalents, a certain amount of trivalents and quadrivalents were observed in all of the investigated genotypes (Figure 1E and F). Besides these, ring octavalents and ring hexavalents were observed in 'Nymph' (Figure 1G and Figure 2A). Even dodecavalents were observed in 'Nymph' (Figure 1H). All of these abnormal chromosome associations at diakinesis-metaphase I implied the occurrence of chromosome interchanges (translocation) in these intersectional hybrids. Specifically, the occurrence of ring octavalents and hexavalents indicated that cyclical translocation must take place in this genotype. This is the first report on cyclical translocation in *Lilium*.

Table 1. Chromosome association at diakinesis-metaphase I in F_1 Oriental x Trumpet lily.

Genotype	No. of cells analyzed	Chromosome association				
		Univalent	Bivalent	No. of bivalents per cell	Trivalent	Quadrivalent
'Gluhwein'	140	1548	852	6.4 (3-9)	20 (14.3%)	12 (8.6%)
'Shocking'	135	2418	361	3.0 (2-5)	12 (8.9%)	16 (11.9%)
'Nymph'*	181	1152	1556	8.9 (7-12)	4 (2.2%)	16 (8.8%)
'Yelloween'	128	2016	502	4.1 (2-6)	6 (4.7%)	8 (6.3%)

*Dodeca-valent and ring octavalent were also observed in this genotype.

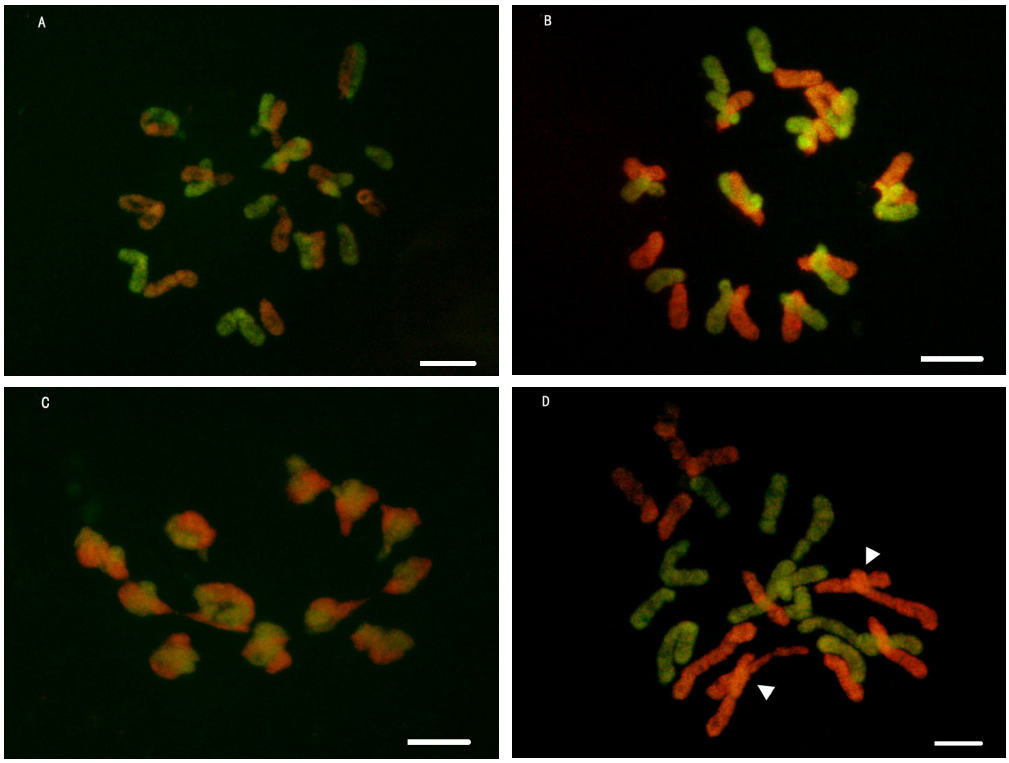

Figure 1. Chromosome association in sterile Oriental x Trumpet lily. **A.** Chromosome pairing in 'Gluhwein' (7 II+10I); **B.**, **C.** chromosome pairing in 'Nymph' (9 II+6I, 12 II); **D.** non-homologous chromosome pairing in 'Gluhwein' (arrow); **E.** trivalent in 'Gluhwein' (arrow); **F.** quadrivalent in 'Shocking' (arrow); **G.** ring octavalent 'Nymph'; **H.** dodeca-valent in 'Nymph'. In all cases, green chromosomes represent Oriental lily chromosomes and red chromosomes represent Trumpet lily chromosomes. Bar = 10 μm.

