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Meiotic chromosome pairing in intergeneric hybrids of colchicaceous ornamentals revealed by genomic in situ hybridization (GISH)

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Abstract Diploid and triploid intergeneric hybrids obtained by crosses among *Gloriosa superba* 'Lutea' ($2n = 2x = 22$), *G.* 'Marron Gold' ($2n = 4x = 44$), *Littonia modesta* ($2n = 2x = 22$), and *Sandersonia aurantiaca* ($2n = 2x = 24$) were analyzed for their meiotic chromosome pairing in pollen mother cells by genomic in situ hybridization (GISH) with digoxigenin-labeled total DNA of one parent as probe. Chromosomes from each parent could be clearly distinguished in pollen mother cells of all the five intergeneric hybrids by GISH. For three diploid hybrids, *L. modesta* × *G. superba* 'Lutea' ($2n = 2x = 22$), *L. modesta* × *S. aurantiaca* ($2n = 2x = 23$) and *S. aurantiaca* × *G. superba* 'Lutea' ($2n = 2x = 23$), 0.04−0.27 autosyndetic bivalents (intragenomic pairing of non-homologous chromosomes) and 0.13−0.36 allosyndetic bivalents (intergenomic chromosome pairing) were observed per pollen mother cell, indicating that there are some homologous chromosomal regions within each genome and among the genomes of *Gloriosa*, *Littonia* and *Sandersonia*. Differences in the average number of allosyndetic bivalents per pollen mother cell among different genome combinations may reflect the evolutionary distances among the three genera, and *Gloriosa* and *Littonia* may be closely related to each other, while *Sandersonia* may have relatively distant relationships with *Gloriosa* and *Littonia*. For two triploid hybrids, *L. modesta* × *G.* 'Marron Gold' ($2n = 3x = 33$) and *S. aurantiaca* × *G.* 'Marron Gold' ($2n = 3x = 34$), no allosyndetic bivalents were observed. Based on the results obtained in the present study, possible utilization of the diploid and triploid intergeneric hybrids for further breeding of colchicaceous ornamentals is discussed.

Keywords Allosyndetic bivalent · *Gloriosa* spp. · Intergenomic relationship · *Littonia modesta* · Meiosis · *Sandersonia aurantiaca*

Introduction

GISH analysis is a powerful tool for discriminating chromosomes from different genomes and allows identification of the genome constitution in allopolyploids (Lim et al. 2003; Pendinen et al. 2012) and wide hybrids (Ji et al. 2004; Marasek et al. 2004). In addition, GISH analysis is one of the most effective means to study intra- or intergenomic relationships by visualizing intra- or intergenomic chromosome pairing during meiosis of allopolyploids and wide hybrids. Cao et al. (2000) observed a high chromosome pairing affinity between *Lolium perenne* and *Festuca mairei* by GISH analysis and demonstrated that chromosomes

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of *Lolium* and *Festuca* may be genetically equivalent and reciprocal mixing of the genomes may be possible. Ge and Li (2007) investigated intragenomic chromosome homology in the B genome of *Brassica nigra* and their homoeology with chromosomes of the A genome of *B. rapa* and the C genome of *B. oleracea* in trigenomic triploid hybrids of different origins by GISH analysis and provided an evidence for the hypothesis that the three basic diploid genomes of the cultivated *Brassica* species evolved from one common ancestral genome with a lower chromosome number. Furthermore, Yao et al. (2010) analyzed trigenomic triploid hybrids among *B. juncea*, *B. carinata* and *B. maurorum* by dual-color GISH and concluded that intergenomic homoeology was higher than intragenomic homology in *Brassica*.

