Stellingen

1. The distantly related species *Alstroemeria aurea* and *A. inodora* possess comparable chromosome morphology. (this thesis)
2. FISH studies have shown that the highly repetitive specific DNA sequences A001-I and D32-13 are distributed in a similar non-random way in the genomes of the species *A. aurea* and *A. inodora*. (this thesis)
3. The colocalisation of the species specific repeat A001-I and the conserved 18S-25S rDNA repeat in the heterochromatic regions of *A. aurea* indicates that at least two different types of repetitive sequences have accumulated in these regions during the course of evolution of *A. aurea*. (this thesis)
4. The abundant homoeologous chromosome pairing and recombination in the hybrid *A. aurea* x *A. inodora* show that introgression of traits in a backcross breeding program is possible. (this thesis)
5. In interspecific hybrids, recombination frequencies between homoeologous chromosomes are much higher in the absence of homologous chromosomes. This phenomenon has probably played an important role in the evolution of allopolyploid species.
6. Genomic *in situ* hybridization (GISH) is a powerful technique to accurately determine after meiosis the restitution mechanism of 2n-gametes of interspecific hybrids. (this thesis)
7. Cytologically, the distal region of the short arm of chromosome 2 of *A. aurea* shares homoeology with the distal region of the long arm of chromosome 2 of *A. inodora*. (this thesis)
8. Met de huidige beeldbewerkingsprogramma's wordt de verleiding groot de onderzoeksresultaten te verfraaien.
9. Reorganisaties maken de verschillen tussen vast en tijdelijk personeel kleiner.
10. Rekeningrijden is geen oplossing voor het fileprobleem.
11. De fopspeen is een prachtige uitvinding.

Stellingen behorende bij het proefschrift 'A molecular cytogenetic analysis of introgression in *Alstroemeria*'.

Silvan Kamstra, Wageningen, 1 juni 1999.

A molecular cytogenetic analysis of introgression in *Alstroemeria*

Een moleculair cytogenetische analyse van introgressie in *Alstroemeria*

Promotor: dr. ir. E. Jacobsen
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A molecular cytogenetic analysis of introgression in *Alstroemeria*

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in het openbaar te verdedigen
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Contents

1 General Introduction

Transfer of valuable genes or chromosomes from alien species and genera into cultivated plants has greatly contributed to the improvement of crop plants. One of the best known examples is the successful transfer of rye chromosome segments into bread wheat. Especially transfer of the short arm of rye chromosome 1R, which confers resistance to several foliar diseases, including genes for powdery mildew, yellow rust, stem rust, and leaf rust along with genetic factors for wide adaptation and high yield performance, has revolutionised wheat production in the world (for references see Jiang *et al.* 1994). In fact, introgression of desirable genes and chromosome segments from alien species into cultivars is often used in breeding of almost all important crops. The traditional approach of introgression of desired genes through recurrent backcrossing of a hybrid with one of its parents is highly laborious and success is not always guaranteed.

There are several reasons why introgression of desirable genes from alien species is laborious and frustrating: a) the occurrence of pre- or post fertilisation crossing barriers between the parental species can hinder hybridization. These barriers can be circumvented to some extent by applying embryo rescue (Williams *et al.* 1987; Sharma *et al.* 1996), bridge crossings or somatic fusion techniques; b) once obtained, interspecific or intergeneric hybrids or their progeny may be highly or completely sterile, obstructing or even preventing the possibilities of further backcrossing; c) several generations of recurrent backcrosses are needed to eliminate undesired alien characteristics. Furthermore, obtained backcross progenies might not possess only the desired parental characteristics, due to, for example, tight linkage to unwanted characteristics. Successful introgression can also be hampered by the selective elimination of the alien genomes or chromosomes or by preferential transmission of undesired chromosomes in the backcross progenies (Jiang *et al.* 1993), the introduction of undesired characteristics together with the desired ones by tight linkage or lack of (homoeologous) recombination between the donor and the recipient genome. Homoeologous recombination is believed to be one of the requirements for stable introgression of desired characteristics and elimination of linked undesired ones.

In spite of all these bottlenecks, as yet, there are no effective alternatives to fully exploit the utilisation of valuable genes from wild relatives, except through cloning of individual genes combined with transformation. Nevertheless, recently developed molecular cytogenetic tech-

niques have offered new ways to visually monitor the process of introgression of alien DNA into crop plants. One of the widely used techniques is fluorescent *in situ* hybridization (FISH), which involves hybridization of labelled DNA sequences, ranging from total genomic DNA (genomic *in situ* hybridization (GISH)) to cloned single copy sequences, to chromosome preparations for the identification of genomes and chromosome (segments) (for method see Leitch *et al.* 1992). For GISH, total genomic DNA of one parental species is labelled and hybridized, in combination with an access amount of unlabelled total genomic DNA of the other parent, in order to identify both parental genomes. The great potential of the GISH technique for introgression research is that it enables an accurate and rapid visualisation of alien genetic material in hybrids and backcross progenies, as was demonstrated for a wide range of plant species (Rayburn and Gill 1985; Schwarzacher *et al.* 1989; Le *et al.* 1989; Heslop-Harrison *et al.* 1990; Kenton *et al.* 1992; Parokonny *et al.* 1992; 1994; 1997; Mukai *et al.* 1993; Anamthawat-Jonsson *et al.* 1993; Ørgaard and Heslop-Harrison, 1994; Wolters *et al.* 1994; Jacobsen *et al.* 1995; Garriga Calderé *et al.* 1997; Takahashi *et al.* 1997; among others). Thus, FISH and GISH techniques can be used to accurately determine the genome constitution of hybrids and their offspring. The monitoring of the process of introgression offers a means to select for the presence of particular alien chromatin in backcross progenies, and this in turn speeds up the process and increases its efficiency.

With the ability to differentiate the parental genomes in hybrids, GISH and FISH provide very accurate tools for analysing homoeologous pairing and recombination (Miller *et al.* 1994; King *et al.* 1994; 1998; Benavente *et al.* 1996; Takahashi *et al.* 1997; Cuadrado *et al.* 1995; Parokonny *et al.* 1994; 1997). In comparison to classical cytological techniques, GISH to meiotic cells will result in a more accurate estimation of homoeologous pairing and recombination in hybrids. However, it is generally not possible to identify all individual chromosomes, mostly because chromosomes are morphologically similar. Chromosome identification is indispensable to reveal whether pairing configurations involve homoeologous or non-homo(eo)logous chromosomes and to establish pairing preferences of individual chromosomes. The use of specific repeats can be helpful for chromosome identification. FISH can be used to determine the physical location of these repeats on the chromosomes. If unique banding patterns for each chromosome are obtained, it can be used to identify individual chromosomes, during mitosis as well as meiosis. For example, the conserved 18S-25S rDNA sequence is widely applied to identify the NOR-bearing chromosomes (Leitch and Heslop-Harrison 1992;

Mukai *et al.* 1991; Heslop-Harrison *et al.* 1994; among others). The identification of individual chromosomes allows chromosome pairing to be studied in more detail (Cuadrado *et al.* 1995).

The advantages of GISH and FISH in introgression studies have been demonstrated in a number of plants (Parokonny *et al.* 1994; 1997; Jacobsen *et al.* 1995; Takahasi *et al.* 1997). In these examples, however, most individual chromosomes were not identified. In view of understanding the fundamental process of introgression and to determine the fate of individual chromosomes it is essential to use parents that are differentiated and cytogenetically characterised. These characteristics can then be used to accurately analyse the offspring of these plants. In addition, the identification of individual chromosomes enables ways to select for particular chromosomes and provides essential information for further breeding. For this purpose, a crop species that has large and easily identifiable chromosomes would be preferable. *Alstroemeria* (an ornamental) is such a species.

Alstroemeria

Besides being a relatively new cut flower crop in the Netherlands (Goemans, 1962), *Alstroemeria* has many attractive features for introgression studies. The parental species of the present-day cultivars originate from South America (mainly Chile and Brazil), and the genomes of many of the species are highly differentiated (Buitendijk and Ramanna, 1996; Kuipers *et al.* 1997). All species possess large genomes, with DNA values (2C values) ranging from 36.6 to 78.9 pg (17600 to 38000 Mb) in diploid genotypes (Buitendijk *et al.* 1997). Interspecific hybrids are difficult to obtain, which is mainly due to the presence of post-fertilisation barriers. However, by applying embryo rescue techniques numerous hybrids are obtained (Buitendijk *et al.* 1995, De Jeu and Jacobsen, 1995, Lu and Bridgen, 1997). F_1 hybrids are generally highly sterile, but unreduced (2n) gametes occur in variable frequencies and these formed the basis of the present-day polyploid (3x or 4x) cultivars (Ramanna, 1992). Since there is a wide range of genetic variation among the wild species of *Alstroemeria*, they are important sources for cultivar improvement. Within the wild species potentially valuable characteristics are assumed to be present such as virus resistance, winter flowering, many horticultural traits (e.g. flower colour, thickness of the stem, vase life, pot plant growing, *in vitro* rhizome multiplication) and hardiness.

Cytologically, the *Alstroemeria* species have interesting characteristics such as a relatively small number ($2n=2x=16$) of large chromosomes. Although there is a two fold difference in

DNA content among the species, all species studied so far possess similar bimodal karyotypes which means that the chromosomes can be categorised into two groups: one set of (four or five) metacentrics and one set of (three or four) acrocentrics. The chromosomes of several species showed distinct C-banding patterns, whereas others did not (Buitendijk and Ramanna 1996). In addition, GISH showed clear differences between the species of Chilean and Brazilian origin, which was confirmed by southern hybridization using total genomic DNA of several species (Kuipers *et al.* 1997), indicating highly differentiated genomes between these species. Furthermore, GISH revealed specific banding patterns (GISH banding) on the chromosomes of several species, which corresponded to heterochromatic regions of the chromosomes (Kuipers *et al.* 1997), suggesting that these heterochromatic regions were of different sequence origin compared to the euchromatic regions. The isolation and physical localisation of several highly repetitive species-specific DNA sequences confirmed the presence of specific repeats at these regions (De Jeu *et al.* 1997). The large chromosomes, combined with the ability to differentiate parental genomes make *Alstroemeria* an interesting species for introgression research.

Scope of this thesis

The aim of this thesis was to gain better insight into the fate of *Alstroemeria* chromosomes in a recurrent backcross procedure in order to study the process of introgression. For this, the genomes of two distantly related species *A. aurea* and *A. inodora* were characterised using GISH and FISH with several probes in such a detail that the genome constitution of interspecific hybrids and their backcross offspring can be analysed at the individual chromosome level. Thus the consequences of distant interspecific hybridization and backcrossing in *Alstroemeria* can be elucidated. In addition, both techniques were also applied to assess the frequency of homoeologous chromosome pairing in the F_1 hybrid and the backcross progenies, and also the types of gametes which are produced in the F_1 hybrids and the backcross progenies were evaluated in order to predict the consequences of these phenomena for introgression in *Alstroemeria*. The species *A. aurea* is assumed to possesses virus resistance. In order to transfer this characteristic into *A. inodora*, the F_1 hybrid was backcrossed with *A. inodora*. Both *A. aurea* and *A. inodora* are used extensively in *Alstroemeria* breeding (Buitendijk, 1998). The hybrids between these two species are highly sterile, producing very low amounts of stainable pollen, making it difficult to backcross them.

In chapter 2, the physical localisation of four different repetitive DNA sequences on somatic chromosomes of the species *A. aurea* and *A. inodora* is described in order to identify all individual chromosomes. The reliability of chromosome identification was tested in the interspecific hybrid between the two species.

Chapter 3 describes the results on the successful backcrossing of the highly sterile F_1 hybrid *A. aurea* x *A. inodora* to the parent *A. inodora* (BC_1). In addition to confirming the cytological constitution of the BC_1 plants through FISH and establishing the types of gametes that were functional in the hybrid, metaphase I chromosome associations of the hybrid *A. aurea* x *A. inodora* were studied to reveal the occurrence of homoeologous chromosome pairing and recombination.

In chapter 4, results on sequential FISH and GISH analysis of somatic chromosomes of the BC_1 plants are described enabling the construction of detailed karyotypes of all plants. Based on these karyotypes, the extent and position of homoeologous recombination events in the hybrid were determined. In addition, the recombinant chromosomes were used to determine which chromosomes of the two parental species were actually homoeologous.

Chapter 5 describes the meiotic behaviour of all individual chromosomes in four allotriploid BC_1 plants, all of which contained two genomes of *A. inodora* and one of *A. aurea*. Special attention was paid to the (preferential) pairing behaviour of homologous chromosomes and to the behaviour of recombinant chromosomes as compared to non-recombinant ones. The consequences of the pairing behaviour of particular chromosomes on the distribution during the later stages of meiosis and gamete constitution were determined.

In chapter 6, the chromosome organisation of the two species *A. aurea* and *A. inodora* as revealed by molecular cytogenetics, the use of GISH and FISH to study meiosis and homoeologous recombination in hybrids and their offspring, and the impact of GISH and FISH for enhancing the efficiency and speed of introgression are discussed in relation to the results obtained on *Alstroemeria*.

2 Physical localisation of repetitive DNA sequences in *Alstroemeria*: karyotyping of two species with species-specific and ribosomal DNA

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ABSTRACT

Fluorescence *in situ* hybridization (FISH) was used to localise two species-specific repetitive DNA sequences, A001-I and D32-13, and two highly conserved 25S and 5S rDNA sequences on the metaphase chromosomes of two species of *Alstroemeria*. The Chilean species, *Alstroemeria aurea* ($2n$=16), has abundant constitutive heterochromatin, whereas the Brazilian species, *Alstroemeria inodora*, has hardly any heterochromatin. The *A. aurea* specific A001-I probe hybridized specifically to the C-band regions on all chromosomes. The FISH patterns on *A. inodora* chromosomes using species-specific probe D32-13 resembled the C-banding pattern and the A001-I pattern on *A. aurea* chromosomes. There were notable differences in number and distribution of rDNA sites between the two species. The 25S rDNA probe revealed 16 sites in *A. aurea* that closely colocalised with A001-I sites and 12 sites in *A. inodora* that were predominantly detected in the centromeric regions. FISH karyotypes of the two *Alstroemeria* species were constructed accordingly, enabling full identification of all individual chromosomes. These FISH karyotypes will be useful for monitoring the chromosomes of both *Alstroemeria* species in hybrids and backcross derivatives.

INTRODUCTION

The genus *Alstroemeria* is endemic in Chile and Brazil. All species known at present are diploid ($2n=2x=16$) and possess large chromosomes (4-20 μm) and a large amount of nuclear DNA (ranging from 36.5 to 78.9 pg/2C nucleus) (Buitendijk *et al.* 1997). Despite this large variation in DNA content, most species show fairly similar karyotypes consisting of four pairs of (sub) metacentric and four pairs of acrocentric chromosomes (Rustanius *et al.* 1991; Stephens *et al.* 1993; Buitendijk and Ramanna 1996). Some of the Chilean species contain conspicuous C-bands consisting of constitutive heterochromatin. Despite comparable genome sizes, the four Brazilian species investigated so far lack prominent C-bands (Buitendijk and Ramanna 1996; unpublished data). Of the species used in the present study, the Brazilian species *Alstroemeria inodora* has no C-bands but has a genome size similar to that of *Alstroemeria aurea*, a Chilean species with prominent C-bands.

In most *Alstroemeria* species, only four pairs of chromosomes (1, 2, 7, and 8) can be unequivocally identified on the basis of length, arm ratios, and C-banding pattern. In interspecific hybrids, owing to the similar karyotypes of the parental species, it is not even always possible to identify individual chromosomes, although genomic *in situ* hybridization can distinguish the parental genomes in hybrids between Chilean and Brazilian species (Kuipers *et al.* 1997). However, fluorescence *in situ* hybridization (FISH) has been used in many plants to accurately identify chromosomes, using species-specific repetitive sequences, ribosomal genes, and even unique sequences (Leitch and Heslop-Harrison 1991; Anamthawat-Jónsson and Heslop-Harrison 1993; Mukai *et al.* 1990, 1991; Linares *et al.* 1996; Lubaretz *et al.* 1996; Jiang and Gill 1994; among others). The large genome sizes of the *Alstroemeria* species indicate the presence of vast amounts of repetitive DNA sequences in these genomes. However, not much is known about these sequences and their chromosomal locations. species-specific repetitive sequences have been isolated from several *Alstroemeria* species (De Jeu *et al.* 1997) and these can be used as probes in FISH experiments to determine their chromosomal location. The physical location of these sequences can be useful for genome and chromosome identification in, for example, interspecific hybrids and backcross derivatives.

