Meiotic behaviour of individual chromosomes in allotriploid *Alstroemeria* hybrids

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Chromosome association and chiasma formation were studied in pollen mother cells at metaphase I of four allotriploid BC1 plants ($2n=3x=24$) obtained from the backcross of the hybrid *Alstroemeria aurea* × *A. inodora* with its parent *A. inodora*. We distinguished the chromosomes of both parental species by genomic *in situ* hybridization (GISH), whereas the individual chromosomes were identified on the basis of their multicolour FISH banding patterns obtained after a second hybridization with two species-specific satellite repeats as probes. All the four BC1 plants possessed two genomes of *A. inodora* and one of *A. aurea*. Variable numbers of recombinant chromosomes, resulting from meiotic recombination in the interspecific

hybrid, were present in these plants. The homologous *A. inodora* chromosomes generally formed bivalents, leaving the homoeologous *A. aurea* chromosomes unassociated. High frequencies of trivalents were observed for the chromosome sets that contained recombinant chromosomes, even when the recombinant segments were small. Chromosome associations in the trivalents were restricted to homologous segments. The implications of the absence of homoeologous chromosome pairing on gamete constitution and prospects for introgression in *Alstroemeria* are discussed.

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Introduction

Allotriploid (AAB) plants contain two genomes of one species and one genome from a different species. Generally, they are obtained from crossing an allotetraploid (eg AABB) with one of the diploid parents (eg AA). Such backcross derivatives, also referred to as sesquidiploids, occasionally produce balanced haploid ($n=x$), diploid ($n=2x$) and triploid ($n=3x$) gametes, as well as unbalanced gametes with an aneuploid number of chromosomes. The unbalanced gametes of allotriploids have been used to produce monosomic additions in several crops, such as *Nicotiana* (Suen *et al*, 1997), *Brassica* (Chen *et al*, 1997), *Beta* (Mesbah *et al*, 1996), *Allium* (Peffley *et al*, 1985, Shigyo *et al*, 1996) and *Lolium* (Morgan, 1991).

Alstroemeria (family *Alstroemeriaceae*, order *Liliales*) is a relatively young ornamental crop. Most cultivars have originated through hybridization of diploid species ($2n=2x=16$) that are endemic to Chile and Brazil (Goemans, 1962). Interspecific hybrids, including inter-Chilean species hybrids and hybrids between Chilean and Brazilian species, are often highly sterile but unreduced ($2n$) gametes occur in variable frequencies and formed the basis of the present-day triploid ($2n=3x=24$) and tetraploid ($2n=4x=32$) cultivars (Ramanna, 1992). To evaluate the breeding prospects

for introgression of desired traits from *A. aurea* into *A. inodora*, Kamstra *et al* (1999a, b) backcrossed the interspecific diploid ($2n=2x=16$) hybrid of *A. aurea* × *A. inodora* with *A. inodora*. In the BC1 progeny, aneuploids as well as allotriploids were observed. The allotriploids resulted from unreduced ($2n$) gametes produced by the F1 hybrid and, consequently, contained two genomes of *A. inodora* (P) and one of *A. aurea* (A) (Kamstra *et al*, 1999b). Identification of all chromosomes in the BC1 plants was achieved on the basis of genomic and fluorescent *in situ* hybridization (GISH and FISH). GISH, which enabled differentiation between the *A. inodora* and *A. aurea* chromatin, revealed the existence of four to 10 recombinant chromosomes in the different BC1 plants (Kamstra *et al*, 1999a, b). The number, size and position of the exchanged segments in these chromosomes varied between plants. In addition, structurally altered chromosomes were detected in some of the BC1 plants (Kamstra *et al*, 1999b). These chromosomes contained hybridization sites for species-specific probes at unexpected locations, not found in parental species (Kamstra *et al*, 1997), indicating the presence of chromosomal rearrangements between the *A. aurea* and *A. inodora* genome (Kamstra *et al*, 1999b).