Gloriosa spp., *Littonia modesta* and *Sandersonia aurantiaca* are tuberous plants belonging to the family Colchicaceae and cultivated as ornamental plants because of their beautiful, unique flowers and good vase life (Nakamura et al. 2005). In order to obtain wide variability in horticultural traits and to develop novel cultivars, we have tried intergeneric hybridization among these plants and produced a number of hybrid plants in various combinations via ovule culture (Kuwayama et al. 2005; Nakamura et al. 2005; Amano et al. 2007, 2008, 2009). All the intergeneric hybrids were clearly distinguishable from the corresponding parents and had novel morphological characteristics, some of which were horticulturally attractive.

In a preliminary study, we examined chromosome behavior during meiosis in pollen mother cells (PMCs) of some diploid intergeneric hybrids among *G. superba*, *L. modesta* and *S. aurantiaca* by acetic-orcein staining (Amano 2008). Although a few bivalents were observed at metaphase I in all the hybrids, it is unclear whether these bivalents are autosyndetic (intragenomic pairing of non-homologous chromosomes) or allosyndetic (intergenomic chromosome pairing). Recently, Nakazawa et al. (2011) successfully applied GISH analysis for identifying the genome constitution of some intergeneric hybrids of colchicaceous ornamentals. In the present study, therefore, we analyzed meiotic chromosome pairing in diploid and triploid intergeneric hybrids by GISH in order to obtain some information on the intergenomic relationships among *Gloriosa*, *Littonia* and *Sandersonia*.

Materials and methods

Plant materials

The parents of intergeneric hybrids, *G. superba* 'Lutea' (Gsu; $2n = 2x = 22$), *G.* 'Marron Gold' (Gma; $2n = 4x = 44$), *L. modesta* (Lit; $2n = 2x = 22$) and *S. aurantiaca* (Sau; $2n = 2x = 24$), diploid intergeneric hybrids, *L. modesta* × *G. superba* 'Lutea' (Lit × Gsu-2; $2n = 2x = 22$), *L. modesta* × *S. aurantiaca* (Lit × Sau-1; $2n = 2x = 23$) and *S. aurantiaca* × *G. superba* 'Lutea' (Sau × Gsu-1; $2n = 2x = 23$), and triploid intergeneric hybrids, *L. modesta* × *G.* 'Marron Gold' (Lit × Gma-1; $2n = 3x = 33$) and *S. aurantiaca* × *G.* 'Marron Gold' (Sau × Gma-6; $2n = 2x = 34$) (Kuwayama et al. 2005; Amano et al. 2007, 2008, 2009), were used in the present study. All plants were cultivated in the greenhouse according to Amano et al. (2008).

Chromosome preparation

Young anthers were collected one week before anthesis. They were pre-treated with 0.5 % (w/v) amiprophos-methyl (Hayashi Pure Chemical Industries, Osaka, Japan) in water for 3 h at room temperature (20−25 °C), fixed in a 3:1 (v/v) mixture of absolute alcohol and glacial acetic acid, and stored at −20 °C. Chromosome preparations for GISH were made according to Nakazawa et al. (2011). Anthers were washed with distilled water for 10 min, cut into ca. 5 mm square pieces, and digested in an enzyme mixture containing 0.6 % (w/v) Cellulase Onozuka RS (Yakult Honsha, Co., Tokyo, Japan), 0.3 % (w/v) Pectolyase Y-23 (Seishin, Co., Tokyo, Japan) and 0.5 % (w/v) Macerozyme R-200 (Yakult Honsha, Co., Tokyo, Japan) for 80 min at 37 °C. PMCs were spread out in a drop of a 3:1 (v/v) mixture of absolute alcohol and glacial acetic acid. Prepared slides were air dried and stored at −20 °C.

Probe preparation and GISH analysis

Total genomic DNA was isolated from young leaves by the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1985). Genomic DNA of Lit and Sau was labeled with digoxigenin-dUTPs by using the DIG Nick Translation Mix (Roche Diagnostics GmbH, Mannheim, Germany) and used as a probe.