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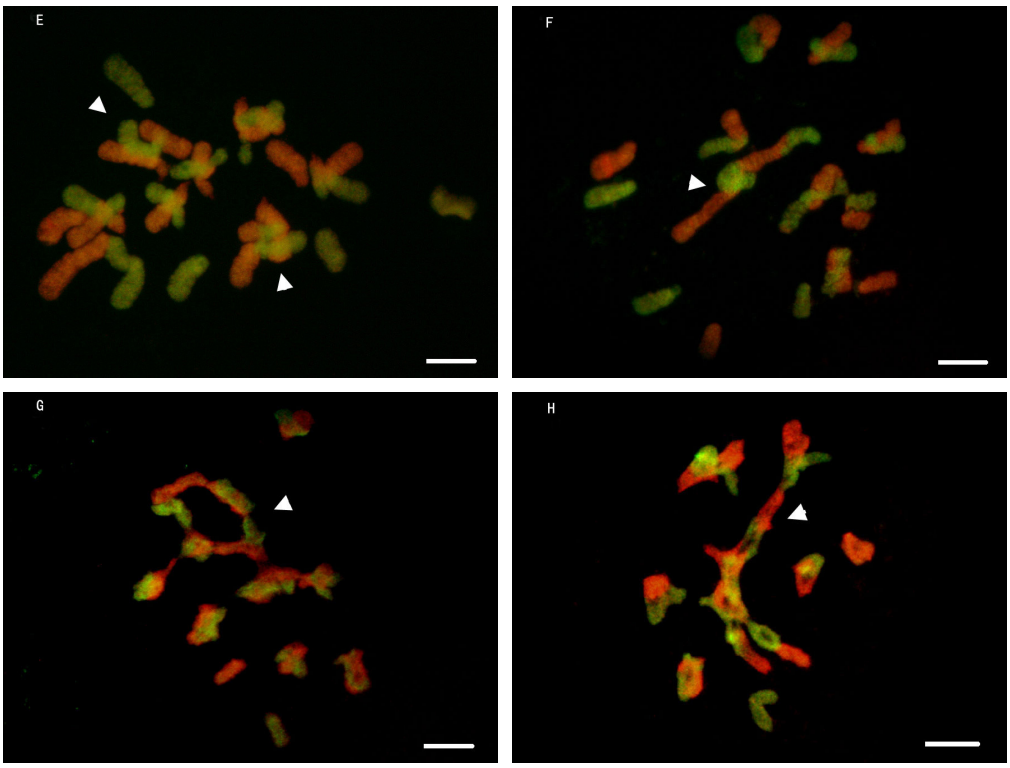
Figure 1. Continued.



Chromosome behaviors at anaphase I-telophase I

More than 260 cells at anaphase I-telophase I were checked using the conventional staining method for each cultivar. A large number of abnormal chromosome segregations were observed (Table 2). Among these abnormal chromosome behaviors, laggard chromosome (Figure 2B) was the most common, which occurred in all the investigated genotypes. The percentage of lagging chromosome varied from 54 (‘Nymph’) to 90% (‘Shocking’). Besides the lagging chromosomes, anaphase bridges were also common in these four interspecific hybrids. In the cases of ‘Gluhwein’ and ‘Nymph’, 29 and 24% PMCs had chromosome bridges, respectively. Compared with ‘Gluhwein’ and ‘Nymph’, the percentage of chromosome bridges in ‘Shocking’ and ‘Yelloween’ was relative lower (13 and 2%). Two different configurations of bridges were observed: one bridge and two bridges (Figure 2C and D). In this investigation, one bridge was the most frequent, and observed in all genotypes ranging from 2 (‘Yelloween’) to 26% (‘Gluhwein’). The configuration of two bridges was only observed in ‘Gluhwein’ and ‘Nymph’ with very low frequency (3 and 1%). Besides the one-bridge and two-bridge configurations, another special chromosome bridge, ‘thick bridge’, was also detected in some PMCs in ‘Shocking’, which is usually caused by chromosome stickiness involving many chromosomes or entire genomes (Figure 2E and F).