This paper describes the meiotic analysis of four BC1 plants using FISH and GISH sequentially applied to spread pollen mother cells at metaphase I to telophase II. To use the allotriploids for further backcrossing, it was essential to analyse meiosis in order to determine the types of gametes that are produced and to predict the consequences of observed meiotic disturbances for further backcrossing. The aims of this investigation were:

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(i) to study the meiotic associations of each individual chromosome in the allotriploids and to estimate pairing frequencies; (ii) to confirm the previously hypothesized structural rearrangements between the chromosomes of both parental species on the basis of metaphase I configurations of the structurally altered chromosomes; (iii) to analyse the behaviour of individual genomes and chromosomes, especially the *A. aurea* chromosomes, during different meiotic stages.

Materials and methods

Plant material

Four allotriploid plants BC1-1 (95SK04), BC1-2 (95SK17), BC1-3 (96SK402) and BC1-4 (96SK403) were derived from the backcross between the hybrid *A. aurea* × *A. inodora* (AP) and its parent *A. inodora* (P) (Kamstra *et al*, 1999a). A combination of GISH for genome identification and FISH for individual chromosome identification was used to assess the karyotypes of these allotriploids (PPA) with two *A. inodora* (P) genomes and one *A. aurea* (A) genome (Kamstra *et al*, 1999b; Figure 1). Chromosomes were classified according to Kamstra *et al* (1999b).

Meiotic preparations of the BC1 plants

Anthers were fixed in 3:1 ethanol:acetic acid for approximately 1 h and stored in 70% ethanol at -20°C until use. Before cell spreading, the material was digested with a mixture of pectolytic enzymes (0.4% pectolyase Y23, 0.4% cellulase RS and 0.4% cytohellicase in 0.1 M citrate buffer, pH 4.5) for 2–3 h at 37°C . The softened anthers were squashed in fresh 45% acetic acid. Preparations containing pollen mother cells from metaphase I to anaphase II were selected. Sequential *in situ* hybridization experiments (GISH and FISH with two species-specific probes) were performed on most slides.

Genomic *in situ* hybridization (GISH)

Probe preparation and GISH were performed as previously described (Kuipers *et al*, 1997; Kamstra *et al*, 1999b). In short, two differentially labelled genomic DNA probes of the parental species *A. aurea* (biotin-labelled) and *A. inodora* (digoxigenin-labelled) were pre-annealed for 2 h at 58°C , hybridized overnight to denatured slides, and detected using Cy3- (Jackson ImmunoResearch Laboratories) and FITC/Alexa 488-conjugated antibodies (Molecular Probes Inc.).

Fluorescent *in situ* hybridization (FISH)

The repeats D32-13 (specific for *A. inodora*; EMBL accession number AJ228814) and A001-I (specific for *A. aurea*; EMBL accession number Y10977) were labelled by PCR with biotin-16-dUTP or digoxigenin-11-dUTP, respectively, and hybridized to the meiotic cells as described in Kamstra *et al* (1997, 1999a, b). The biotin- and digoxigenin-labelled probes were detected using Cy3- (Jackson ImmunoResearch Laboratories) and FITC/Alexa 488-conjugated antibodies (Molecular Probes Inc.).

Results

All the four allotriploids BC1-1, BC1-2, BC1-3 and BC1-4 ($2n = 3x = 24$) possess two genomes of *A. inodora* and one of *A. aurea*. These plants contain several recombinant

chromosomes, which are described in a previous paper (Kamstra *et al*, 1999b). In total, chromosome association frequencies were analysed in 542 pollen mother cells (PMCs) at metaphase I in the four allotriploids to establish the pairing frequencies. Pairing was studied per set of three homo(eo)logous chromosomes. This was analysed for all eight sets of homoeologous chromosomes in all plants. In addition, chromosome behaviour at later meiotic stages was established.