In situ hybridization was carried out according to Nakazawa et al. (2011). Standard stringency conditions in GISH were applied to distinguish chromosomes of each genome according to Ji et al. (2004). Total DNA of one parent was used as a probe and 50-fold excess of salmon sperm DNA (Funakoshi, Co. Ltd., Tokyo, Japan) was used instead of blocking DNA. Hybridization signals of the probe were detected using anti-digoxigenin-rhodamine (Roche Diagnostics GmbH, Mannheim, Germany). Chromosomes were counterstained with 1 % (w/v) 4′-6-diamino-2-phenylindole (DAPI) in the antifade solution (Vector Laboratories, Inc., CA, USA). Chromosomes were examined under a fluorescent microscope (OPTIPHOT-2 9 2F EFD2, Nikon Corp., Tokyo, Japan) equipped with a CCD camera (VB-6010, Keyence Corp., Osaka, Japan). One hundred and 50 PMCs were observed for diploid and triploid intergeneric hybrids, respectively. Images were processed by the Photoshop Element 10 (Adobe Systems Inc., CA, USA). Differences in the average number of allosyndetic bivalents among different diploid intergeneric hybrids were determined by *t* test according to Molnár and Molnár (2010).

Results

Chromosomes from each parent could be clearly distinguished in PMCs at metaphase I of meiosis by GISH for all the five intergeneric hybrids analyzed in the present study.

For three diploid hybrids, Lit × Gsu-2, Lit × Sau-1 and Sau × Gsu-1, most PMCs contained only univalents, but both allosyndetic and autosyndetic bivalents were sometimes observed in each hybrids. In Lit × Gsu-2, allosyndetic bivalents of *Gloriosa* (G)—*Littonia* (L) genomes (Fig. 1A) were formed with an average of 0.36 per PMC corresponding to 1.70 % of the total number of chromosome conformations, while autosyndetic bivalents of G and L genomes were formed with average numbers of 0.26 and 0.27 per PMC, respectively (Table 1). In Lit × Sau-1, an average number of allosyndetic bivalents between L—