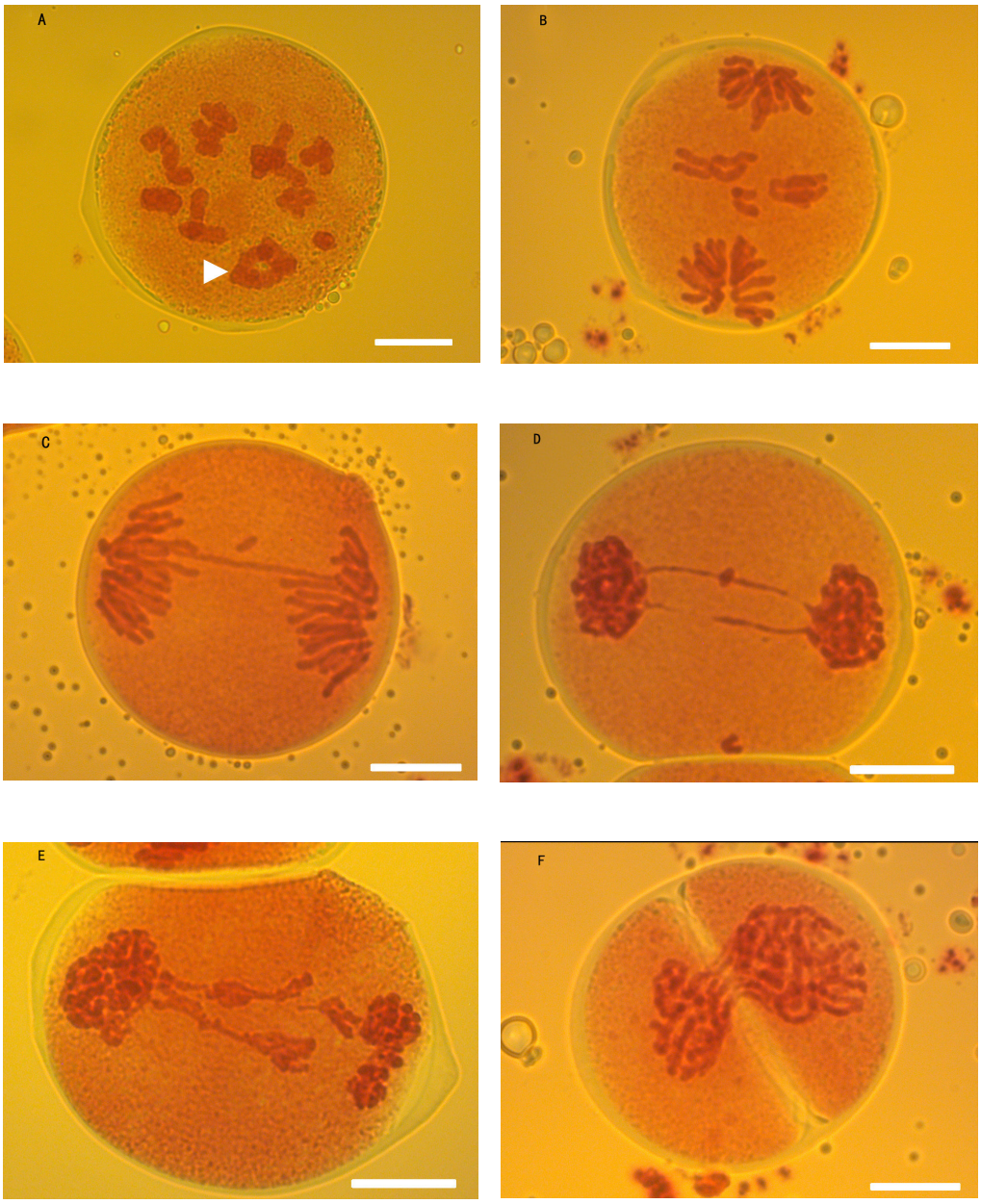


Figure 2. Abnormal meiotic chromosome behaviors in lily hybrids. **A.** Ring hexavalent; **B.** lagging chromosomes at anaphase I; **C.** one bridge-fragment; **D.** two bridges; **E. F.** thick chromosome bridge; **G.** lagging chromosome at anaphase II; **H. I. J.** anaphase II bridge; **K.** irregular orientation segregation; **L.** tetrad wih micronuclei; **M. N. O.** polyads with different numbers, size, and micronuclei; **P.** pollen grains of different sizes. **Q. R.** Scattered univalent in the cytoplasm. Bars = 20 µm.

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Figure 2. Continued.