In this study, we applied FISH to the Chilean and the Brazilian species, *A. aurea* and *A. inodora*, respectively. These species are extensively used in *Alstroemeria* cut flower breeding

programs. The specific aims of the present investigation were (i) to develop FISH karyotypes of these species by using two species-specific DNA sequences and the 25S and 5S ribosomal RNA genes (rDNA) as probes; and (ii) to verify the characteristic FISH patterns as diagnostic tools for chromosome identification in the hybrid *A. aurea* x *A. inodora*.

MATERIAL AND METHODS

Plant material

Two accessions of *A. aurea* Graham (A1 and A3), one accession of *A. inodora* Herb. (P2), and the interspecific hybrid *A. aurea* x *A. inodora* (A1P2-2) were used in this investigation. Details of the accessions and the hybrid origin are described elsewhere (Buitendijk and Ramanna 1996).

DNA probes

The species-specific repetitive DNA sequences D32-13 and A001-I contain a 191-bp sequence specific for *A. inodora* and a 217-bp insert specific for *A. aurea* (De Jeu *et al.* 1997; unpublished data). The two rDNA sequences were the 5S rDNA pBC1.1 with a 497-bp sequence of *Arabidobsis thaliana* (Cambell *et al.* 1992) and the 25S rDNA with a 700-bp fragment of *Petunia* (van Blokland *et al.* 1994).

For multicolour FISH experiments, all sequences were labelled either by nick translation according to the instructions of the manufacturer (Boehringer Mannheim), or by PCR labelling (De Jeu *et al.* 1997) with either biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim).

FISH (Fluorescent *in situ* hybridization)

FISH was performed according to Kuipers *et al.* (1996), with some modifications. Chromosome preparations were pretreated with RNase A (100 µg/ml) and pepsin (5 µg/ml), fixed with 4% (w/v) paraformaldehyde for 10 min, dehydrated in an ethanol series, and air-dried. The hybridization mixtures contained 50% deionized formamide, 10% (w/v) sodium dextran sulphate, 2 x SSC (1x SSC: 0.15 M NaCl plus 0.015 M sodium citrate), 0.25% (w/v) SDS, 1.25-2.5 ng/µl of each differently labelled probe, and 4 µg/µl sonicated herring sperm DNA. The mixtures were denatured for 10 min at 70°C, and then placed on ice for at least 5 min; 40 µl of the hybridization mixture was applied per slide. Slides were denatured at 85°C for 5 min and hybridization was performed for at least 16 h at 37°C in a humid chamber. The hybridization stringency was about

77%. The slides were washed for 30 min in 2 x SSC, for 15 min in 0.1 x SSC at 42°C, for 30 min in 2 x SSC, and then for 5 min in buffer 1 (0.1 M Tris HCl plus 0.15 M NaCl, pH 7.5).

Digoxigenin-11-dUTP was detected with 20 µg/ml anti-dig-FITC (fluorescein isothiocyanate; Boehringer Mannheim) and 20 µg/ml rabbit-anti-sheep-FITC (Vector Laboratories). Biotin-16-dUTP was detected with 4 µg/ml streptavidin-Cy3 (Jackson Immuno Research Laboratories), 10 µg/ml biotionylated-anti-streptavidin (Vector Laboratories), and again 4 µg/ml streptavidin-Cy3. For each detection step the slides were incubated with 200 µl blocking buffer (1% blocking reagent (Boehringer Mannheim) in buffer 1) for 5 min, incubated for 1 h with the appropriate antibodies in 100 µl blocking buffer, and washed 3 times in buffer 1 at 37°C. Preparations were counterstained with 1 µg/ml DAPI (4,6-diamidino-2-phenylindole) and mounted in 5 µl of Vectashield (Vector Laboratories). Slides were examined with a Zeiss Axiophot fluorescence microscope with appropriate filter sets for DAPI, FITC, and Cy3. Photographs were taken on 400 ASA colour negative film. All double and triple exposures were made with a triple band pass filter, using separate excitation filters for each fluorochrome. Negatives were scanned at 500 dpi and the digital images optimized for contrast and brightness using routine image processing software.

Sequential hybridization of slides

Cover glasses were carefully removed after FISH by washing for 1-4 h in 4 x SSC with 0.2% Tween 20. Probe DNA was dissociated from the chromosomes with 70% formamide in 2 x SSC at 70°C for 3-5 min. Slides were then dehydrated for 3 min in each 70, 90, and 100% ethanol and air-dried. New hybridization mixture (40 µl) was then added to the slides, followed by denaturation and detection steps as described above.

C-banding following FISH

After removing the cover glasses, the preparations could be C-banded following refixing in ethanol-acetic acid (3:1) for 1 h and 100% ethanol for 1 h, and air-drying overnight at 37°C. C-banding was performed according to Buitendijk and Ramanna (1996).

Silver staining

To detect the actively transcribed ribosmal sites, chromosome preparations were silver stained as described by De Jong *et al.* (1991).

RESULTS

Chromosome differentiation through banding techniques and FISH

Alstroemeria aurea

The karyotype of *A. aurea* (accession A1) was investigated for structural differentiation of individual chromosomes through banding techniques, as well as for *in situ* hybridization patterns of repetitive DNA sequences. Chromosome banding was performed with C-banding, DAPI-fluorochrome staining, and silver staining. Sequential staining of the same chromosome preparations revealed that the patterns obtained with C-banding and DAPI-fluorochrome staining were identical; therefore, only the C-banding patterns are depicted in Figure 2.1. Prominent C-bands were present in all chromosomes, except for chromosome 1. Among the C-banded chromosomes, the three (sub)metacentrics had terminal bands either on one (chromosome 2) or both (chromosomes 7 and 8) arms, whereas the four acrocentrics (chromosomes 3, 4, 5, and 6) had only prominent interstitial bands (Figures 2.1 and 2.2a).

The banding pattern resulting from silver staining corresponded to the C-bands. Additionally, the short arms of the four acrocentrics that lacked C-bands (chromosomes 3, 4, 5, and 6) showed clear silver deposits upon AgNO3 incubation. The presence of many silver-staining sites in the genome of *A. aurea* was also evident from the interphase nuclei. Since the banding patterns resulting from silver staining of metaphase chromosomes colocalised completely with the *in situ* hybridization patterns of 25S rDNA sequences (discussed below), these sites are not shown separately in Figure 2.1.

In situ hybridization patterns on *A. aurea* chromosomes were investigated for three repetitive DNA sequences, viz., the *A. aurea* specific satellite repeat A001-I and the 25S and 5S ribosomal repeats. Sequential hybridization with these repetitive probes and C-banding revealed that the A001-I probe specifically hybridized to regions corresponding to all the C-bands of *A. aurea* (Figures 2.1 and 2.2b). The 25S rDNA probe hybridized to all C-banding regions in addition to the short arms of all acrocentric chromosomes (Figures 2.1 and 2.2c). One of the interstitial C-bands on the long arm of chromosome 4 (4L) did not show any signal. In all, there were 16 different 25S rDNA sites present in *A. aurea*. The hybridization pattern of the 5S rDNA probe was quite different. Four hybridization sites were detected: one major site on each of the

chromosomes 2L and 5L, and one minor site on each of the chromosomes 7L and 8L (Figures 2.1a and 2.2c).

Besides *A. aurea* accession A1, a different accession, A3, was also hybridized with the A001-I, 25S rDNA, and 5S rDNA probes for verification of the hybridization patterns. The patterns were similar in the two *A. aurea* accessions, although some differences in the hybridization patterns were observed. Two additional minor polymorphic hybridization sites on chromosomes 1L and 2S (Figure 2.2b, arrows) were found in accession A3. In this accession, an inversion was present in chromosome 5 (Figure 2.2b, arrows).

Alstroemeria inodora

Compared with the karyotype of *A. aurea*, completely different banding patterns were obtained for *A. inodora* with C-banding, DAPI-fluorochrome staining, and silver staining. A prominent feature was that the large blocks of interstitial and terminal C-bands observed in *A. aurea* were absent in *A. inodora*. Instead, small and faintly stained C-bands were present only in the proximal parts of all *A. inodora* chromosomes (Figure 2.1b).

DAPI-fluorochrome staining revealed dull fluorescence of these proximal bands and one clear interstitial band on chromosome 3L (arrow in Figures 2.1b and 2.2d). Upon silver staining, interphase nuclei showed from one to three conspicuous nucleoli. At prometaphase, one large nucleolus-like structure was associated with several chromosomes. As a result of this association, no banding patterns of individual chromosomes were obtained with silver staining.

Using FISH, the physical localisation of three different repetitive sequences, D32-13, 25S rDNA, and 5S rDNA, was determined. The species-specific probe D32-13 hybridized to all chromosomes either at interstitial or distal sites (Figures 2.1b and 2.2e). The distal hybridization sites were present on one acrocentric and on all (sub)metacentric chromosomes, either on one (chromosomes 1, 2, 6, and 8) or both (chromosome 7) arms. The interstitial hybridization sites were found on three acrocentric (chromosomes 3, 4 and 5) and one submetacentric (chromosome 2) chromosomes.

The 25S rDNA probe hybridized to all chromosomes of *A. inodora*. Proximal hybridization sites were found on all chromosomes, with the exception of chromosome 1. Additional distal hybridization sites were detected on four chromosomes (1, 3, 4, and 7) (Figures 2.1b and 2.2f). In total, 12 different 25S rDNA sites were found in *A. inodora*.

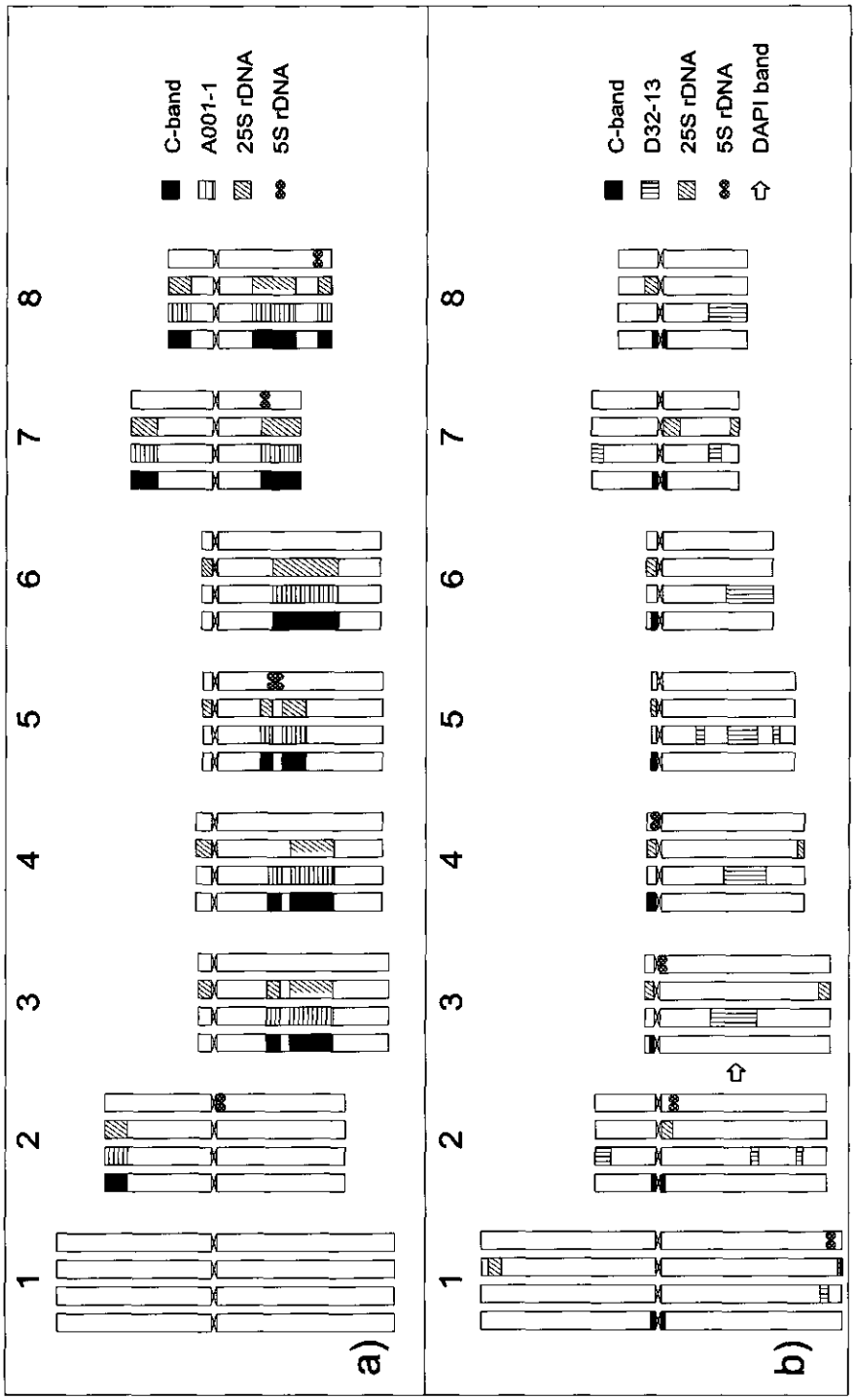

Figure 2.1: FISH karyotypes of *A. aurea* accession A1 (a) and *A. inodora* accession P2 (b). The four specimens of each of the eight chromosomes in the two karyotypes show C-banding pattern, and FISH with the species-specific sequences (either A001-I or D32-13), 25S rDNA, and 5S rDNA (see legends). The chromosomes are in order of descending length.

With regard to 5S rDNA, two major sites (chromosomes 2 and 4) and two minor sites (chromosomes 1 and 3) were detected on the chromosomes of *A. inodora*.

FISH patterns of all probes in the hybrid

To verify the suitability of the hybridization patterns of the probes for chromosome identification, all probes were hybridized to the chromosomes of the F_1 hybrid of *A. aurea* x *A. inodora*. The species-specificity of probes A001-I and D32-13 was deduced from the fact that no cross-hybridization was observed. This allowed a clear differentiation of the two genomes in the hybrid (Figure 2.2g). Based on the hybridization patterns of the species-specific probes, all individual chromosomes were clearly identified in the hybrid, except for chromosome 1 of each parental species (Figure 2.2g). Also, chromosomes 3 and 4 of *A. aurea* were not clearly identified. However, the ribosomal probes, especially 25S rDNA, allowed clear identification of all the individual chromosomes of both species. The hybridization sites of D32-13, 25S rDNA, and 5S rDNA on chromosome 1 of *A. inodora* distinguished this chromosome from chromosome 1 *A. aurea*, which lacked hybridization sites of any of the probes (Figures 2.1, 2.2g and 2.2i). Although chromosomes 3 and 4 of *A. aurea* could not be identified after hybridization with the A001-I probe, the 25S rDNA probe could distinguish these two chromosomes based on the number of hybridization sites (Figures 2.1a and 2.2i). The prominent 5S rDNA site on chromosome 5 served as a diagnostic marker for this chromosome.

Along with metaphase chromosomes, interphase nuclei revealed remarkable differences in the hybridization patterns of the two species-specific probes A001-I and D32-13. Whereas the hybridization of D32-13 was dispersed throughout the nucleus (Figure 2.2h; red, Cy3), that of A001-I was condensed into large blocks in the nucleus (Figure 2.2h; green, FITC).

DISCUSSION

Our FISH karyotypes of *A. aurea* and *A. inodora* were based on the distributions of two species-specific repeats and the 5S and 25S ribosomal repeats. Their combined patterns were sufficiently differentiated to allow full identification of all chromosomes in the metaphase complements. As to *A. aurea*, where pronounced C-band patterns distinguish only six of its eight chromosome pairs (Buitendijk and Ramanna 1996), the combined hybridization patterns of the 25S rDNA probe and the A001-I probe on acrocentric chromosomes 3 and 4 were suffi-

cient to identify all chromosome pairs in the karyotype. Furthermore, the 5S rDNA sites on chromosomes 2 and 5 provided additional markers for these chromosomes.