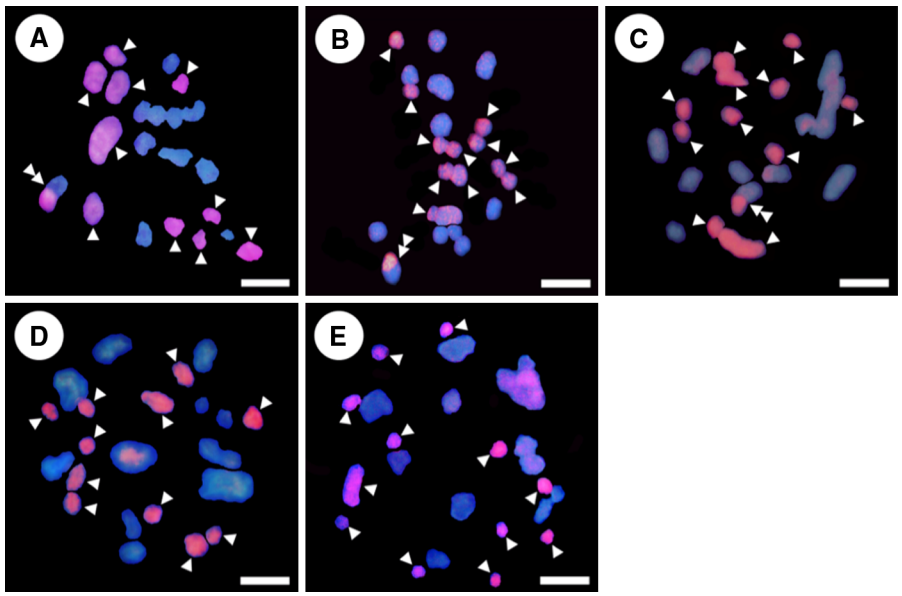

Fig. 1 GISH analysis of PMCs during meiosis of intergeneric hybrids of colchicaceous ornamentals. **A** Lit × Gsu-2: univalents of *Littonia* probed with digoxigenin-dUTPs and detected with anti-digoxigenin-rhodamine, were *red* (*arrowheads*). *Double-arrowhead* indicates an allosyndetic bivalent of *Littonia—Gloriosa* genomes. **B** Lit × Sau-1: univalents of *Sandersonia* probed with digoxigenin-dUTPs and detected with anti-digoxigenin-rhodamine, were *red* (*arrowheads*). *Double-arrowhead* indicates an allosyndetic bivalent of *Littonia—Sandersonia* genomes. **C** Sau × Gsu-1: univalents of *Sandersonia* probed with digoxigenin-dUTPs and detected with anti-digoxigenin-rhodamine, were *red* (*arrowheads*). *Double-arrowhead* indicates an allosyndetic bivalent of *Gloriosa—Sandersonia* genomes. **D** Lit × Gma-1: univalents of *Littonia* probed with digoxigenin-dUTPs and detected with anti-digoxigenin-rhodamine, were *red* (*arrowheads*). **E** Sau × Gma-6: univalents of *Sandersonia* probed with digoxigenin-dUTPs and detected with anti-digoxigenin-rhodamine, were *red* (*arrowheads*). *Bars* 10 μm. (Color figure online)

Sandersonia (S) genomes (Fig. 1B) was 0.13 per PMC corresponding to 0.57 % of the total number of chromosome conformations, while average numbers of autosyndetic bivalents of L and S genomes were 0.05 and 0.22 per PMC, respectively (Table 1). In Sau × Gsu-1, an average number of allosyndetic bivalents of G−S genomes (Fig. 1C) was 0.18 per PMC corresponding to 0.79 % of the total number of chromosome conformations, while average numbers of autosyndetic bivalents of G and S genomes were 0.04 and 0.23 per PMC, respectively (Table 1).

Table 2 shows the results of *t* test describing differences in the average number of allosyndetic bivalents among different diploid hybrids. The average number of allosyndetic bivalents in Lit × Gsu-2 (G−L genomes) was significantly higher than those in Sau × Gsu-1 (G−S genomes) and in Lit × Sau-1 (L−S genomes) at $P = 0.05$ and $P = 0.01$ levels, respectively. No significant difference in the average numbers of allosyndetic bivalent was observed between Lit × Sau-1 (L−S genomes) and Sau × Gsu-1 (G−S genomes).

For two triploid hybrids, Lit × Gma-1 and Sau × Gma-6, no allosyndetic bivalents were observed in PMCs at metaphase I of meiosis. Most PMCs of Lit × Gma-1 contained only 11 univalents of L genome and 11 bivalents of G genome (Table 3; Fig. 1D). Similarly, only 12 univalents of S genome and 11 bivalents of G genome were observed in most PMCs of Sau × Gma-6 (Table 3; Fig. 1E). In both hybrids, univalents of G genome were sometimes observed.

Discussion

The success to distinguish different genomes by GISH in allopolyploids and wide hybrids largely depends on the sequence homology (Ji et al. 2004). Genomes sharing 80–85 % or less sequence homology can generally be discriminated by standard GISH conditions (Schwarzacher et al. 1989). On the other hand, increased stringency conditions in combination with an excess of blocking DNA are required to discriminate genomes sharing up to 90−95 % of sequence homology (Parokonny et al. 1997). In our previous study on GISH analysis of mitotic chromosomes, parental chromosomes in colchicaceous intergeneric hybrids could be clearly discriminated under standard

Table 1 Chromosome associations in PMCs at metaphase I of meiosis in diploid intergeneric hybrids of colchicaceous ornamentals as indicated by GISH analysis