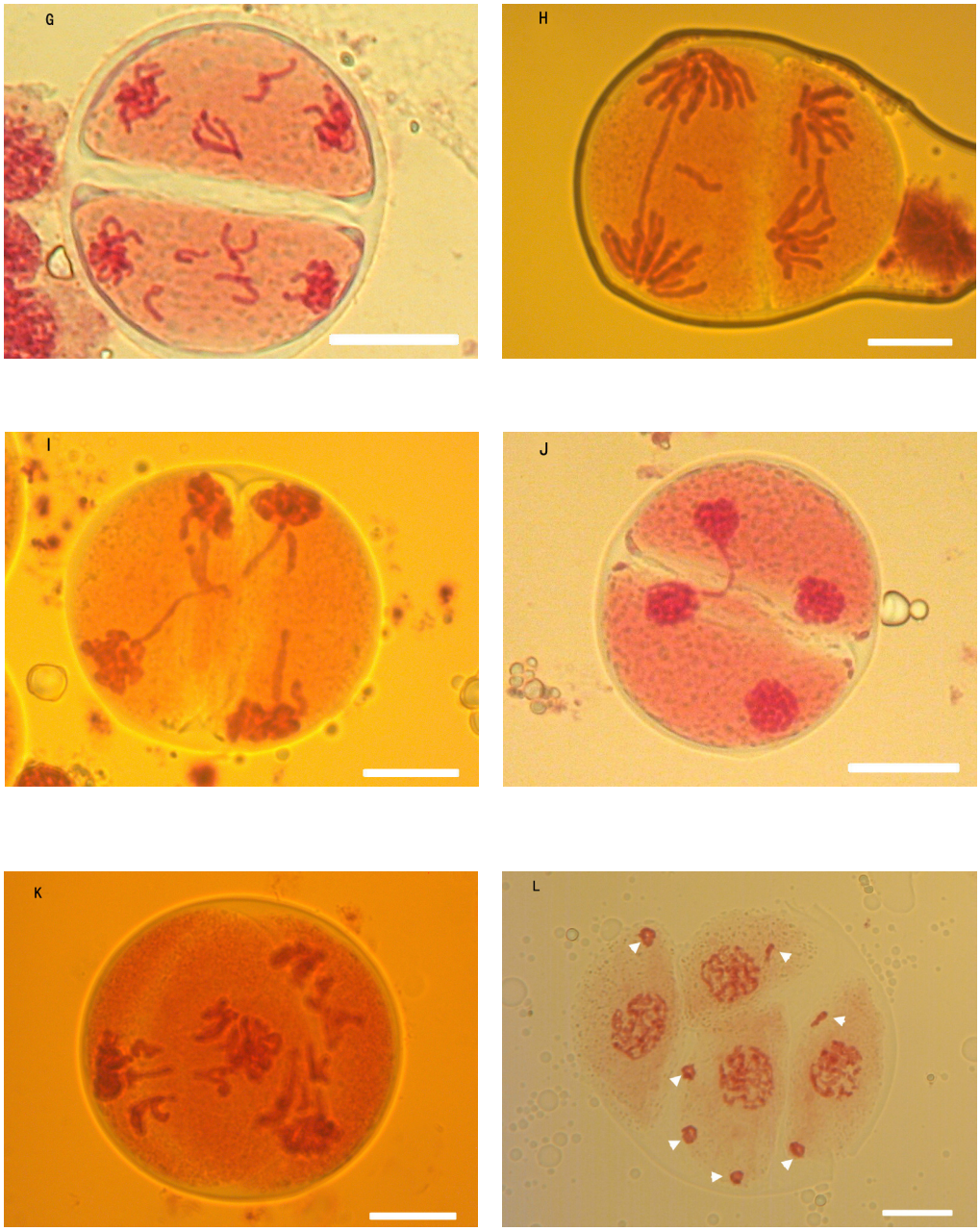

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Figure 2. Continued.