In *A. inodora*, a species that lacks prominent C-bands, the karyotype requires FISH signals from either the species-specific probe D32-13 or the 25S rDNA probe to allow full identification of all chromosomes. As these probes showed dissimilar distributions of hybridization sites on individual chromosomes, they could serve as FISH markers for specific chromosome regions. The 5S rDNA sites did not reveal any diagnostic markers, as those observed were quite small and not always detectable.

In addition to identifying all chromosomes of both species, several interesting remarks can be made about the physical location of the repeats in both species.

FISH with the species-specific probes revealed strikingly similar patterns in the two species. These repetitive probes were found at interstitial sites on the acrocentric chromosomes and at terminal sites on the (sub)metacentric chromosomes. Although the two *Alstroemeria* species are distantly related, one being Chilean and the other Brazilian, these results indicate that their chromosomal composition and organisation is similar. Moreover, the physical distances between the species-specific sites and the centromeres are comparable in most chromosomes. Consequently, in both species, distinct blocks of species-specific repetitive DNA are present at specific sites in their genomes. These observations support the suggestion that distinct classes of repetitive DNA are dispersed within plant genomes in a nonrandom fashion (Flavell 1982; Kenton 1991; Pedersen and Linde-Laursen 1994; Schweizer and Loidl 1987).

In contrast to the hybridization patterns of the species-specific probes, which were similar in the two species, the patterns of the ribosomal probes showed remarkable differences between them. In *A. aurea*, the 25S rDNA probe hybridized at proximal and interstitial sites on all acrocentric chromosomes and at distal sites on the metacentric chromosomes. However, in *A. inodora*, the 25S rDNA probe was found only at distal and proximal sites on the chromosomes. Of the 16 25S rDNA sites in *A. aurea* and the 12 25S rDNA sites in *A. inodora* only 4 were shared by both species. The number and location of the 25S rDNA sites varied considerably between the species. In *A. aurea* and *A. inodora*, four 5S rDNA sites were detected. Of these 5S rDNA sites, only one is present in both species. Variation in the number and location of rDNA sites between species (or tribes) has been reported for other plants (Castilho and Heslop-Harrison 1995; Maluszynska and Heslop-Harrison 1993; Ørgaard and Heslop-Harrison 1994; Badaeva *et al.* 1996; among others). Schubert and Wobus (1985) have described the

so-called "jumping" NORs in interspecific *Allium* hybrids. The rDNA sites that are variable between the two *Alstroemeria* species could be the result of a similar mechanism.

The number of rDNA sites has been determined in many plant species, including barley (Leitch and Heslop-Harrison 1991), wheat (Mukai *et al.* 1991), *Allium* (Ricroch *et al.* 1992), rye (Cuadrado *et al.* 1995), and *Brassica* (Maluszynska and Heslop-Harrison 1993). In these studies, rDNA sites were confined to one or a few chromosome pairs, in contrast to the *Alstroemeria* species, where the 16 sites detected in *A. aurea* and the 12 sites detected in *A. inodora*, were observed on all chromosomes. Furthermore, in *A. aurea* all the rDNA sites were detected with silver staining, indicating that the ribosomal sequences at all these sites are actively transcribed. This is in contrast to most plants species, where usually only one or a few ribosomal sites are actively transcribed.

A striking feature revealed by *in situ* hybridization in *A. aurea* is the colocalisation on metaphase chromosomes of the A001-I repeat and the 25S rDNA at the heterochromatin bands. Hybridization of rDNA at the heterochromatin bands has been reported in other plant species, such as *Zingeria biebersteiniana* (Bennett *et al.* 1995) and barley (Pedersen and Linde-Larson 1994). It seems that during the course of evolution of *A. aurea*, two totally different types of repetitive sequences have accumulated in the same chromosomal regions. Moreover, both nontranscribing (A001-I) and transcribing (25S rDNA) sequences, as revealed by silver staining, are present in these regions. More information about the colocalisation of the species-specific A001-I and 25S rDNA sequences is to be expected from FISH to extended DNA fibres of interphase nuclei (Fransz *et al.* 1996).

The physical localisation of repetitive sequences in *Alstroemeria* enabled the identification of all chromosomes in an interspecific hybrid between *A. aurea* and *A. inodora*. Both species-specific probes could be used in multicolour FISH experiments to specifically identify the *A. aurea* and *A. inodora* chromosomes, and the ribosomal sequences provided additional chromosome markers. This type of differentiation of parental chromosomes in an interspecific hybrid in combination with genomic *in situ* hybridzation (Kuipers *et al.* 1997) offers unparalleled opportunity to accurately detect and elucidate the consequences of introgression in *Alstroemeria*. Furthermore, to assess homoeologous chromosome pairing and crossing-over during meiosis in the interspecific hybrids, simultaneous hybridization of the species-specific sequences will be most valuable. With the long-term objective of elucidating the consequences

of the introgression of complete chromosomes or chromosome fragments of *A. aurea* and *A. inodora*, the interspecific hybrid A1P2-2 will be backcrossed to the parents.

3 Homoeologous chromosome pairing in the distant hybrid *Alstroemeria aurea x A. inodora* and the genome composition of its backcross derivatives determined by fluorescent *in situ* hybridization with species-specific probes

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ABSTRACT

A distant hybrid between two diploid species ($2n=2x=16$), *Alstroemeria aurea and A. inodora*, was investigated for homoeologous chromosome pairing, crossability with *A. inodora* and chromosome transmission to its BC_1 offspring. Fluorescent *in situ* hybridization (FISH) with two species-specific probes, A001-I (*A. aurea*-specific) and D32-13 (*A. inodora*-specific), was used to analyse chromosome pairing in the hybrid and the genome constitution of its BC_1 progeny plants. High frequencies of associated chromosomes were observed in both genotypes of the F_1 hybrid, A1P2-2 and A1P4. In the former, both univalents and bivalents at metaphase I were found, whereas the latter plant also showed tri- and quadrivalents. Based on the hybridization sites of DNA probes on the chromosomes of both parental species, it was established that hybrid A1P4 contains a reciprocal translocation between the short arm of chromosome 1 and the long arm of chromosome 8 of *A. inodora*. Despite regular homoeologous chromosome pairing in 30% of the pollen mother cells, both hybrids were highly sterile. They were backcrossed reciprocally with one of the parental species, *A. inodora*. Two days after pollination, embryo rescue was applied and eventually six BC_1 progeny plants were obtained. Among these, two were aneuploids ($2n=2x+1=17$) and four were triploids ($2n=3x=24$). The aneuploid

plants had originated when the interspecific hybrid was used as a female parent indicating that *n*-eggs were functional in the hybrid. In addition, 2*n*-gametes were also functional in the hybrid, resulting in the four triploid BC_1 plants. Of these four plants, three had received 2*n*-pollen grains from the hybrid and one a 2*n*-egg. Using FISH, homoeologous crossing-over between the chromosomes of the two parental species in the hybrid was clearly detected in all BC_1 plants. The relevance of these results for the process of introgression, and the origin of *n*- and 2*n*-gametes are discussed.

INTRODUCTION

More than a hundred species have so far been listed in the genus *Alstroemeria*. Most of them occur in two geographically distant South American regions, namely Chile and Brazil (Aker and Healy, 1990). Most of the species are diploid ($2n=2x=16$), possess large chromosomes and asymmetric karyotypes (Tsuchiya and Hang, 1989; Buitendijk and Ramanna, 1996) and have large genome sizes (Buitendijk *et al.* 1997). The genomes of the species are highly differentiated, especially between Chilean and Brazilian species. Such differentiation is evident at three levels: (i) sexual hybridization between species generally is not possible without the help of embryo rescue (Buitendijk *et al*,. 1995; De Jeu and Jacobsen, 1995); (ii) compared to homologous chromosome pairing in the parental species, homoeologous chromosome pairing in the interspecific hybrids is highly reduced (Ramanna, 1991, 1992; Lu and Bridgen, 1997); (iii) the genomes of individual species clearly differ with respect to the molecular organization of repetitive DNA sequences among the Chilean species as well as between the Chilean and Brazilian species (Kuipers *et al.* 1997).

Once obtained, interspecific *Alstroemeria* hybrids usually are vigorous and also highly sterile, producing no or very few viable pollen grains (Goemans, 1962; Ramanna and Buitendijk, unpubl. obs.). In these hybrids a disturbed meiosis is often observed, which is probably associated with the high degree of sterility. Nevertheless, viable gametes occur rarely in the hybrids and have been used by the cut flower breeders to produce numerous, mainly complex polyploid cultivars (Ramanna, 1991). The viable gametes can also be used in breeding programs in order to, for example, introgress alien chromosomes or chromosome segments into other species. Because one of the most reliable ways to achieve stable introgression of desired characteristics is homoeologous recombination, it is interesting to study whether

homoeologous chromosome pairing and recombination occur in interspecific *Alstroemeria* hybrids.

The large and easily identifiable chromosomes of the *Alstroemeria* species contain multiple ribosomal DNA sites and sites for species-specific tandem repeats which can be used as cytological markers to identify individual chromosomes and genomes (Kamstra *et al.* 1997). For *A. aurea*, a Chilean species, and *A. inodora*, a Brazilian species, FISH karyotypes were constructed using two species-specific probes and two ribosomal probes which allowed identification of all individual chromosomes (Kamstra *et al.* 1997). The species-specific probes, A001-I, specific for *A. aurea*, and D32-13, specific for *A. inodora*, can be used simultaneously in FISH experiments to identify the parental genomes and chromosomes in the interspecific hybrid.

In the present study, we have used FISH with two species-specific probes to analyse homoeologous chromosome pairing in interspecific hybrids between the Chilean species *A. aurea* and the Brazilian species *A. inodora*. With the long-term objective to introgress segments of *A. aurea* into *A. inodora*, it is essential to know whether, and in what frequency, recombination occurs between homoeologous chromosomes because this is a prerequisite for stable introgression. In addition, the interspecific hybrid was backcrossed to *A. inodora*. The backcross progeny was analysed with FISH to test whether these were the result of the backcross and to gain more insight into the type of viable gametes that are produced in this highly sterile hybrid.

MATERIALS AND METHODS

Plant material and backcrossing of the interspecific hybrid

The accessions A1P2-2 and A1P4 of the hybrid *A. aurea* x *A. inodora* were produced through embryo rescue techniques (Buitendijk *et al.* 1995). These hybrids were backcrossed with the *A. inodora* parent (P2 or P4, respectively). The hybrids and *A. inodora* were diploid ($2n=2x=16$). Two days after pollination, embryo rescue was applied as described by De Jeu and Jacobsen (1995). The ovules were dissected 2-3 days after pollination, placed on MS90 medium (Murashige and Skoog, 1962), and six weeks later transferred to MS30 (Murashige and Skoog, 1962) to induce germination of the embryos. Germinated ovules were transferred to AV4 medium (MS salts, 20 g/L sucrose, 0.5 mg/L 6-benzylaminopurine (BAP), pH 6.2) and the plants obtained were grown in a greenhouse.

Chromosome preparations

For meiotic preparations, anthers of the hybrids A1P2-2 and A1P4 were fixed for 1-2 h in ethanol: acetic acid (3:1) and stored in 70% ethanol until use. Anthers containing pollen mother cells (PMCs) were digested with pectolytic enzymes (0.4% pectolyase Y23, 0.4% cellulase RS and 0.4% cytohelicase) for 2-3 h at 37°C. The macerated anthers were squashed in 45% acetic acid or 2% acetocarmine. The preparations were frozen in liquid nitrogen and after removal of the coverslips, briefly washed in absolute ethanol, air-dried and stored at 4°C until further use.

For mitotic preparations, fast growing root tips of backcross plants were collected in 8-hydroxyquinoline, stored overnight at 4°C and fixed in ethanol: acetic acid (3:1). Chromosome preparations were made as described by Kuipers *et al.* (1997). Chromosome numbers were counted in all BC_1 plants obtained.

Lactophenol-acid-fuchsin stain was used to estimate the frequency of viable pollen grains in the hybrids and some BC_1 plants. At least 500 pollen grains were counted per plant. Only fully stained pollen grains were scored as viable.

DNA probes and labelling

Two species-specific DNA sequences were used in this study to identify the parental chromosomes in the F_1 hybrid and its derivatives. The probes, A001-I and D32-13, specific for *A. aurea* and *A. inodora*, respectively, (De Jeu *et al.* 1997; Kamstra *et al.* 1997; Kuipers *et al.* 1999) hybridize to all chromosomes of either *A. aurea* or *A. inodora* (Kamstra *et al.* 1997), except for chromosome 1 which was detected by neither probe. The hybridization patterns for both probes on each individual chromosome of the two species are indicated in Figure 3.1. In addition, two ribosomal probes pTa71(18S-5.8S-25S rDNA; Gerlach and Bedbrook, 1979) and pTa794 (5S rDNA; Gerlach and Dyer, 1980) were used in some experiments to study the chromosomes in more detail.

All probes were labelled by PCR with either digoxigenin-11-dUTP or biotin-16-dUTP (both from Boehringer Mannheim) except pTa71, which was labelled by nick translation with biotin-16-dUTP.

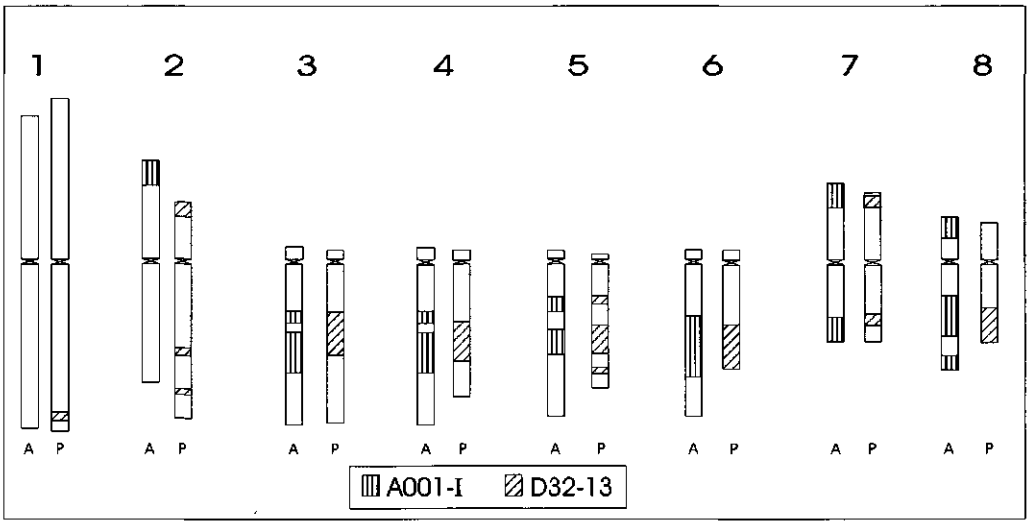


Figure 3.1: Ideogram showing the hybridization patterns of both species-specific probes A001-I and D32-13 for each individual chromosome of A. aurea (A) and A. inodora (P) (For details see Kamstra et al. 1997).

Fluorescent *in situ* hybridization (FISH)

The pretreatment and hybridization steps applied to meiotic and mitotic preparations were performed as described in Kamstra *et al.* (1997). For meiotic preparations the pretreatment with pepsin was increased from 10 min to 25 min at 37°C. Digoxigenin-11-dUTP and biotin-16-dUTP labelled probes were detected with FITC- and Cy3-conjugated antibodies, respectively (for details see Kamstra *et al.* 1997).

Chromosome preparations were counterstained with DAPI (4',6-diamidino-2'-phenylindole). Slides were examined using a Zeiss Axiophot fluorescence microscope and photographs were taken on 400 ASA colour negative film. All triple exposures were made with a triple band pass filter for DAPI, FITC and Cy3, using separate excitation filters for each fluorochrome. Negatives were scanned at 500 dpi and processed using routine image processing software.

RESULTS

Meiotic behaviour and crossability of the F_1 hybrid *A. aurea* x *A. inodora*

FISH with the probes A001-I and D32-13 to spread preparations of pollen mother cells revealed a clear distinction of the *A. aurea* and *A. inodora* chromosomes (Figure 3.2a-f). As a result, homoeologous associations as well as univalents could be identified. Most surprising in A1P2-2 was the large variation in homoeologous associations ranging from two to eight bivalents per complement. The average number of bivalents per pollen mother cell (PMC) was 6.7.