Hybrid line	Genome constitution[a]	Chromosome number (2*n*)	No. of cells observed	Average number of chromosome conformations per PMC (range)[b]									
				IG	IL	IS	IIG–L	IIG–S	IIL–S	IIG–G	IIL–L	IIS–S	Total
Lit × Gsu-2	LG	$2x = 22$	100	10.12 (5–11)	10.10 (5–11)	–	0.36 (0–4)	–	–	0.26 (0–3)	0.27 (0–3)	–	21.11 (16–22)
Lit × Sau-1	LS	$2x = 23$	100	–	10.72 (7–11)	11.46 (7–12)	–	–	0.13 (0–1)	–	0.05 (0–2)	0.22 (0–2)	22.58 (20–23)
Sau × Gsu-1	SG	$2x = 23$	100	10.74 (7–11)	–	11.36 (8–12)	–	0.18 (0–2)	–	0.04 (0–2)	–	0.23 (0–2)	22.55 (20–23)

[a] *G*, *L* and *S Gloriosa*, *Littonia* and *Sandersonia* genomes, respectively

[b] *IG*, *IL* and *IS* univalents of *Gloriosa*, *Littonia* and *Sandersonia*, respectively. *IIG–L*, *IIG–S* and *IIL–S* allosyndetic bivalents of *Gloriosa*−*Littonia*, *Gloriosa*−*Sandersonia* and *Littonia*−*Sandersonia* genomes, respectively. *IIG–G*, *IIL–L* and *IIS–S* autosyndetic bivalents of *Gloriosa*, *Littonia* and *Sandersonia* genomes, respectively

Table 2 Results of *t* test describing differences in the average number of allosyndetic bivalents among different diploid intergeneric hybrids of colchicaceous ornamentals

	t value	*P*-value
Lit × Gsu-2 (IIG–L) − Lit × Sau-1 (IIL–S)	2.6904	0.008040**
Lit × Gsu-2 (IIG–L) − Sau × Gsu-1 (IIG–S)	2.0304	0.044079*
Lit × Sau-1 (IIL–S) − Sau × Gsu-1 (IIG–S)	0.9390	0.348916

IIG–L, *IIG–S* and *IIL–S* allosyndetic bivalents of *Gloriosa*−*Littonia*, *Gloriosa*−*Sandersonia* and *Littonia*−*Sandersonia* genomes, respectively

* significant difference at $P = 0.05$, ** significant difference at $P = 0.01$

stringency conditions without an excess of blocking DNA (Nakazawa et al. 2011). Similar GISH conditions also allowed clear discrimination of parental chromosomes in PMCs at metaphase I of meiosis in the present study, indicating that the sequence homology among G, L and S genomes is relatively low.

On the other hand, allosyndetic bivalents as well as autosyndetic bivalents were observed in all the three diploid intergeneric hybrids investigated in the present study, although their average numbers per PMC were low (0.13−0.36 for allosyndetic bivalents and 0.04−0.27 for autosyndetic bivalents). These results indicate that there are some homologous chromosomal regions within each genome and among the genomes of *Gloriosa*, *Littonia* and *Sandersonia*. For allosyndetic bivalents, the average number of IIG-L was significantly higher than those of IIG-S and IIL-S, indicating that the genome affinity between G−L genomes may be higher than G−S and L−S genomes. An informal classification of Colchicaceae incorporating the classifications by Nordenstam (1998) and Dahlgren et al. (1985) includes all the three genera, *Gloriosa*, *Littonia* and *Sandersonia*, into the tribe Iphigenieae. However, a phylogenetic analysis by sequencing three plastid regions revealed that *Gloriosa* and *Littonia* form a clade, whereas *Sandersonia* forms the different clade with *Ornithoglossum* in this tribe (Vinnersten and Reeves 2003). Therefore, the difference in the average number of allosyndetic bivalents among different genome combinations may reflect the evolutionally distances among *Gloriosa*, *Littonia* and *Sandersonia*. *Gloriosa* and *Littonia* may be closely related to each

Table 3 Chromosome associations in PMCs at metaphase I of meiosis in triploid intergeneric hybrids of colchicaceous ornamentals as indicated by GISH analysis