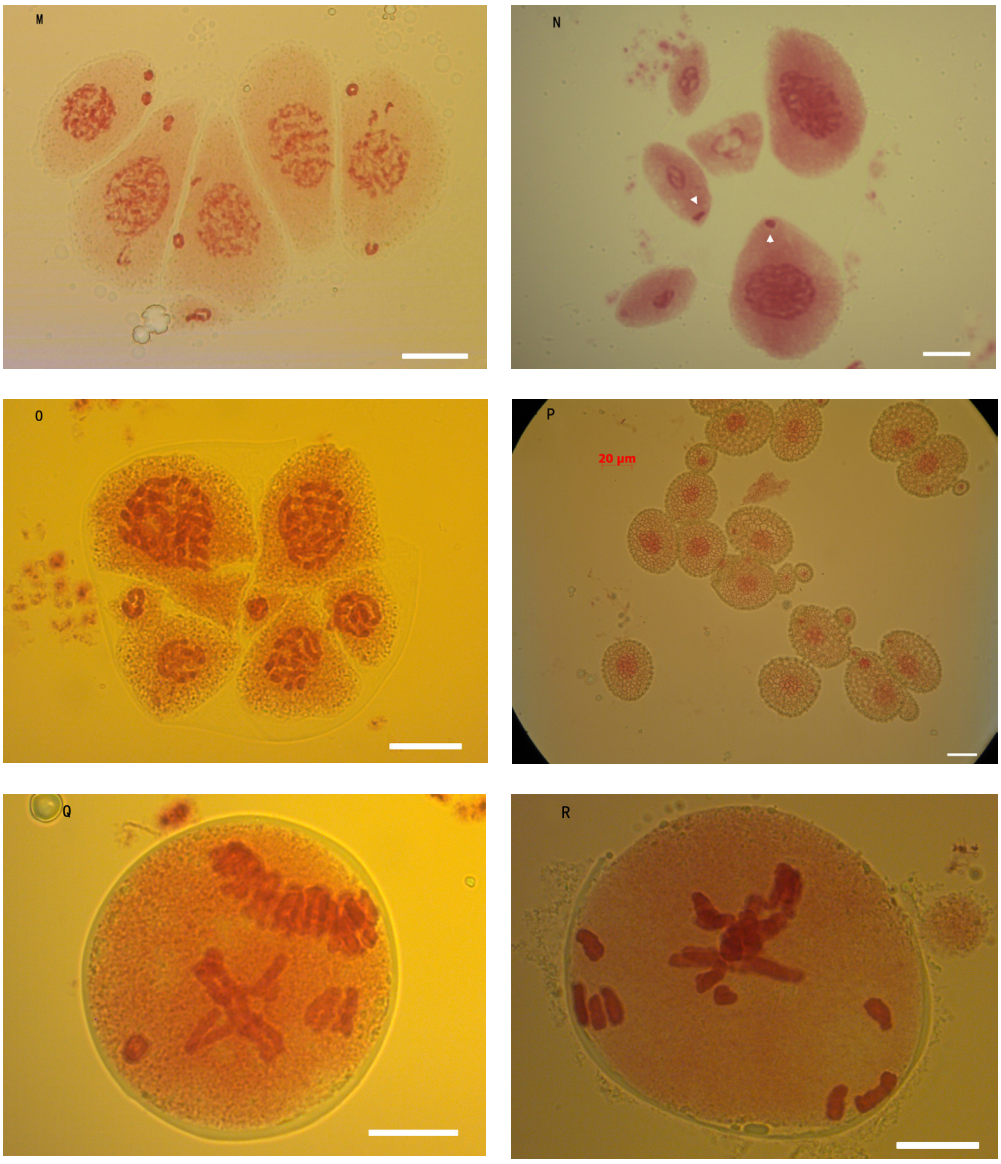

Table 2. Chromosome behaviors at anaphase I-telophase I in F_1 Oriental x Trumpet lily.

Genotype	No. of cells analyzed	PMCs with laggards	No. of PMCs with bridge	
			One bridge	Two bridges
'Gluhwein'	285	171 (60%)	73 (26%)	9 (3%)
'Shocking'	267	240 (90%)	35 (13%)	0
'Nymph'	274	147 (54%)	63 (23%)	3 (1%)
'Yelloween'	265	228 (86%)	5 (2%)	0

Chromosome behaviors at anaphase II-telophase II

At anaphase II, lagging chromosomes were also very common in these four genotypes (Table 3, Figure 2G). The percentage of laggards varied from 43 to 89%. Compared with anaphase I, chromosome bridge-fragments were relatively rare, but observed in some PMCs of 'Nymph', 'Shocking' and 'Gluhwein', and only the configuration of the one bridge-fragment was observed (Figure 2H, I, and J). In some PMCs, irregular chromosome segregation (Figure 2K) was also found.

Table 3. Chromosome behaviors at anaphase II-telophase II in F_1 Oriental x Trumpet lily.

Genotype	No. of cells analyzed	PMCs with laggards	PMCs with bridge and fragment
'Gluhwein'	218	128 (59%)	22 (10%)
'Shocking'	214	190 (89%)	6 (3%)
'Nymph'	210	90 (43%)	12 (6%)
'Yelloween'	207	180 (87%)	0

Chromosome behaviors at tetrad

At the tetrad stage, a remarkable feature in these four genotypes was the occurrence of a micronucleus in many PMCs (Table 4, Figure 2L, M, and N). The frequency of PMCs with a micronucleus varied from 31 ('Nymph') to 86% ('Yelloween'). In addition, polyads with different numbers and sizes of spores and pollen grains of different sizes were also observed in some cells (Figure 2O and P).