In 30% of the PMCs the maximum number of eight bivalents (Table 3.1) was observed, indicating that every A. aurea chromosome was associated with its homoeologous counterpart in the A. inodora genome. In some cells univalents were found to which both species-specific probes hybridized (Figure 3.2b, yellow arrowheads), indicating that these resulted from precocious separation of bivalents .

Unlike A1P2-2, pollen mother cells of A1P4 often contained one multivalent chromosome association, consisting of a tri- or quadrivalent (Figure 3.2e-f; Table 3.1). An obvious explanation for these multivalents was the presence of a reciprocal translocation, because the same (homoeologous) pairs of chromosomes 1 and 8 of *A. aurea* and *A. inodora* were always involved. Confirmation came from FISH analysis using the species-specific probes and both ribosomal probes (pTa71 and pTa794) to mitotic preparations as this revealed two normal and two translocation chromosomes. Identification was based on the following criteria (see Kamstra *et al.* 1997).

(1) No hybridization sites for any of the probes used were detected on chromosome 1 of *A. aurea*. In addition, the morphology of this chromosome was not altered.

(2) Chromosome 1 of *A. inodora* (1P) normally containing the minor D32-13 and pTa794 hybridization sites on the long arm and a diagnostic pTa71 site on the short arm, underwent two morphological alterations. One of its arms became shorter (Figure 3.2g-h) and a prominent D32-13 site was observed on this arm, whereas the expected pTa71 site was not present.

(3) Chromosome 8 of *A. aurea* (8A) retained its original shape with three hybridization sites for A001-I.

(4) Chromosome 8 of *A. inodora* (8P) was morphologically altered with a long arm about twice as long compared to the parental species. On this long arm a distal pTa71 hybridization site was detected instead of the expected D32-13 site (Figure 3.2g-h).

Table 3.1: Chromosome associations at metaphase I in two plants of the F_1 hybrid, *Alstroemeria aurea x A. inodora*, analysed by FISH

Hybrid	No. of cells analysed	% of cells with complete chromosome pairing	Aaverage number of chromosome configurations per metaphase I			
			I	II	III	IV
A1P2-2	167	30[a]	2.5	6.7	-	-
A1P4	15	20[b]	2.1	5.7	0.4	0.3

a) Percentage of cells containing eight bivalents.
b) Percentage of cells containing eight bivalents or six bivalents and one quadrivalent (see text).

Both hybrids A1P2-2 and A1P4 had a very low frequency (3%) of stainable pollen indicating that they were highly male-sterile. Nevertheless, a fairly extensive attempt was made to backcross both genotypes to the parent *A. inodora* (Table 3.2). Hybrid A1P2-2 was used both as female and as male parent in combination with accession P2. After 6 weeks of ovule culture, a few ovules germinated and produced callus structures; however, most of these structures eventually died. Six plants were obtained from the backcrosses of the *A. aurea* x *A. inodora* hybrid (A1P2-2); three each in both directions of crossing. All were grown to maturity in a greenhouse where some of them flowered after 18 months from the time the initial crosses were made. Unlike the F_1 hybrid, the pollen stainability in these BC_1 plants was considerably higher (19% in 96SK402, 22% in 95SK04 and 58% in 95SK17). In contrast to A1P2-2, backcrossing was not successful in the case of A1P4.

Analysis of BC_1 plants

The somatic chromosome numbers were determined in all six BC_1 plants. Four plants possessed triploid chromosome numbers ($2n=3x=24$) and two were aneuploids ($2n=2x+1=17$) (Table 3.3). Considering the chromosome numbers (Figure 3.3a-f), it was evident that all plants were backcross derivatives. Furthermore, the different ploidy levels of the six progeny plants indicated that both *n*- and *2n*-gametes were functional in these crosses. The two aneuploid plants, 95SK11 and 95SK18, had originated from the cross in which the F_1 hybrid, A1P2-2, was used as the female, indicating that haploid gametes were functional in the F_1 hybrid.

Table 3.2: Parentage and the origin of six BC_1 plants produced through ovule culture from backcrossing the F_1 hybrid (*Alstroemeria aurea* x *A. inodora*) to the parent, *A.inodora*.

Female parent (code)	Male parent (code)	No. of crosses made	No. of germiated ovules	No. of BC_1 plants obtained	Codes of BC_1 plants
A. inodora (P2)	*A. aurea* x *A. inodora* (A1P2-2)	105	44	3	(95SK17, 96SK402, 95SK403)
A. aurea x *A. inodora* (A1P2-2)	*A. inodora* (P2)	107	7	3	(95SK04, 95SK18, 95SK11)
A. aurea x *A. inodora* (A1P4)	*A. inodora* (P4)	87	-	-	

The presence of chromosomes of both parental species in the BC_1 plants was confirmed with FISH using the two species-specific probes (Figure 3.3a-f). In all plants, except 95SK17, chromosomes were observed to which both species-specific probes hybridized (Figure 3.3a-f, arrows). These chromosomes are probably the result of meiotic recombination in the F_1 hybrid. The number of recombinant chromosomes ranged from none (in 95SK17) to five (in 95SK402) (Table 3.3). In addition, some of the other chromosomes of the BC_1 plants lacked expected hybridization sites, suggesting that these chromosomes were also recombinant (Table 3.3). FISH with species-specific probes on the triploid BC_1 plants proved that functional 2*n*-gametes were formed in the F_1 hybrid. In all these plants one intact genome of *A. inodora* was present, except for 95SK17 in which it seems that two complete genomes of *A. inodora* were present. One complete genome of *A. inodora* is expected because this was the recurrent backcross parent. Considering the direction of the backcrosses, 2*n*-pollen was functional in the case of 95SK17, 96SK402 and 96SK403 and 2*n*-eggs were functional in the case of 95SK04 (Table 3.3). In addition, both aneuploids were derived from functional *n*-eggs. One of the aneuploid plants, 95SK11, possessed spontaneously doubled somatic cells at a low frequency (Fig. 3.3a).

Table 3.3: Observed total number of chromosomes and the number of chromosomes containing hybridization signals for the species-specific probes in six BC_1 plants

BC_1 plant	No. of chromosomes (2*n*)	No. of chromosomes with			
		A001-I and D32-13	A001-I[a] only	D32-13[a] only	No signals[b]
Hybrid (A1P2-2)[c]	16	-	7	7	2
95SK11	17	1	3 (1)	12 (2)	1
95SK18	17	1	4 (1)	10 (1)	2
95SK17	24	0	6 (0)	15 (0)	3
95SK04	24	4	5 (1)	12 (1)	3
96SK402	24	5	3 (0)	13 (0)	3
96SK403	24	3	5 (0)	13 (0)	3

a) Numbers between brackets indicate the number of chromosomes with an unexpected number of hybridization sites.
b) In some cases a small signal for D32-13 probe was observed but the signal was not present in all cells analysed.
c) See Kamstra *et al.* (1997).
d) Frequently 34 chromosomes per cell were counted instead of 17 chromosomes.

Two of the BC_1 plants, 95SK04 and 95SK17, were successfully backcrossed to *A. inodora* (P2). This was an indication that BC_2 plants can be obtained through further backcrossing of the BC_1 plants.

DISCUSSION

In this study, we have shown that FISH with species-specific probes can be used to study chromosome associations in the interspecific hybrid *A. aurea* x *A. inodora* and to analyse its backcross (BC_1) progeny. The simultaneous detection of these two probes enabled us to distinguish chromosomes belonging to either parent of the hybrid, thus allowing an accurate estimation of the number of bivalents per PMC and showing homoeologous recombination.

The parental species, being of different geographical origin are highly diverged (Aker and Healy, 1990). For example, the variation in the physical location of the ribosomal DNA sites, the abundant presence of species-specific tandem repeats on the chromosomes of both *A. aurea* and *A. inodora* (Kamstra *et al.* 1997) and the variation at the repetitive DNA sequence level (Kuipers *et al.* 1997) indicate that the genomes/chromosomes have diverged considerably. In addition, using GISH it is possible to distinguish both parental genomes in the hybrid (Kuipers *et al.* 1997). Despite this high degree of genome differentiation, high frequencies of bivalents were observed at metaphase I (MI) of the interspecific hybrid. This indicates that a certain degree of homology is still present between the chromosomes of both parental species. This homology is sufficient to enable chromosome pairing, as up to eight bivalents were observed in 30% of the PMCs of the hybrid, suggesting that for each *A. aurea* chromosome a homoeologous counterpart is present in *A. inodora*. However, the morphological comparison of karyotypes of the chromosomes of the parental species (Kamstra *et al.* 1997) did not establish which chromosomes are homoeologous. Based on further analysis this was determined for all chromosomes of *A. aurea* and *A. inodora* (Kamstra *et al.* 1999b).

The observed variation in the number of associated chromosomes (range 2-8) per PMC combined with observed meiotic abnormalities such as laggards and bridges at anaphase I (data not shown), indicate that the chromosomes of both species have diverged considerably. Variation in the number of associated chromosomes at MI was also observed in other hybrids between *Alstroemeria* species (Tsuchiya and Hang, 1989; Ramanna, 1991; Lu and Bridgen, 1997).

A reciprocal translocation was present between chromosomes 1P and 8P in hybrid A1P4. This chromosome aberration was not present in the parent *A. inodora* (accession P4) and chromosome pairing in this parent seemed to be normal, suggesting that the translocation occurred spontaneously during either the process of embryo rescue or the prolonged duration of *in vitro* culture of the plant material. The presence of this translocation might explain to some extent why no progeny was obtained from the hybrid A1P4.

The high frequencies of homoeologous associated chromosomes per PMC indicate considerable recombination between homoeologous chromosomes of *A. aurea* and *A. inodora*. This was clearly visible at anaphase I stages (Figure 3.2c) and also in the karyotypes of the BC_1 plants (Figure 3.3a-f), because in the BC_1 plants several chromosomes possessed hybridization signals either for A001-I as well as D32-13 or altered hybridization patterns (Table 3.3). Therefore these species-specific probes are useful to detect homoeologous recombination. However, because of the lack of hybridization sites for the species-specific probes to certain chromosome regions, it is possible that some recombination events were not detected through FISH. The large number of these recombinant chromosomes in the BC_1 plants confirms that homoeologous recombination occurs in the interspecific hybrid. This is important for the introgression of (small) chromosome segments from *A. aurea* into *A. inodora*, which is one of the main objectives of producing these BC_1 plants.

Despite the low frequencies of stainable pollen grains in the interspecific hybrid, progeny from the interspecific hybrid *A. aurea* x *A. inodora* was obtained after extensive backcrossing and embryo rescue. Only a few plants were obtained from the 299 crosses in total (approximately 7500 ovules were cultured *in vitro*), indicating that the survival rate of the gametes is very low. However, from the number of germinated ovules that never developed into plants (Table 3.2), it can be concluded that some gametes actually do survive until this stage, but for unknown reasons they are not able to survive. Therefore, it is interesting to determine what kind of gametes actually survived and resulted in BC_1 progeny.

The two different ploidy levels (diploid and triploid) among the various BC_1 plants indicate that viable *n*- and 2*n*-gametes are produced in the hybrid. In both reciprocal crosses, using the hybrid either as male or female parent, plants were obtained, indicating that the hybrid produced viable male and female gametes. Both *n*- and 2*n*-eggs were functional when the hybrid was used as a female parent and only 2*n*-pollen grains were functional in the male

parent. However, the number of BC_1 plants is too small to conclude whether these observed differences between male and female gametes are meaningful.

Both diploid BC_1 plants, which received *n*-gametes from the hybrid, contain one additional chromosome ($2n=2x+1=17$). Probably this extra chromosome is the result of nondisjunction at metaphase I/anaphase I or a lagging chromosome during meiosis in the F_1 hybrid. These aneuploid plants showed abnormal development, which is probably caused by the presence of an extra chromosome.

The triploid BC_1 plants received 2*n*-gametes from the hybrid. In theory, these 2*n*-gametes could also have been produced in the backcross parent *A. inodora*. However, the backcross parent *A. inodora* produces regular-shaped pollen grains, which is indicative of *n*-gametes. Furthermore, in all BC_1 plants only one complete genome (eight different chromosomes) of *A. inodora* was detected with FISH (additional GISH studies showed that also BC_1 plant 95SK17 contains one complete genome of *A. inodora* (Kamstra *et al.* 1999b), instead of the two genomes that would result from 2*n*-gamete formation in the backcross parent *A. inodora*.

2*n*-gametes have been found in other interspecific *Alstroemeria* hybrids (Ramanna, 1992; Ramanna and Buitendijk, unpubl. obs.). Ramanna (1991) stated that the polyploid nature of the present cultivars is mainly the result of 2*n*-gamete formation in the hybrids. It is believed that because of failure of chromosome pairing in the interspecific hybrids, the 2*n*-gametes originate through an equational division of the entire chromosome complement of the hybrid (Ramanna, 1983). This is the first division restitution (FDR) mechanism. Based on the number of chromosomes (especially the centromeres) of each of the parental species in the BC_1 plants, it can be concluded they obtained FDR gametes from the hybrid, which is confirmed by a combined GISH and FISH analysis of the BC_1 plants (Kamstra *et al.* 1999b).

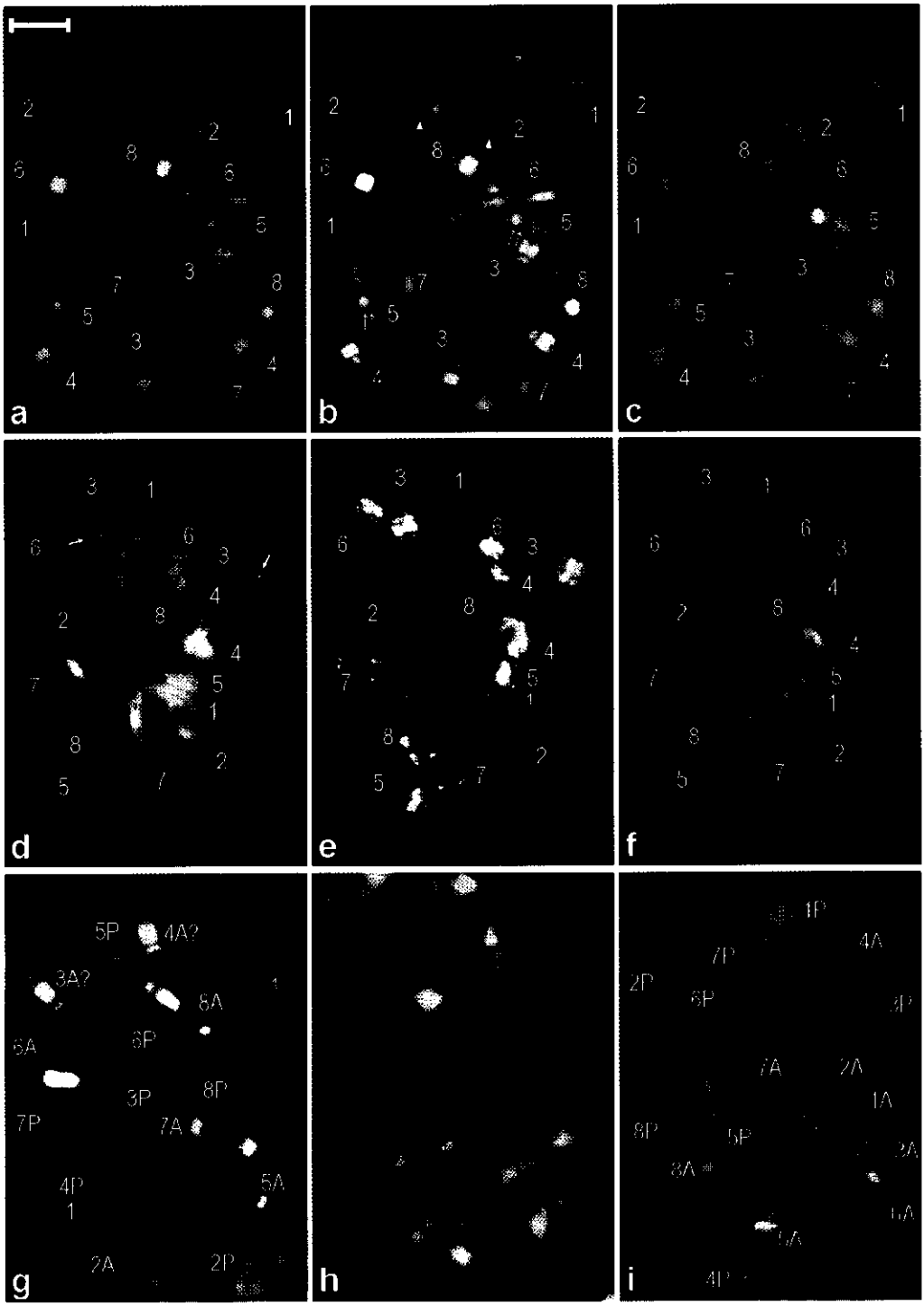
The BC_1 plants were made with the main objective of introgressing *A. aurea* chromosomes into *A. inodora*. Only the triploid BC_1 plants seem usable for this purpose, because the aneuploid BC_1 plants show abnormal plant development and do not flower. The triploid plants, on the other hand, produce abundant flowers and stainable pollen which indicates that they can be used to produce further backcross populations. In addition, the number of recombined chromosomes in the BC_1 plants as a result of homoeologous recombination indicates that it is possible to introgress chromosome segments of *A. aurea* into *A. inodora*.