Hybrid line	Genome constiution[a]	Chromosome number (2*n*)	No. of cells observed	Average number of chromosome conformations per PMC (range)[b]								
				IG	IL	IS	IIG–L	IIG–S	IIL–S	IIG–G	IIL–L	IIS–S	Total
Lit × Gma-1	LGG	$3x = 33$	50	0.04 (0–2)	11 (11)	–	0	–	–	10.98 (10–11)	0	–	22.02 (22–23)
Sau × Gma-6	SGG	$3x = 34$	50	0.04 (0–2)	–	12 (12)	–	0	–	10.98 (10–11)	–	0	23.02 (23–24)

[a] *G*, *L* and *S* *Gloriosa*, *Littonia* and *Sandersonia* genomes, respectively

[b] *IG*, *IL* and *IS* univalents of *Gloriosa*, *Littonia* and *Sandersonia*, respectively. *IIG–L*, *IIG–S* and *IIL–S* allosyndetic bivalents of *Gloriosa*−*Littonia*, *Gloriosa*−*Sandersonia* and *Littonia*−*Sandersonia* genomes, respectively. *IIG–G* bivalents derived from homologous chromosome pairing of *Gloriosa*. *IIL–L* and *IIS–S* autosyndetic bivalents of *Littonia* and *Sandersonia* genomes, respectively

other, while *Sandersonia* may have relatively distant relationships with these two genera.

In the present study, all the three diploid intergeneric hybrids produced allosyndetic bivalents during meiosis of PMCs, indicating that intergenomic recombination may occur among *Gloriosa*, *Littonia* and *Sandersonia*. Gametes with intergenomic recombination could contribute to introgression breeding, although the gametes should have fertility for this purpose. Actually, these diploid hybrids showed very low or no pollen fertility (0−2.4 %) as assessed with acetocarmine staining (Amano et al. 2008, 2009). Although chromosome doubling has generally been used for fertility restoration of wide hybrids (Van Tuyl 1989; Van Tuyl et al. 1992; Isshiki and Taura 2003; Dunn and Lindstrom 2007), this approach could not contribute much to introgression breeding, because amphidiploids obtained via chromosome doubling usually produce 2*x*-gametes without intergenomic recombination (Ramanna and Jacobsen 2003; Van Tuyl and Lim 2003). On the other hand, it has been reported for *Alstromeria* (Kamstra et al. 1999) and *Lilium* (Lim et al. 2003; Zhou et al. 2008) that some interspecific hybrids spontaneously produced fertile 2*x*-gametes with intergenomic recombination via the first division restitution and these 2*x*-gametes were used for introgression breeding. In addition, artificial induction of 2*x*-gametes by nitrous oxide gas or high temperature treatments during meiosis has been reported for *Populus adenopoda* (Lu et al. 2013) and interspecific hybrids in *Lilium* (Barba-Gonzalez et al. 2006; Hasegawa et al. 2013). Therefore, we are now examining such treatments for inducing unreduced 2*x*-gametes in the diploid hybrids investigated in the present study.

For the two triploid intergeneric hybrids, allosyndetic bivalents were never observed in PMCs during meiosis. Most PMCs contained only univalents of L or S genome and bivalents of G genome, which might be derived from homologous chromosome pairing. Because of very low homology among three genomes, homologous chromosomes of G genome might paired prior to form allosyndesis. Thus, the triploid hybrids are inadequate as materials for introgression breeding via intergenomic recombination. On the other hand, fertile allotriploid hybrids have been used to produce alien chromosome addition lines in several genera such as *Allium* (Vu et al. 2012) and *Lilium* (Lim et al. 2003). Amano (2008) and Amano et al. (2009) reported that the triploid intergeneric hybrids in Colchicaceae showed 7.0−8.3 % of pollen fertility as assessed with acetocarmine staining and produced varied sizes of pollen grains, which might be resulted from abnormal chromosome segregation and cytokinesis. Therefore, the triploid hybrids investigated in the present study are possible materials for producing intergeneric chromosome addition lines in Colchicaceae. Backcross pollination using the triploid hybrids and subsequent ovule culture are now in progress.

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