Metaphase I chromosome associations in the BC1 plants

We first analysed the number of univalents (I), bivalents (II) and trivalents (III) in PMCs at metaphase I of the four allotriploids BC1-1, BC1-2, BC1-3 and BC1-4. Figure 2 shows examples obtained from BC1-3 that are representative for all the four plants. Unequivocal identification of individual chromosomes was based on sequential GISH and FISH patterns (Figure 2a, b). In all plants, the three homo(eo)logous chromosomes of each set either formed one bivalent + one univalent or one trivalent. The trivalents were observed for all eight chromosome sets per plant in variable frequencies, ranging from 2.0 to 100% (Table 1). Bivalent associations were mainly confined to the two homologous *A. inodora* chromosomes, whereas their *A. aurea* homoeologue was present as a univalent (eg Figure 2b). Three univalents were not observed for any of these chromosome sets.

Sets of three homo(eo)logous chromosomes, containing one or two recombinant chromosomes (recombinant sets), predominantly formed trivalents (Figure 2a). The frequency of trivalents in recombinant sets ranged from 40.3% for the chromosomes 6 in BC1-3 to 100% for both recombinant sets (1 and 5) in BC1-2 (Table 1), which is significantly higher than for non-recombinant sets (two-sample one-tailed *t*-test, assuming equal variances; $P = 1.6 \times 10^{-15}$).

For all recombinant sets, GISH clearly revealed that the chromosome associations in the trivalents were restricted to homologous segments (Figure 2a, c), that is, no associations between homoeologous parts were detected. In general, the *A. aurea* segments in these trivalents were unpaired (Figure 2a), except for the recombinant sets in which two homologous *A. aurea* segments were present such as set 8 of BC1-1 or set 4 of BC1-3 (Figure 2a, trivalent of 4A/5P + 5P + 5P/4A).

In the case of nonrecombinant sets, we observed far more bivalent + univalent configurations than trivalent configurations, with the two *A. inodora* chromosomes forming the bivalent and the *A. aurea* chromosome the univalent (Figure 2a, b). The frequency of bivalent + univalent ranged from 42.1 to 97% (Table 1). The occurrence of trivalents in the nonrecombinant sets, which ranged from 3 to 57.9% per set (Table 1), indicated pairing and recombination between the homoeologous *A. inodora* and *A. aurea* chromosomes (see Figure 2a, b: trivalent of 1P-1P-1A). Compared to all other nonrecombinant sets of chromosomes, the sets of chromosomes 1 of BC1-4 and BC1-3 formed significantly (χ^2 ; $P < 0.05$) more trivalents (Table 1).

Metaphase I configurations of the structurally altered chromosomes

In the BC1-3 a pseudo-iso-chromosome 2A/2P was present, containing two homologous segments of the

Figure 1 Karyotypes of the four allotriploids. (a) BC₁-1, (b) BC₁-2, (c) BC₁-3, (d) BC₁-4. *A. aurea* and *A. inodora* chromatin is indicated in dark grey and white, respectively. All plants contain recombinant chromosomes. The bars next to the chromosomes of recombinant sets indicate that for these chromosome segments homologous segments are present. Light grey boxes: A001-I hybridization sites on *A. aurea* chromosomes. Lined boxes: D32-13 hybridization sites on *A. inodora* chromosomes.

short arm of 2P distally on both arms. This recombinant chromosome was considered to be the result of two crossovers between assumed translocated homoeologous

segments in the parental hybrid (Kamstra *et al*, 1999b; Figure 2d4). To confirm the nature of the pseudo-iso-chromosome, we analysed chromosome configura-

Table 1 Observed frequency (%) of metaphase I associations (trivalent (III) or bivalent + univalent (II+I)) per nonrecombinant or recombinant set of three homo(eo)logous chromosomes

Genotype	No. of PMCs analysed	Chromosome associations	Observed frequency (%) per set of three homo(eo)logous chromosomes							
			1	2	3	4	5	6	7	8
BC ₁ -1	57	III	94.7*	80.7*	84.2*	7.0	93.0*	3.5	15.8	82.5*
		II+I	5.3	19.3	15.8	93.0	7.0	96.5	84.2	17.5
BC ₁ -2	294	III	100*	6.1	3.0 ^a	3.0 ^a	100*	4.8	5.4	4.1
		II+I	0	93.9	97.0	97.0	0	95.2	94.6	95.9
BC ₁ -3	134	III	30.6	91.0*	97.0*	93.3*	81.3*	40.3*	10.4	11.2
		II+I	69.4	9.0	3.0	6.7	18.7	59.7	89.6	88.8
BC ₁ -4	57	III	57.9	12.3	14.0	84.2*	98.2*	10.5	75.4*	14.0
		II+I	42.1	87.7	86.0	15.8	1.8	89.5	24.6	86.0

^aSets 3 and 4 could not be distinguished in all MI cells. Data of these sets were added resulting in identical trivalent frequencies.