The development of molecular cytogenetic methods for the identification of individual chromosomes and genomes (for a review see Jiang and Gill, 1994) has provided very valuable

cytological markers to monitor the consequences of hybridization and the process of introgression using backcross derivatives. In particular, the aspects of homoeologous chromosome pairing, position and extent of crossing-over and the gamete composition can be more critically monitored, as was clearly demonstrated for *Alstroemeria* in the present study.

Colour figures

Figure 2.2: DAPI-banding patterns and sequential hybridization of the species-specific probes and the rDNA probes on metaphase chromosomes of the species *A. aurea* and *A. inodora* and multicolour FISH with the repetitive sequences on metaphase chromosomes of the interspecific hybrid of *A. aurea* x *A. inodora*. Reddish colours correspond to biotin-labelled probes detected with Cy3-conjugated antibodies and yellow-green colours correspond to digoxigenin-labelled probes detected with FITC-conjugated antibodies. All chromosomes are indicated by numbers. Scale bar = 10 μm for all panels. (a-c) The same metaphase of *A. aurea* with (a) DAPI counterstain, (b) the biotin labelled species-specific A001-I probe (arrows indicate chromosome polymorphisms; note: the positions of the large and small bands on chromosome 5 are inverted), and (c) biotin labelled 25S rDNA and digoxigenin labelled 5S rDNA. (d-f) The same metaphase of *A. inodora* with (d) DAPI counterstain, (e) the digoxigenin labelled D32-13 probe counterstained with propidium iodide, and (f) biotin labelled 25S rDNA. (g-i) Simultaneous *in situ* hybridization on the interspecific hybrid with biotin labelled D32-13 and digoxigenin labelled A001-I probes on (g) metaphase and (h) interphase nuclei and with (i) biotin labelled 25S rDNA and digoxigenin labelled 5S rDNA.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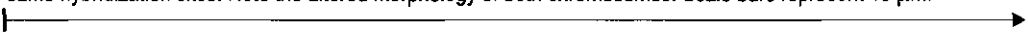
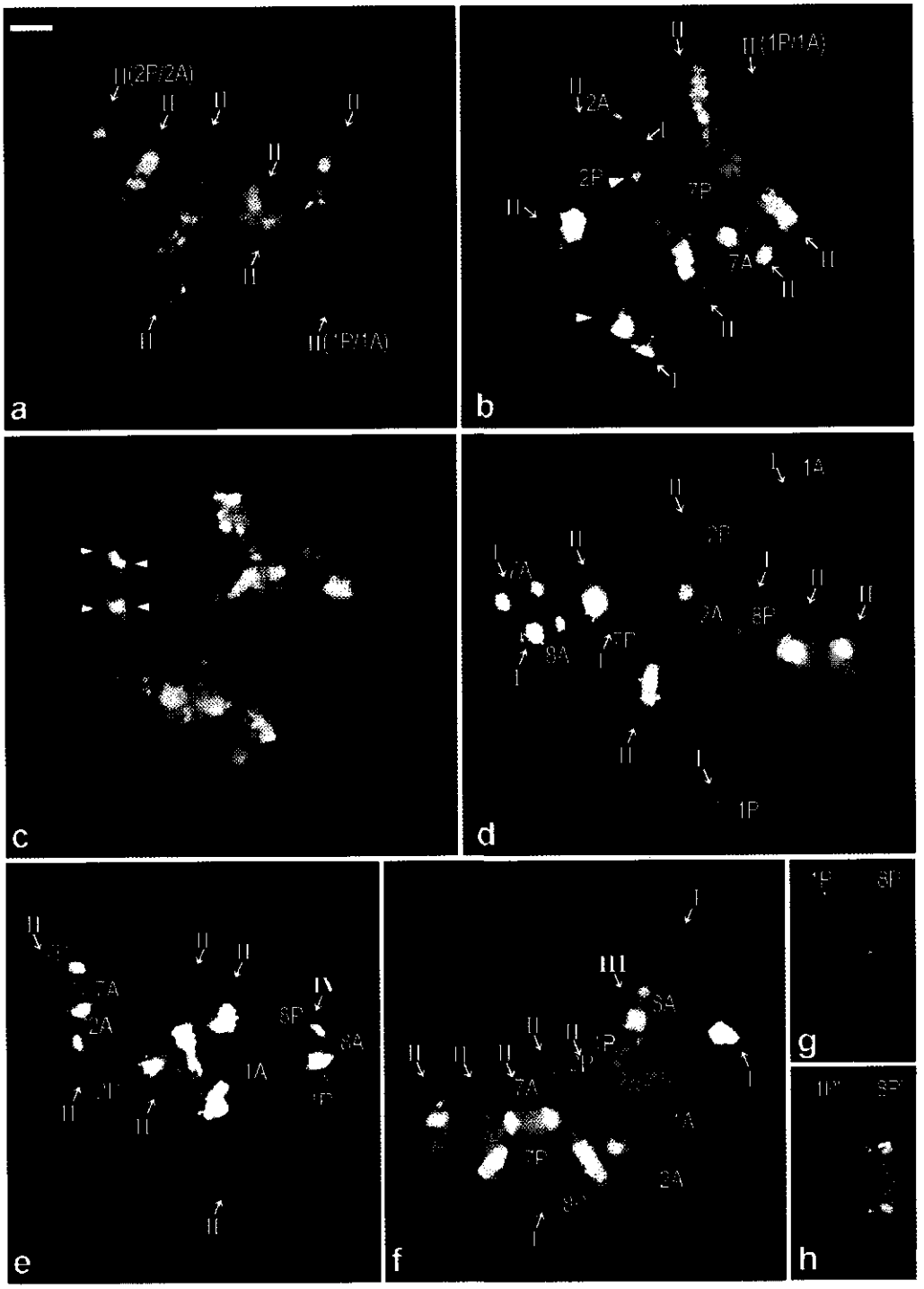


Figure 3.2: FISH using two species-specific DNA probes to meiotic metaphase I /anaphase I spreads of the *A. aurea* x *A. inodora* interspecific hybrids A1P2-2 and A1P4. The D32-13 probe was labelled with biotin and detected with Cy3 conjugated antibodies (red). The A001-I probe was labelled with digoxigenin and detected with FITC conjugated antibodies (green). DAPI (blue) was used as counterstain. Chromosome configurations are indicated in each panel (I=univalent, II=bivalent, III=trivalent and IV=quadrivalent). Also chromosomes which could be identified on the basis of their hybridization patterns are indicated. (a-c) FISH to metaphase I and anaphase I spreads of hybrid A1P2-2. (a) Complete chromosome pairing (eight bivalents) in A1P2-2. (b) Seven bivalents and two univalents at metaphase I. The univalents already possess exchanged segments (yellow arrows), indicating precocious separation of a bivalent. (c) Anaphase I of hybrid A1P2-2. With both species-specific probes it is possible to detect crossover in the lagging chromosome pair (yellow arrow). (d-f) FISH to metaphase I spreads of hybrid A1P4. The chromosomes 1A, 1P, 8A and 8P are indicated in each panel. Note the four univalents (d), trivalent + univalent (f) and quadrivalent (e) configurations of these chromosomes. (g-h) Normal and altered mitotic chromosomes 1P and 8P of hybrid A1P2-2 (g) and A1P4 (h) hybridized with biotinylated pTa71 probe detected with Cy3 conjugated antibodies. The arrows indicate the same hybridization sites. Note the altered morphology of both chromosomes. Scale bars represent 10 μm.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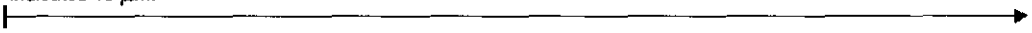
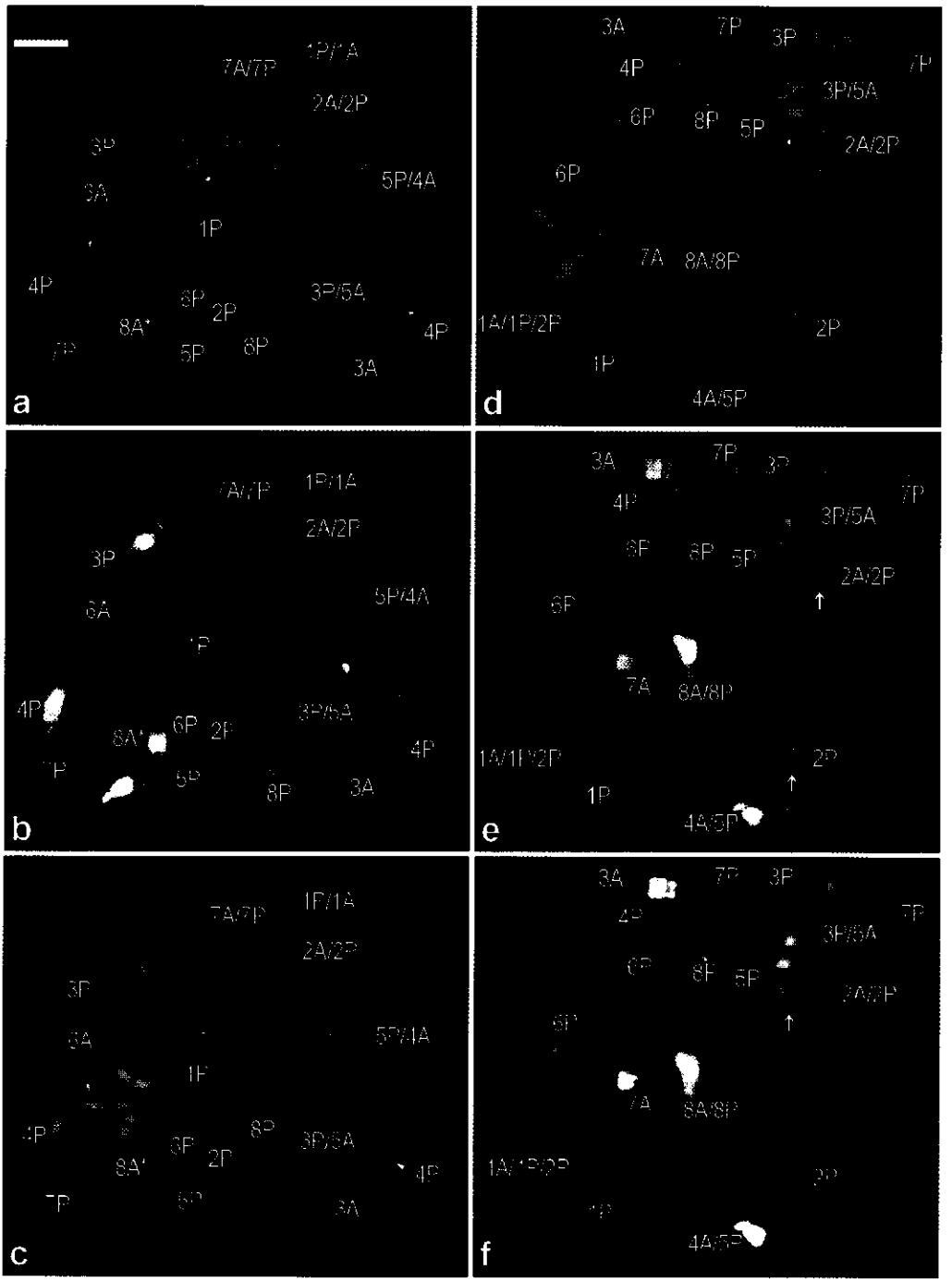


Figure 4.1: Sequential fluorescent *in situ* hybridization onto chromosome spreads of the two aneuploid BC_1 plants 95SK18 (a-c) and 95SK11 (d-f). The small coloured arrowheads indicate the locations of the different probes; A001-I (blue), D32-13 (green), pTa71 (red), and pTa794 (yellow). All individual chromosomes are indicated. The large yellow arrows indicate the crossover points between chromosomes of *A. aurea* and *A. inodora*. (a) Chromosomes of BC_1 plant 95SK18 after genomic *in situ* hybridization. The digoxigenin labelled DNA of *A. inodora* was detected with anti-dig-FITC (yellow), and biotin labelled DNA of *A. aurea* was detected with streptavidin-Cy3 (red). (b) Reprobing of the same preparation with the species-specific probes A001-I (red) and D32-13 (green) identified most of the chromosomes. Note the absence of the distal A001-I site on chromosome 8A/8P. (c) Simultaneous hybridization of the two rDNA sequences pTa71 (red) and pTa794 (green). Note that some of the signals of the species-specific probes from the second reprobing experiment are still present. However, the additional signals obtained after the third probing can be clearly identified. Two chromosomes of 4P are clearly identified by their large proximal pTa794 sites. Note that the distal pTa71 site of 1P identifies the short arm of 1P and also the recombinant 1P/1A. (d) GISH identifies the *A. inodora* (yellow) and *A. aurea* (red) chromosomes in BC_1 95SK11. (e) Sequential hybridization of the same preparation with the species-specific probes A001-I (green) and D32-13 (red). Most chromosomes were identified by their specific hybridization pattern. Note the crossover point between the short-arm of 2A and the long arm of 2P (white arrow) in chromosome 2A/2P. The other white arrow indicates the distal D32-13 on the long arm of 2P. The purple arrows indicate two similar sites of the D32-13, which is clearly the site of this probe on the short arm of 2P. One of these sites is detected on the recombinant chromosomes 1A/1P/2P. (f) Reprobing of the same metaphase spread with the rDNA probes, pTa71 (red) and pTa794 (green). Some of the signals of the species-specific probes are still visible in the preparation, but the additional sites for both rDNA probes are clearly visible and allow identification of some of the chromosomes. The white arrow indicates the proximal pTa794 sites on the long arm of 2A/2P. Scale bar indicates 10 μm.