Table 4. Chromosome behaviors at tetrad in F_1 Oriental x Trumpet lily.

Genotype	No. of cells analyzed	Tetrad with micronuclei	Polyad
'Gluhwein'	310	163 (53%)	12 (4%)
'Shocking'	280	235 (84%)	28 (10%)
'Nymph'	300	93 (31%)	0
'Yelloween'	290	249 (86%)	23 (8%)

DISCUSSION

Chromosome pairing in hybrids is used to assess genomic relationships between species and provides an important starting point in alien chromosome introgression. Furthermore, the degree of differentiation between hybridizing taxa can be estimated by analyses of chromosome pairing behaviors and other meiotic abnormalities (Rieseberg et al., 2000). In the present study, many chromosome pairing configurations (univalent, bivalents, and multivalents) were observed in sterile diploid OT lily cultivars. In the case of 'Nymph,' the mean frequency of bivalents per cell was 8.9, which was the highest in these four genotypes. The number of bivalents varied from 2 to 5 per cell in PMCs of 'Shocking', and the mean percentage of bivalents per cell was 3.0%, which was the lowest in the investigated genotypes. Taking the abnormal chromosome behaviors in other meiotic stages into consideration, it indicated that the relationship of parents of 'Shocking' may be more remote than those of 'Nymph'. In addition, 12 bivalents per

cell were also observed in very few PMCs of 'Nymph' (Figure 1C), which usually occurs in fertile genotypes. It implied that some pollen grains of 'Nymph' may be fertile.