*Indicates sets containing recombinant chromosomes (see Figure 1 for the sizes and locations of exchanged segments).

tions at metaphase I of this chromosome with its homo(eo)logues. The types of observed chromosome configurations are shown in Figures 2c1–3 and 2d1–3. In the trivalents, the pseudo-iso-chromosome was frequently present as a ring (Figure 2c2, 2d2). At low frequencies (2.2%), this chromosome was observed as a ring univalent (Figure 2c3, 2d3). This observation is in agreement with the assumed chromosome morphology.

Meiotic transmission of the *A. aurea* and *A. inodora* chromosomes

At anaphase I all nonrecombinant *A. inodora* chromosomes, and most of the recombinant *A. inodora* and *A. aurea* chromosomes, segregated regularly to both poles in all BC₁ plants (Figures 2e–i). In contrast, the nonrecombinant *A. aurea* chromosomes and some of the recombinant chromosomes remained in the equatorial plane. At later stages, their chromatids disjoined and migrated to both poles (Figures 2e–i). At late anaphase I/ telophase I, most of the *A. aurea* chromatids, although still lagging, were equally distributed to both poles in most cells (Figure 2j). In some telophase I cells the metacentric chromatids were divided in the centromeric regions (Figure 2j, chromosome 1A). At anaphase II, the *A. inodora* chromosomes were evenly distributed to both poles, whereas the *A. aurea* chromosomes were lagging in the equatorial plane and seemed to migrate randomly to both poles. Tetrad stages showed microspores with a different number of A001-I sites, thus confirming the unequal distribution of the *A. aurea* chromosomes in the

gametes. Many of the gametes possessed one to three micronuclei, in which most of the *A. aurea* chromosomes were present (data not shown).

At anaphase I of the BC₁-2 and BC₁-3 plants, bridges were frequently observed, especially in the latter (in 75% of all AI cells). Usually the bridges, which occurred in the nonrecombinant chromosomes 8P in BC₁-3 (Figure 2e, f), were accompanied by an acentric fragment (Figure 2h, i), indicating crossovers in a paracentric inversion loop.

The low frequencies of associated homoeologous chromosomes at metaphase I indicated that homoeologous crossover occurs in the BC₁ plants. Evidence for this was observed at anaphase I. Since most of the individual chromosomes could be identified even at anaphase I, it was possible to confirm which chromosomes actually contained novel recombinations (Figure 2h, i; yellow arrows). Although only few anaphase I cells could be analysed in detail, the occurrence of homoeologous recombination seemed to be similar, as predicted from the homoeologous associations at metaphase I. For example, at anaphase I of the plant BC₁-3, more novel crossovers were observed in the chromosomes 1 than in the smaller chromosomes 7 and 8, which was expected from the pairing behaviour of these chromosomes at metaphase I (Table 1). As a consequence of the crossover and the involvement in trivalent chromosome configurations, the homoeologous recombination allowed these chromosomes to segregate regularly with their homo(eo)logues at anaphase I. This was observed for all chromosomes containing novel homoeologous recombinations.