a
7A/7P
1P/1A
2A/2P
5P/4A
3A
1P
4P
6P
2P
3P/5A
8A*
5P
6P
3A
4P
b
7A/7P
1P/1A
2A/2P
3P
3A
5P/4A
1P
4P
8A*
6P
2P
3P/5A
4P
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8P
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2A/2P
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8A*
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8A/8P
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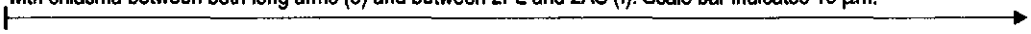
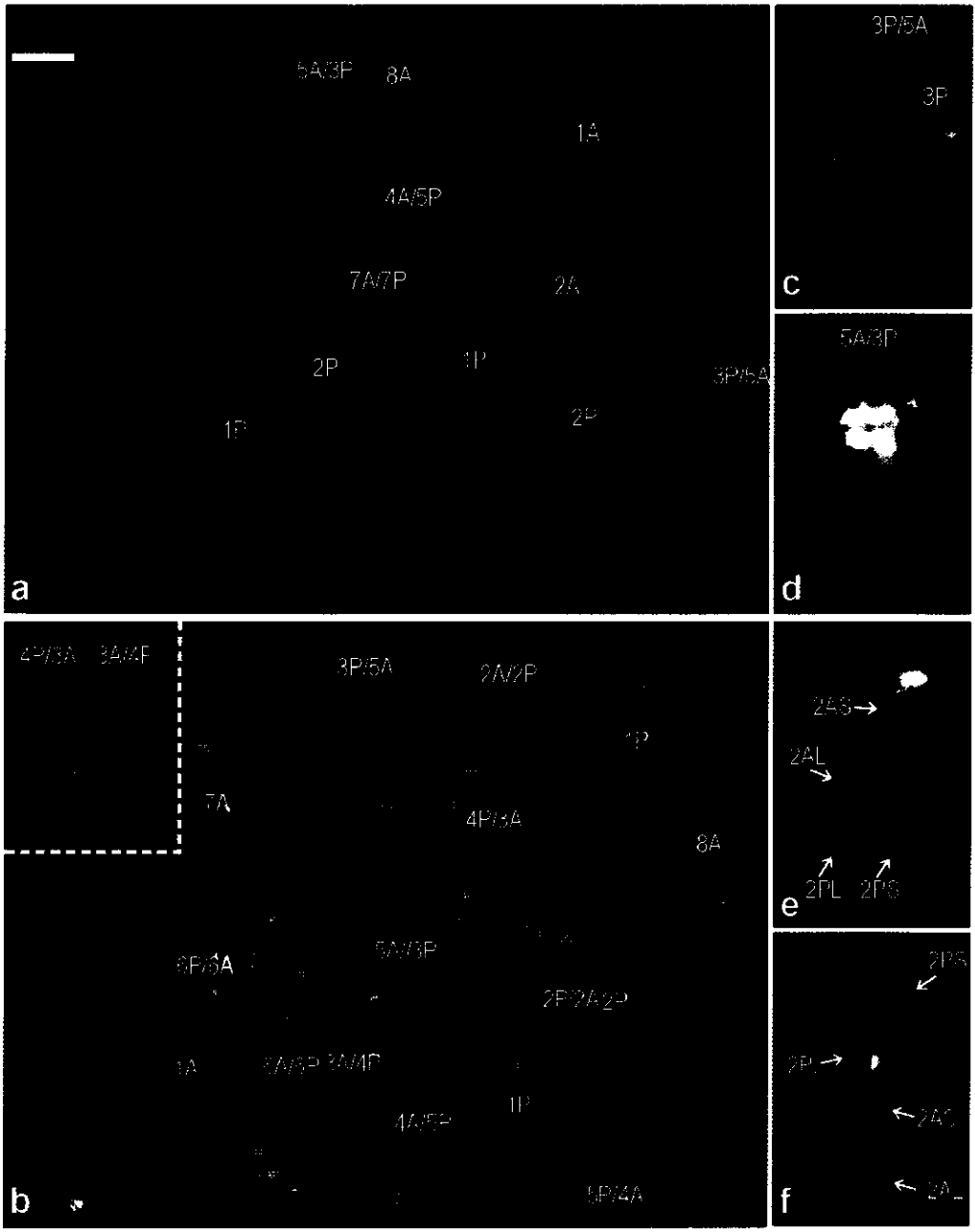


Figure 4.2: (a-b) GISH onto metaphase spreads of two triploid BC_1 plants 96SK403 (a) and 96SK402 (b). Arrows indicate crossover points. Note the differences in the number of crossover points in the two BC_1 plants. Insert (b) shows the three reciprocal crossovers in chromosomes 3A/4P and 4P/3A in 96SK402. (c-d) The three homoeologous chromosomes 3P/5A (c), 3P (c) and 5A/3P (d) after the second reprobing with the rDNA probes of BC_1 plant 96SK403. The species-specific probes are still present after the second reprobing, but are useful to accurately identify these chromosomes. The differently coloured arrowheads indicate A001-I (blue), D32-13 (green), pTa794 (yellow) and pTa71 (red). Note that the large interstitial A001-I site (large blue arrowhead) in chromosome 5A/3P (d), is at a distal location in 3P/5A (arrowhead) (c), which is an indication for an ancient inversion between the homoeologous chromosomes 3P and 5A. (e-f) Two bivalents of the homoeologous chromosomes 2P and 2A hybridized with the A001-I (green) and D32-13 (red), indicating the ancient translocation in these chromosomes. Bivalent with chiasma between both long arms (e) and between 2PL and 2AS (f). Scale bar indicates 10 μm.


5A/3P
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1A
4A/5P
7A/7P
2A
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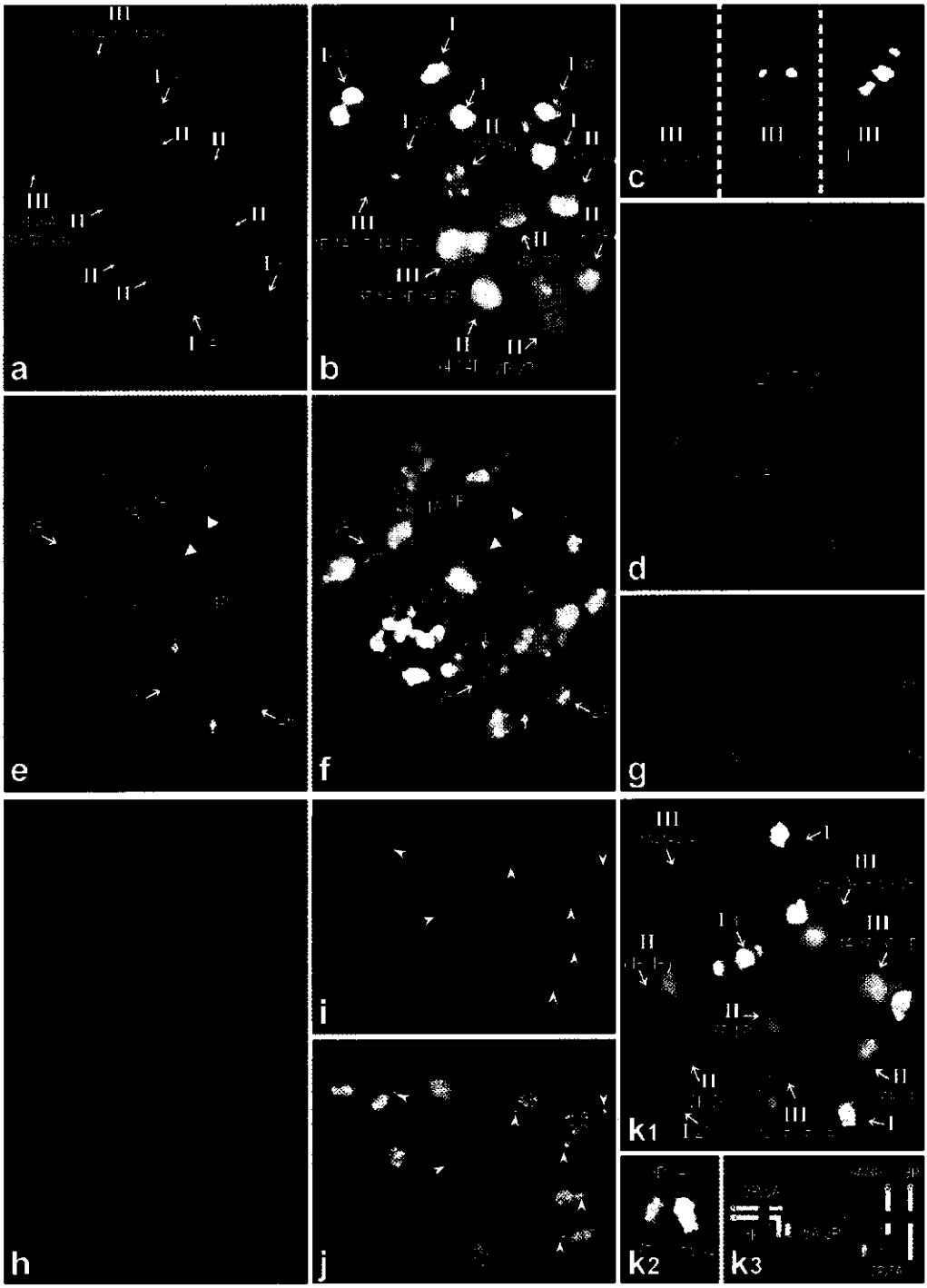
Figure 5.2: Analysis of meiosis in BC_1-2 (a-j) and BC_1-4 (k). GISH was applied to differentiate chromosomes belonging to both genomes; *A. aurea* chromatin (red) was detected by Cy3-conjugated antibodies and *A. inodora* chromatin (yellow) by FITC/Alexa 488-conjugated antibodies. FISH with the two species-specific probes, D32-13 and A001-I, was applied (sometimes sequentially) to identify all individual chromosomes. D32-13 and A001-I were detected by Cy3-conjugated (red) and FITC/Alexa 488-conjugated (green) antibodies, respectively. DAPI (blue) was applied as counterstain. The blue arrows indicate *A. aurea* chromosomes or chromatids. (a) GISH applied to metaphase I. Chromosome configurations are indicated. Note the two recombinant sets which are involved in two trivalents and that the associations in these trivalents are between homologous chromosome segments. (b) Metaphase I after FISH with D32-13 (red) and A001-I (green). Obtained hybridization patterns allow identification of all individual chromosomes in the chromosome configurations, except for the two acrocentric chromosomes 3A and 4A. (c) Three examples of observed homoeologous associations (trivalents) detected through FISH with D32-13 (red) and A001-I (green). (d) GISH applied to anaphase I. All *A. inodora* chromosomes (yellow) segregate to both poles, whereas the *A. aurea* chromosomes remain lagging in the equatorial plane. Note the two recombinant chromosomes of *A. aurea* (1A and 5A) which segregate regularly. Some *A. aurea* chromosomes divide into chromatids. (e-f) GISH and sequential FISH analysis with D32-13 and A001-I applied to anaphase I. Bridge + a-centric fragment between the (non-recombinant) short arms of the chromosomes 1P (white arrowheads). Note the novel homoeologous recombination (yellow arrows) in chromosome 2P and 2A detected through GISH and the differently sized exchanged segments. As a result of this novel exchange these chromosomes segregate regularly to both poles, like the *A. inodora* chromosomes, whereas all other non-recombinant *A. aurea* chromosomes segregate equationally into chromatids. (g) GISH applied to late anaphase I. All *A. inodora* chromosomes (yellow) + the recombinant *A. aurea* (yellow + red) chromosomes have moved to both poles, whereas the *A. aurea* chromatids (red) migrate to both poles. (h) GISH applied to anaphase II. All *A. inodora* chromatids have already migrated to both poles, whereas the *A. aurea* chromatids are delayed in their migration and segregate randomly to the poles. (i-j). FISH with A001-I (yellow) and D32-13 (red) probes applied to gametes of BC_1-2. The arrows indicate the presence of micronuclei revealed by (j) DAPI counterstaining. Note the A001-I signals in these micronuclei and the varying number of A001-I sites in the nucleus of these gametes. (k) FISH with D32-13 and A001-I applied to metaphase I of BC_1-4. Note the four trivalent chromosome configurations. The trivalent for chromosomes 1 is formed through homoeologous association, the other trivalents through homologous association between recombinant chromosome segments. Note the trivalent configuration of the chromosomes 3P/5A, 5A/3P and 3P (insert) and the schematic drawing of this configuration (yellow and red indicate *A. inodora* and *A. aurea* chromatin, respectively. A001-I and D32-13 sites are indicated with green and purple, respectively). This configuration proofs the assumed inverted segment in chromosome 3P/5A (see text).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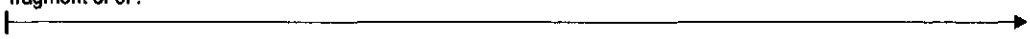
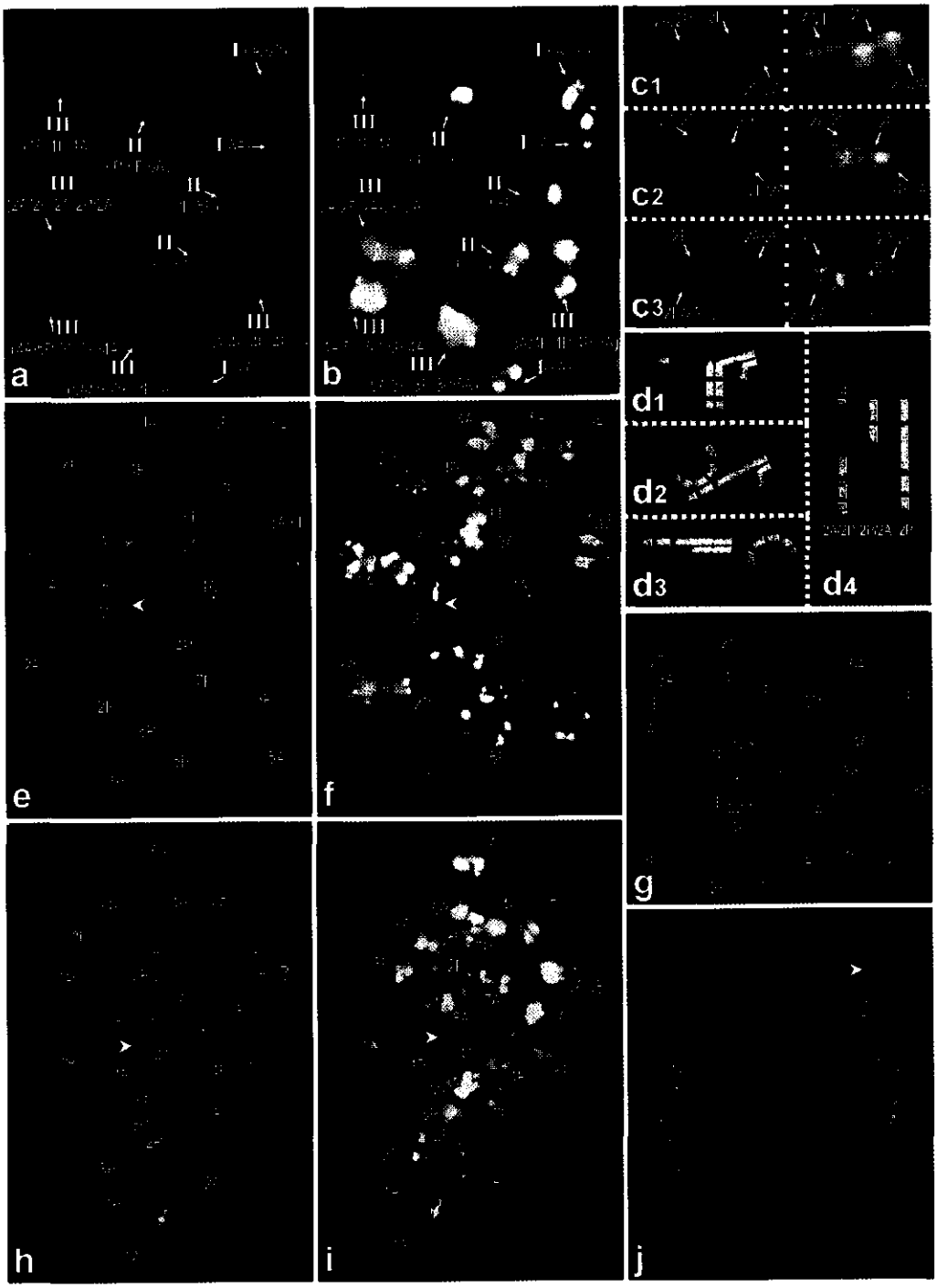