Pachytene loops are proof of the presence of inversions, but pachytene chromosomes are technically difficult or impossible to analyze in most organisms, whereas observation of bridge-fragment configurations has been extensively applied as indirect evidence for the presence of paracentric inversions. On the basis of this evidence, a large number of plants were reported to have inversions, such as *Aesculus* (Upcott, 1936), *Tulipa* (Upcott, 1937), *Fritillaria* (Frankel, 1937), *Zea mays* (Morgan, 1950; Ting 1965), barley (Kreft, 1968), *Agave* (Brandham, 1969), soybeans (Ahmad et al., 1977), and *Gasteria* (Brandham, 1977). However, cytological evidence from paired chromosomes suggests that inversion is not responsible for all bridge-fragment configurations. This category includes sister chromatid bridges and variation of fragment size (Newman, 1966). These configurations can be explained by U-type exchanges (Jones and Brumpton, 1971; Couzin and Fox, 1973). This is an indication that the inversion in plants may be overestimated by some researchers in the previous studies. In the present investigation, a sister chromatid bridge (anaphase II bridge) was observed (Figure 2H, I, and J). At the same time, most fragment sizes in the bridge-fragment configuration at anaphase I remained stable in this study. All of these indicated that not only inversion but also U-type exchange took place in these interspecific lily hybrids. Inversions and U-type exchanges are both chromosomal structural aberrations that will result in gamete sterility.

The spindle apparatus is normally bipolar, and its bipolar symmetry plays a crucial role in the alignment of metaphase chromosomes and their poleward movement during anaphase. Meiotic plant spindle are initiated from the chromosomes by self-assembly (Baskin and Cande, 1990) and then assembled around the mass of chromosomes, and initially appear as poorly organized, often multipolar structures (Yu et al., 1999). Multiple spindle poles are visible at early prometaphase I. During mid-prometaphase I, the multiple spindle poles become concentrated into two poles, and the typical bipolar meiotic spindle is complete at metaphase I (Suzuki and Tanaka, 1999; Kitamura et al., 2009). Multiple spindles are a meiotic abnormality in which more than one spindle occurs in meiosis. In some PMCs, not all the chromosomes congregated at a single metaphase plate in metaphase I. Some of them (univalent) were scattered in the cytoplasm or remained in several small groups (Figure 2Q and R). In this case, not only bipolar spindles formed, but several mini-spindles were also induced by chromosomes that scattered in the cytoplasm. Multiple spindles are usually associated with abnormal cytokinesis, which will lead to polyad formation (Tilquin et al., 1984). It is obvious that most of the microspores and the pollen grains formed from them do not contain a normal haploid set of chromosomes, and they are non-functional. Abnormal chromosome segregation, microspores with micronuclei, and polyad formation are the manifestations of this irregularity (Caetano-Pereira and Pagliarini, 2001). In the present study, irregular chromosome segregation (Figure 2K), microspores with micronuclei, and polyads were all observed (Figure 2L to O). All of these suggest that multiple spindles must have occurred in meiosis of the lily hybrids.

In some PMCs of 'Nymph', a certain percentage of chromosome stickiness was detected (Figure 2E and F). Chromosome stickiness is characterized by an intense clustering of chromosomes (Rao et al., 1990). Sticky chromosomes may result from the defective functioning of one or two types of specific non-histone proteins involved in chromosome organization, which are needed for chromatid separation and segregation. The altered functioning of these proteins will lead to stickiness of the chromosomes, which can cause chromosome aberrations

by the physical stretching and breaking of chromatids at the sticky sites (Gaulden, 1987). The phenotypic manifestation of stickiness may be highly variable, ranging from a mild phenomenon (Figure 2E), when only a few chromosomes of the genome are involved, to an intense phenomenon (Figure 2F) involving the entire genome complements (Consolaro and Pagliarini, 1996; Pagliarini et al., 2000). At the end, the pollen will be sterile because of the chromosome pyknosis (Pagliarini et al., 2000).

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