Figure 2 Fluorescent *in situ* hybridization applied to metaphase I and anaphase I spreads of BC₁-3. GISH was applied to differentiate both parental genomes, *A. aurea* (red) and *A. inodora* (green), and sequential FISH with D32-13 (red) and A001-I (green) allowed identification of all individual chromosomes. DAPI (blue) was used as a counterstain. The blue arrows indicate *A. aurea* chromosomes or chromatids. (a,b) GISH and sequential FISH with D32-13 and A001-I allowed identification of all chromosomes involved in the chromosome configurations. Note that the recombinant chromosome 6A/6P is present as a univalent. Homoeologous pairing was observed in the trivalent of non-recombinant chromosomes 1. (c1–3) Different chromosome configurations for the homo(eo)logous chromosomes 2A/2P, 2P/2A and 2P prove the presence of two homologous segments on both arms of 2A/2P (see text). (d1–3) Schematic drawings of the chromosome configurations shown in (c1–3). Red and yellow indicate *A. aurea* and *A. inodora* chromatin, respectively, and purple indicates the D32-13 hybridization sites. (d4) Ideograms of the different chromosomes 2 present in BC₁-3. Note the pseudo-iso-chromosome 2A/2P. (e,f) GISH and FISH applied sequentially to the same anaphase I. In these stages all chromosomes could be identified, however, due to recombination most chromosomes possess different recombined segments and therefore only the centromeric regions of these chromosomes are identified. The bridge (white arrow) between the long arms of the nonrecombinant chromosomes 8P was often observed in combination with a small a-centric fragment (white arrows in (h,i)). (g) Regular migration of the *A. inodora* chromosomes to both poles, whereas four chromosomes (blue arrows) remain in the equatorial plane. (h,i) A novel recombination (yellow arrows) between two homoeologous chromosome 1A and 1P. Note the a-centric fragment of chromosome 8P (white arrow) and the lagging *A. aurea* chromosomes. (j) Telophase I. Note segregation of *A. aurea* chromatids and the centromere breakage in one of the chromatids of chromosome 1A. White arrow indicates the small a-centric fragment of 8P.

Discussion

Compared to meiotic analysis by GISH alone (cf. Parokonny *et al*, 1994, 1997; King *et al*, 1994; Benavente *et al*, 1996), sequential GISH/FISH analysis allowed identification of parental genomes through GISH, and simultaneous identification of all individual chromosomes in the same cell, based on FISH with repetitive probes. This enabled to study the pairing behaviour of all individual chromosomes in allotriploid *Alstroemeria* hybrids.

Meiotic chromosome behaviour

A strong correlation was found between the high percentage of trivalents, in which only homologous chromosome segments were associated, and the occurrence of recombinant chromosomes in the BC1-plants. These recombinant chromosomes originated during meiosis in the diploid *A. aurea* × *A. inodora* hybrid, where high frequencies of homoeologous chromosome pairing (80% heteromorphic bivalents) and recombination were observed (Kamstra *et al*, 1999a, b). Apparently, the genomes of *A. aurea* and *A. inodora*, although distinguishable in GISH which suggests substantial (repetitive) DNA divergence, remain related to such an extent that homoeologous pairing does occur when homologous chromosomes are absent. A similar situation was observed for the diploid interspecific hybrid ($2n = 2x = 16$) between *Astroemeria ligtu* L. ($2n = 2x = 16$) and *A. pelegriana* L. var. *rosea* ($2n = 2x = 16$), where partial homoeologous chromosome association resulted in bivalent formation and chances for homoeologous recombination (Ishikawa and Ishizaka, 2002). An amphidiploid interspecific hybrid ($4x = 32$) from these species mainly produced bivalents of homologous chromosomes.

The degree of homo(eo)logy between the parental genomes in interspecific hybrids seems fundamental for the occurrence of intergenomic recombination in successive backcross progenies. In general, chromosomes of closely related genomes tend to pair more often than chromosomes of genomes that are more distantly related (Kimber and Yen, 1990). Due to low parental genome homoeology in an allotriploid hybrid between *N. plumbaginifolia* and *N. sylvestris*, monosomic addition lines were obtained in BC1 and BC2 for most *N. sylvestris* chromosomes upon backcrossing to *N. plumbaginifolia*, whereas only a few chromosome translocations were observed (Suen *et al*, 1997). In monosomic *Brassica campestris*-*alboglabra* addition lines, obtained from backcrosses between the less distant resynthesized *B. napus* and *B. campestris*, segmental intergenomic homoeology was observed between the parental genomes that could explain the occurrence of intergenomic introgression in the progenies of these addition lines (Chen *et al*, 1997).