Figure 5.3: Fluorescent *in situ* hybridization applied to metaphase I and anaphase I spreads of BC_1-3. GISH was applied to differentiate both parental genomes, *A. aurea* and *A. inodora*, which were detected by Cy3-conjugated and FITC- and Alexa 488-conjugated antibodies, respectively. Specific hybridization patterns obtained after 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. All monosomic *A. aurea* segments in the recombinant chromosomes are not associated in the trivalents. The recombinant chromosome 6A/6P is present as a univalent. Homoeologous pairing was observed in the trivalent of non-recombinant chromosomes 1. ($c_{1\text{-}3}$) Different chromosome configurations for the homo(eo)logous chromosomes 2A/2P, 2P/2A and 2P using GISH and FISH proof the presence of two homologous segments on both arms of 2A/2P (see text). ($d_{1\text{-}3}$) Schematic drawings of the chromosome configurations shown in ($3c_{1\text{-}3}$). Red and yellow indicate *A. aurea* and *A. inodora* chromatin, respectively, and purple indicates the D32-13 hybridization sites. (d_4). Ideograms of the different chromosomes 2 present in BC_1-3. Note the pseudo-iso-chromosome 2A/2P. (e-f) GISH and FISH applied sequentially to the same anaphase I. Regular segregation of non-recombinant *A. inodora*, recombinant *A. inodora* and recombinant *A. aurea* chromosomes. Non-recombinant *A. aurea* chromosomes are delayed and remain in the equatorial plane. The behaviour of chromosome 6A/6P, which was usually unpaired at metaphase, I is similar as the non-recombinant *A. aurea* chromosomes. 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 non-recombinant 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. Usually these chromosomes were not associated during metaphase I. Note the different hybridization patterns on the chromatids of the same chromosome indicating the expected recombination between homologous segments in the recombinant chromosomes (for example both chromosomes 5P). (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 on of the chromatids of chromosome 1A. White arrow indicates the small a-centric fragment of 8P.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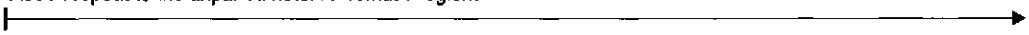
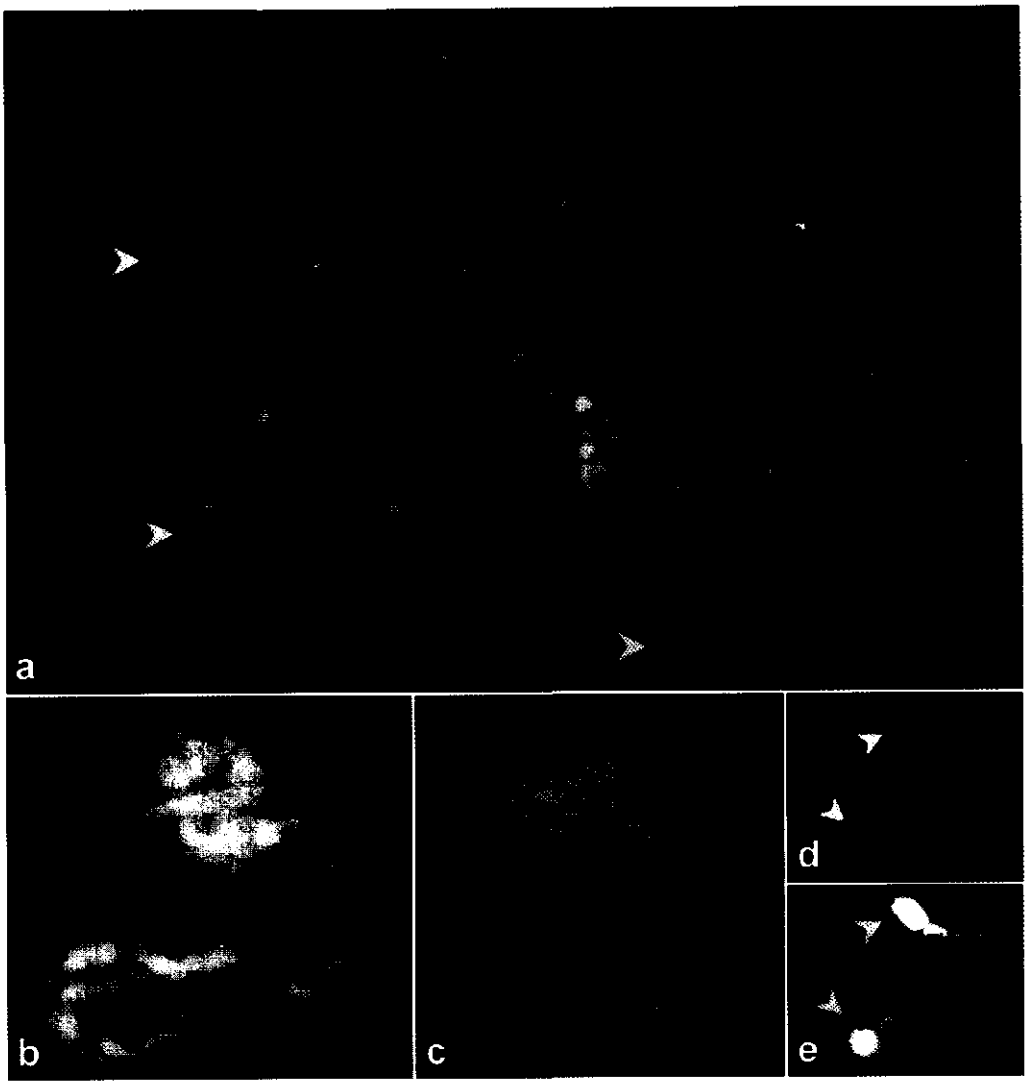


Figure 6.1: GISH applied to early (a) and late (b-c) pachytene spreads of the diploid interspecific hybrid *A. aurea* x *A. inodora*. (*A. aurea* = red; *A. inodora* =yellow). Note that most of the homoeologous chromosomes are paired (see for examples the yellow and red arrows). The heterochromatic regions of *A. aurea* are not paired (blue arrows). (d-e) Bivalent of chromosomes 7A and 7P from Figure 6.1c. GISH reveals almost completely paired chromosomes, except for the large distal heterochromatic regions of 7A (d). Sequential FISH with species-specific probes, D32-13 (red) and A001-I (green) allows identification of the involved homoeologous chromosomes (e). Note the hybridization of the A001-I repeat to the unpaired heterochromatic regions.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4 The extent and position of homoeologous recombination in a distant hybrid of *Alstroemeria*: a molecular cytogenetic assessment of first generation backcross progenies

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ABSTRACT

To estimate the extent and position of homoeologous recombination during meiosis in an interspecific hybrid between two distantly related *Alstroemeria* species, the chromosome constitution of six BC_1 plants was analysed using sequential GISH and FISH analysis. Four different probes were used for the FISH analysis; two species-specific and two rDNA probes. The six BC_1 plants were obtained from crosses between the hybrid *A. aurea* x *A. inodora* with its parent *A. inodora*. GISH clearly identified all chromosomes of both parental genomes as well as recombinant chromosomes. The sequential GISH and FISH analysis enabled the accurate identification of all individual chromosomes in the BC_1 plants, resulting in detailed karyotypes of the plants. The identification of the recombinant chromosomes provided evidence which chromosomes of both species are homoeologous. Two of the BC_1 plants were aneuploid ($2n=2x+1=17$) and four triploid ($2n=3x=24$), indicating that both n- and $2n$-gametes were functional in the F_1 hybrids. Using GISH, it was possible to estimate homoeologous recombination in two different types of gametes in the F_1 hybrid. The position of the crossover points ranged from highly proximal to distal and the maximum number of crossover points per chromosome arm was three. Compared to the aneuploid plants, the triploid plants (which received $2n$-gametes) clearly possessed fewer crossovers per chromosome, indicating a

reduced chromosome pairing/recombination prior to the formation of the 2*n*-gametes. Besides homoeologous recombination, evidence was found for the presence of structural rearrangements (inversion and translocation) between the chromosomes of both parental species. The presence of the ancient translocation was confirmed through the FISH analysis of mitotic and meiotic chromosomes.

INTRODUCTION

When plant species with highly differentiated genomes are hybridized, the homoeologous chromosomes in the resulting F_1 hybrids either do not pair regularly during meiosis or, even if they pair, they tend to form a very low frequency of chiasmata. In some cases, however, a considerable amount of pairing and chiasma formation can occur in distant and intergeneric hybrids. Such hybrids and their progenies will be most useful to gain insight into the basic aspects of the position, extent and consequences of crossing-over between homoeologous pairs of chromosomes. In order to investigate such phenomena in distant hybrids, *in situ* hybridization techniques (Rayburn and Gill, 1985; Schwarzacher *et al.* 1989; Anamthawat-Jonsson *et al.* 1990; Parakonny *et al.* 1994; and others) can be used to differentiate both parental genomes and consequently the position and extent of crossing-over in recombinant chromosomes in the progenies. Both genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) have been successfully used to identify the parental genomes in intergeneric and distant interspecific hybrids (Schwarzacher *et al.* 1989; Parokonny *et al.* 1994, 1997; Wolters *et al*, 1994; Anamthawat-Jonsson *et al.* 1990, 1995; Jacobsen *et al.* 1995; Takahashi *et al.* 1997; Kamstra *et al.* 1997; and others).

The genus *Alstroemeria* consists of diploid ($2n=2x=16$) species, mainly from Chile and Brazil (Bayer, 1987; Aker and Healy 1990). All *Alstroemeria* species possess large genomes, 2C DNA values ranging from 36.5 to 78.9 pg and contain eight pairs of large chromosomes (4 to 20 μm), which can be divided for most species into two groups of four acrocentric and four (sub)metacentric chromosomes (Buitendijk *et al.* 1997). Between the Chilean and Brazilian species distinct differences are observed regarding plant- or flower-morphology and also in the molecular organisation of their genomes. The parental genomes in hybrids between Brazilian and Chilean species can be distinguished by GISH (Kuipers *et al.* 1997). In addition, species-specific repetitive DNA sequences were isolated from several Chilean and Brazilian species (De Jeu *et al.* 1995). These repeats and two ribosomal DNA sequences were used as

probes for FISH to physically map these sequences to chromosomes of different species and to construct detailed karyotypes. Hence it was possible to identify all individual chromosomes of the species *A. aurea* and *A. inodora* (Kamstra *et al.* 1997).

The distant interspecific hybrid *A. aurea* x *A. inodora* was backcrossed with its parent *A. inodora*. This resulted in six first generation backross (BC_1) plants; two aneuploids ($2n=2x+1=17$) and four triploids ($2n=3x=24$) (Kamstra *et al.* 1999a). The triploids were formed by $2n$-gametes of the hybrid probably via a first division restitution (FDR) mechanism (Kamstra *et al.* 1999a). Using FISH with species-specific probes, it was shown that all plants were true BC_1 plants. Furthermore, the BC_1 plants were found to contain chromosomes, which were the result of homoeologous recombination between chromosomes of *A. aurea* and *A. inodora*. Such recombinant chromosomes were expected to be present, because many heteromorphic bivalents were observed at metaphase I stage of the diploid parental hybrid (Kamstra *et al.* 1999a). However, using the species-specific probes only, it was not possible to identify all chromosomes accurately, especially the recombinant chromosomes, in the BC_1 plants.

Therefore, a detailed study was performed using a combination of GISH and FISH with repetitive probes to construct detailed karyotypes of the six BC_1 plants. The specific aims of this study were (i) to analyse the genome constitution of the BC_1 plants and consequently, the genome constitution of some of the viable gametes produced by the interspecific hybrid; (ii) to identify the homoeologous chromosomes in the species *A. aurea* and *A. inodora* by characterisation of the recombinant chromosomes in the BC_1 plants; (iii) to determine the distribution of crossover points along the different chromosome arms; (iv) study the restitution mechanism of the $2n$-gametes in more detail.

MATERIALS AND METHODS

Plant material

The six BC_1 plants were obtained from crosses between the hybrid *A. aurea* x *A. inodora* (A1P2-2) and *A. inodora* (P2). Four were triploid ($2n=3x=24$) and two aneuploid ($2n=2x+1=17$). Details were described in Kamstra *et al.* (1999a).

Fluorescent *in situ* hybridization

DNA probes and labeling

For GISH, total genomic DNA of *A. aurea* and *A. inodora* was extracted from leaves according to Rogers and Bendich (1988), sonicated to obtain fragment sizes of 1 to 5 kb and labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation. Blocking DNA was obtained by autoclaving total genomic DNA of *A. aurea* for 5 min.

The following DNA probes were used for chromosome identification: (i) A001-I (EMBL accession number Y10977), which contains an *A. aurea* specific, highly repetitive sequence (De Jeu *et al.* 1997, Kamstra *et al.* 1997); (ii) D32-13 (EMBL accession number AJ228814), containing a highly repetitive sequence which is specific for *A. inodora* (Kamstra *et al.* 1997, Kuipers *et al.* 1999); (iii) pTa71, containing a fragment of 9 kb of the 18S-5.8S-26S rDNA sequence isolated from wheat (Gerlach and Bedbrook 1979). (iv) pTa794, which contains a 410 bp fragment of the 5S rDNA from wheat (Gerlach and Dyer 1980). The physical location of these sequences on the chromosomes of both *Alstroemeria* species has been described in detail by Kamstra *et al.* (1997). All sequences were amplified and labelled by PCR with either biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim, Germany), except for pTa71, which was labelled with biotin-16-dUTP by nick translation according to the manufacturer (Boehringer Mannheim, Germany).

Genomic in situ *hybridization (GISH)*

For GISH, two differently labelled total genomic DNA probes of *A. aurea* and *A. inodora* were pre-annealed as described in Anamthawat-Jonsson and Reader (1995) with some modifications. Pre-hybridization steps including enzymatic digestions with pepsin and RNase A and fixation in 4% (para)formaldehyde were performed as in Kuipers *et al.* (1997). Prior to hybridization, the slides were denatured with 70% formamide in 2 x SSC at 70°C for 5 min, dehydrated through an ice-cold ethanol series (70%, 90% and 100%) and air dried.

The hybridization mixture [containing 2.5 ng/µl of biotin-16-dUTP labelled *A. aurea* probe, 2.5 ng/µl of digoxigenin-11-dUTP labelled *A. inodora* probe, 100 ng/µl unlabeled blocking DNA of *A. aurea*, 2x SSC, 0.25% SDS, 10% dextran sulphate] was denatured at 100°C for 5 min, pre-annealed for 2-3 h at 58°C and transferred to the denatured slides. Hybridization was performed overnight at 63°C.

Upon hybridization, the slides were washed in 2x SSC at 63°C for 2 x 5 min. and cooled down to 20°C. Detection of the hybridized probes was performed according to Kamstra *et al.* (1997). Biotinylated *A. aurea* probes were detected with streptavidin-Cy3 (Jackson ImmunoResearch Laboratories). Digoxigenin-labelled *A. inodora* probes were detected with anti-digoxigenin-FITC (Boehringer Mannheim) and amplified by rabbit-anti-sheep-FITC (Vector Laboratories). Alternatively, 15 µg/ml anti-fluorescein-ALEXA 488 conjugate raised in rabbit (Molecular Probes Inc) was used as the second antibody to amplify digoxigenin labelled probes instead of the rabbit-anti-sheep-FITC. All DNA was counterstained with DAPI.

Sequential FISH with repetitive probes for chromosome identification

The slides, previously used for GISH analysis, were washed in 4 x SSC containing 0.2% (w/v) Tween-20 for several hours, dehydrated through an ethanol series (70%, 90%, 100%), air dried and hybridized with the repetitive probes. As second probing the two differently labelled species-specific probes, D32-13 and A001-I, were hybridized to the slides. After examination of the slides, they were washed again for several hours in 4 x SCC containing 0.2% (w/v) Tween-20, dehydrated and the third probing of the slides was applied by hybridizing the two differently labelled rDNA sequences, pTa71 and pTa794. Fluorescent *in situ* hybridization with all repetitive sequences was carried out as described by Kamstra *et al.* (1997).

All biotin-16-dUTP and digoxigenin-11-dUTP labelled probes were detected with the appropriate antibodies conjugated with Cy3 and FITC respectively according to Kamstra *et al.* (1997). Biotin-16-dUTP was detected in a three-step detection/amplification; streptavidin-Cy3 (Jackson ImmunoResearch Laboratories) - biotinylated streptavidin (Vector laboratories) -streptavidin-Cy3. Detection of digoxigenin-11-dUTP was performed as described for the GISH experiments.

Slides were examined using an Axiophot fluorescence microscope (Zeiss). Photomicrography and digital imaging were performed as described in Kamstra *et al.* (1997).

Chromosome nomenclature

The individual chromosomes of both parental species are indicated by numbers and the letters A or P for chromosomes of *A. aurea* or *A. inodora*, respectively (see Kamstra *et al.* 1997). All recombinant chromosomes are indicated as a combination of the involved chromosomes, of which the segment containing the centromere will be indicated first, followed by the other segment.