The level of recombination in backcross progenies of interspecific hybrids is often lower than in the original hybrids (Chetelat *et al*, 2000). Reduced homoeologous pairing and recombination were also observed during meiosis of the allotriploid BC1-plants derived from the *A. aurea* × *A. inodora* hybrid when compared to the hybrid itself. This could be fully attributed to preferential homologous pairing in the BC1 plants that contain an additional set of *A. inodora* chromosomes. While suppression of homoeologous pairing and recombination may have a stabilizing effect on the genome constitution

of backcross plants, it may hamper intergenomic introgression for breeding purposes.

Genomic stability of *Alstroemeria* allotriploids

Newly formed (allo)polyploids, often allotetraploids, generally show considerable meiotic complexity, which is exemplified by the frequent occurrence of phenomena such as multivalent pairing and the production of unbalanced gametes (Ramsey and Schemske, 2002). These meiotic aberrations imply that neopolyploid progenies may include high frequencies of aneuploids, pseudoeuploids and genotypes with recombinant homoeologous chromosomes. Restoration of diploid-like meiotic pairing and selection against meiotic configurations that produce unbalanced gametes generally result in the restoration of fertility in successive generations (Ramsey and Schemske, 2002; Comai *et al*, 2003).

Although these characteristics broadly apply to our findings, the situation for the allotriploid *Alstroemeria* hybrids is slightly different. They originated through $2n$ -gamete formation in the interspecific *A. aurea* × *A. inodora* hybrid that was backcrossed with *A. inodora* (Kamstra *et al*, 1999a). Homoeologous pairing in the hybrid yielded varying numbers of recombinant chromosomes (Kamstra *et al*, 1999b), which led to a high frequency of trivalents (Table 1), affecting meiotic stability and fertility in the BC1 plants. Another factor that complicates genomic stability is the fact that the *A. aurea* chromosomes have no counterparts for meiotic pairing. This can be overcome by either a gradual loss of *A. aurea* chromosomes, which are often observed as univalents in meiosis and in micronuclei in gametes of BC1 plants, or limited intergenomic introgression of *A. aurea* chromosome segments through homoeologous recombination. The latter possibility is supported by the observation of low frequencies of homoeologous association in metaphase I and homoeologous crossovers in meiosis of the BC1 plants. Taken together, it is doubtful whether the BC1 plants will evolve into genomically stable allotriploids. A more likely result is a diploid constitution in which *A. aurea* chromosome segments have been introgressed into homoeologous *A. inodora* chromosomes.

Implications for introgression of alien chromosomes

Homoeologous chromosome pairing which may result in introgression was observed at low frequencies in all BC1 plants, with variation among plants and individual chromosomes. In particular, the nonrecombinant *A. aurea* chromosome 1 in BC1-3 and BC1-4 was frequently associated with the homoeologous chromosome 1 from *A. inodora* (Table 1). In *A. aurea* and *A. inodora*, chromosome 1 is large compared to other chromosomes (Buitendijk *et al*, 1997); therefore chromosome size seems to influence the pairing frequency, as was shown in wheat (Bernardo *et al*, 1988; Cuadrado *et al*, 1997).

The random segregation of nonrecombinant *A. aurea* chromosomes at anaphase II and telophase II suggested that BC2 plants may contain random numbers of *A. aurea* chromosomes. Theoretically, monosomic addition lines could be obtained in BC2, as was found for other plant species (Peffley *et al*, 1985; Morgan, 1991; Mesbah *et al*, 1996; Shigyo *et al*, 1996; Chen *et al*, 1997; Suen *et al*, 1997). Many gametes of the BC1 plants contained one to three

micronuclei with *A. aurea* chromosomes. The question remains whether these micronuclei might hamper fertility and whether the chromosomes in the micronuclei can be transmitted to the progeny. The amount of stainable pollen per BC1 plant varied from 30 to 60% (Kamstra et al, 1999a). Despite this, only a few BC2 plants were obtained after extensive backcrossing, indicating that additional factors may reduce the actual viability of gametes of the BC1 plants. Cytogenetic analysis of BC2 plants could reveal the genomic constitution of successful gametes.

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