RESULTS

Genome analysis of the BC_1 plants with GISH

GISH clearly distinguished the chromosomes of the two parental genomes in the hybrid (not shown) and in the BC_1 plants (Figures 4.1a, 4.1d and 4.2a-b). The chromosomes or chromosome segments of *A. inodora* showed uniform yellow/greenish fluorescence (FITC or Alexa 488), whereas the predominantly orange-fluorescing *A. aurea* chromosomes or segments showed a red-orange banding pattern. This banding pattern is caused by the abundant presence of species-specific sequences in the probe (and blocking DNA) of *A. aurea* which correspond, and thus hybridize, to sequences at the heterochromatic regions (GISH banding; Kuipers *et al.* 1997). The orange fluorescence along the other parts of the *A. aurea* chromosomes is likely the result of slight cross hybridization of the *A. inodora* probe to more common sequences.

In all six BC_1 plants recombinant chromosomes were detected through GISH. These chromosomes were recognised by the orange/red (*A. aurea*) and yellow/green (*A. inodora*) fluorescing segments on the same chromosome. The total number of recombinant chromosomes per BC_1 plant varied from 4 to 10 (Table 4.1). Among these chromosomes, most contained a combination of a single *A. aurea*- and a single *A. inodora*-fragment (Table 4.1), indicating a single crossover event during meiosis of the parental hybrid. Other recombinant chromosomes were composed of 3 to 5 different segments (Table 4.1), being the result of (at least) 2 to 4 crossover events. Of the total number of 36 recombinant chromosomes that were found in the six BC_1 plants, 20 were the result of 1 crossover (56%, Table 4.1), whereas one chromosome was detected with 4 crossover points (Figures 4.1d and 4.3c; Table 4.1).

The crossover points were observed along the entire length of the chromosomes, ranging from proximal to distal (Figures 4.1a, 4.1d and 4.2a-b). Multiple crossovers per chromosome occurred either in a narrow segment of a chromosome (Figure 4.2b) or were distributed along the chromosome arm (Figures 4.1d and 4.2b). Measurements of the length of the exchanged segments in these chromosomes showed that the smallest exchanged segments, which could be detected through GISH, were less than 1μm.

Chromosome identification with sequential FISH

Reprobing of the preparations that were used for GISH with the two species-specific probes, A001-I and D32-13 (Figure 4.1b, 4.1e), followed by both rDNA probes, pTa71 and pTa794

Table 4.1: The number of chromosomes transmitted by the gametes of the F_1 hybrid, A1P2-2, to each of the six BC_1 plants and the number of crossover points in the recombinant chromosomes.

BC1 progeny[a]	Number of chromosomes obtained from the hybrid	number of chromosomes with *n* crossover points					
		n=0	*n*=1	*n*=2	*n*=3	*n*=4	total (*n*=1-4)
95SK11 (2*n*=2*x*+1=17)	9	4	4	0	0	1[b]	5
95SK18 (2*n*=2*x*+1=17)	9	4[b]	4	1	0	0	5
95SK04 (2*n*=3*x*=24)	16	8	5	0	3	0	8
95SK17 (2*n*=3*x*=24)	16	12	1	1	2	0	4
96SK402 (2*n*=3*x*=24)	16	6[b]	2	6	2	0	10
96SK403 (2*n*=3*x*=24)	16	12	4	0	0	0	4
total number	82	46	20	8	7	1	36

a) For details on the directions of the crossings see Kamstra et al. (1999a)
b) Some of these chromosomes possess structural rearrangements, but no recombination between both genomes could be detected through GISH.

(Figure 4.1c, 4.1f), revealed additional information, which allowed identification of most of the individual chromosomes present in the BC_1 plants, except for a few recombinant chromosomes. The mainly interstitial and distal hybridization sites of species-specific probes could be used to identify most of the non-recombinant chromosomes of *A. aurea* and *A. inodora*. Some of the recombinant chromosomes were identified with the species-specific probes as well. Additional information was gathered using the rDNA probes, pTa71 and pTa794, which mainly identified chromosomal regions that differed from those detected by the species-specific probes. In addition to GISH, these rDNA probes, especially pTa71, were useful to determine whether the centromeric regions belonged to either *A. aurea* or *A. inodora* chromosomes (Figure 4.1c, 4.1f). This information could be used to determine the restitution mechanism of the 2*n*-gametes that resulted in the triploid BC_1 plants. The distal hybridization sites of pTa71 on 1P, 3P, 4P and 7P and the distinct pTa794 hybridization sites of 4P, 2A and 5A were useful to identify these chromosomal regions in the recombinant chromosomes. The small pTa794 sites on 1P, 2P, 3P, 7A and 8A were inconsistently detected and therefore not taken into account. The combined information of GISH and FISH proved essential to identify

the recombinant chromosomes. A detailed description of the individual karyotypes of all the BC1 plants was made (Figure 4.3b-g).

Identification of homoeologous chromosomes

It was possible to identify most of the *A. aurea* and *A. inodora* segments in the recombinant chromosomes through the sequential FISH and GISH analysis. Since these recombinant chromosomes are assumed to be the result of homoeologous recombination during meiosis of the parental hybrid, the homoeologous chromosomes of *A. aurea* and *A. inodora*, which had paired and recombined during meiosis, were identified. For all eight homoeologous chromosome pairs, except for chromosome 1, at least one chromosome was detected in six BC_1 plants in which both *A. aurea*- and *A. inodora*-segments were identified (underlined in Table 4.2). The order of most of the homoeologous chromosomes of the two species, based on the length and centromere position of these chromosomes, corresponded, except for some of the

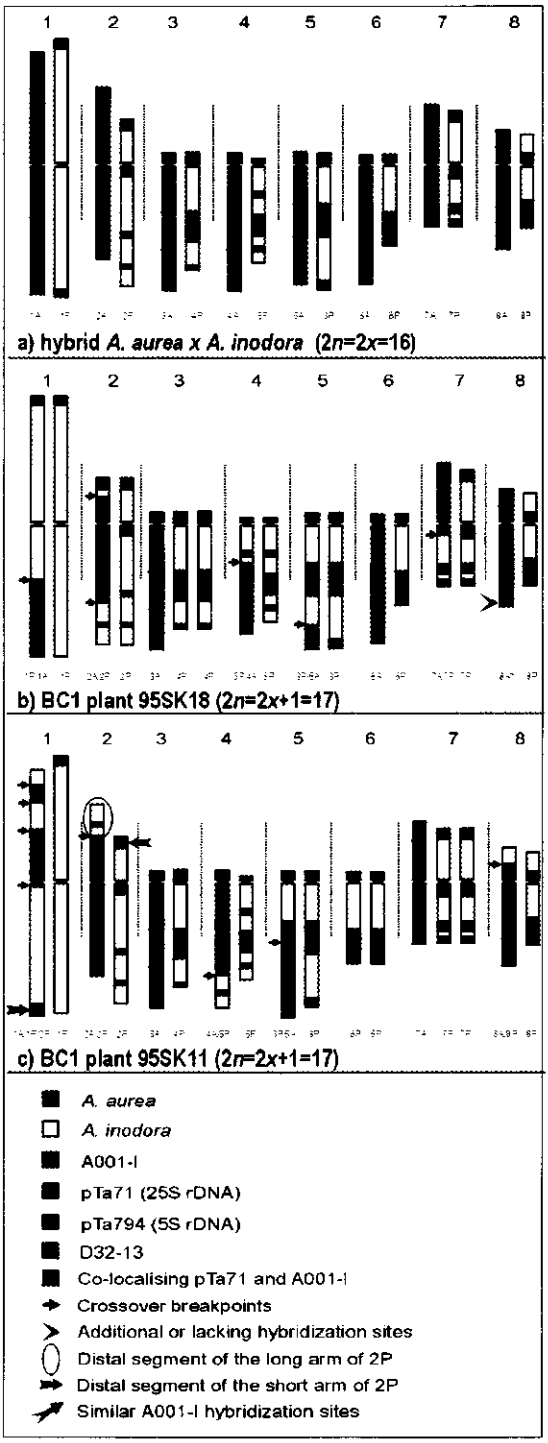
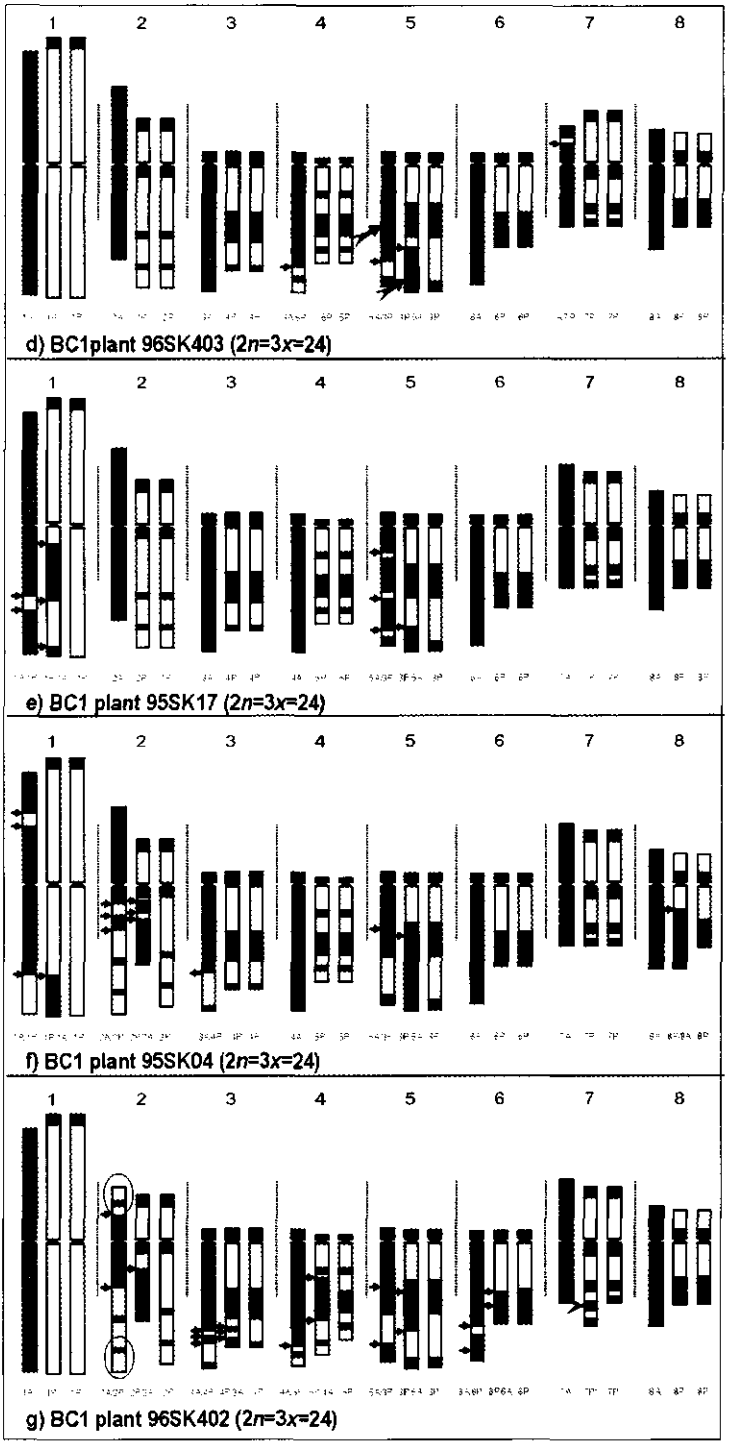


Figure 4.3: FISH karyotypes of the interspecific hybrid *A. aurea* x *A. inodora* (a) and the two diploid (b-c). For all chromosomes it is indicated which part belongs to either *A. aurea* or *A. inodora*. The small arrows indicate the observed crossover points. In addition, the hybridization patterns of the repetitive sequences are depicted for each chromosome.



acrocentric chromosomes (Table 4.2; homoeology of the acrocentrics: 3A - 4P, 4A - 5P, and 5A - 3P).

In addition, for the (sub) metacentric chromosomes it was possible to determine the corresponding homoeologous chromosome arms (this is indicated in Figure 4.3), except for chromosome 1, because chromosome 1 of *A. aurea* lacks identifiable hybridization sites for any of the used probes on both arms.

In two BC_1 plants (95SK11 and 96SK402) recombinant chromosomes were found in which the distal segment of the short arm of chromosome 2A was exchanged with a distal segment of the long arm of chromosome 2P. This

Figure 4.3: (continued) FISH karyotypes of the four triploid BC_1 plants (d-g).

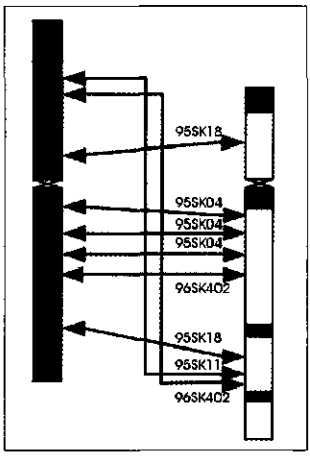


Figure 4.4: Observed cross-overs between chromosome 2A and 2P in the six BC_1 plants. The arrows indicate the positions of the crossover points in the recombinant chromosomes. Note: the distal segment of the short arm of 2A is homoeologous to the distal segment of the long arm of 2P (for legend see Figure 4. 3).

distal segment of 2P was identified by the small interstitial D32-13 hybridization site (for example see Figure 4.1e). However, in other BC_1 plants recombinant chromosomes 2 were found with exchanges between both long arms (in 95SK04, 95SK18, 96SK402) or both short arms (in 95SK18) (Figure 4.4). Apparently, the distal segment of the short arm of chromosome 2A is homoeologous to the distal segment of the long arm of 2P. To confirm this homoeology, meiotic association behaviour of the arms of these chromosomes was studied in 82 metaphase I cells. To this end, FISH with the species-specific probes was applied to the metaphase I spreads. The short arms of both chromosomes 2 possessed easily identifiable D32-13 or A001-I hybridization sites, allowing identification of both arms of these chromosomes. Many bivalents between the two chromosomes 2 were found with a chiasma between the short arm of 2A and the long arm of 2P (Table 4.3, Figure 4.2e, 4.2f), demonstrating the homology between the chromosome segments. From the size of

Table 4.2: Recombinant chromosomes per BC_1 plant, ordered in homoeologous pairs.

95SK11	1A/1P	2A/2P	-	4A/5P	3P/5A	-	-	8A/8P
95SK18	1A/1P	2A/2P	-	5P/4A	3P/5A	-	7A/7P	-
95SK17	1A/1P	-	-	-	5A/3P	-	-	-
	1P/1A	-	-	-	3P/5A	-	-	-
95SK04	1A/1P	2A/2P	3A/4P	-	5A/3P	-	-	-
	1P/1A	2P/2A	-	-	3P/5A	-	-	8P/8A
96SK402	-	2A/2P	3A/4P	4A/5P	5A/3P	6A/6P	-	-
	-	2P/2A	4P/3A	5P/4A	3P/5A	6P/6A	-	-
96SK403	-	-	-	4A/5P	5A/3P	-	7A/7P	-
	-	-	-	-	3P/5A	-	-	-

Underlining indicates that both recombinant segments of these chromosomes were identified. For all other chromosomes only one of the segments could be identified.

Table 4.3: Observed location of chiasmata in bivalents of homoeologous chromosomes 2A and 2P in 82 metaphase I cells of the parental hybrid *A. aurea* x *A. inodora* (A1P2) to prove the existence of an ancient translocation in these two chromosomes.

	Observed chiasma location			
	2PL-2AL	2PS-2AS	2PL-2AS	2PS-2AL
Number of chiasmata	27	9	41	-
Frequency	35.1%	11.7%	53.2%	0

In 30.5% of the metaphase I cells two univalents for these chromosomes were observed.

the exchanged segments in the recombinant chromosomes 2 in the BC_1 plants, it was estimated that approximately the distal 30% of the long arm of 2P shares homology with the distal segment of the short arm of 2A. However, analysis of the chiasmata positions in the bivalents of the two chromosome 2 revealed that 53.2% of the observed chiasmata were between the long arm of 2P and the short arm of 2A, whereas chiasmata between both short arms or both long arms were observed in, respectively, 11.7% and 35.1% of the cells. Thus, the chiasma formation frequency between the different homoeologous chromosome segments of chromosome 2A and 2P was different as would be expected from the relative length of these segments.

In the BC_1 plants many recombinant chromosomes were identified through GISH and FISH. In addition to these chromosomes, structural changes in some of the other chromosomes were detected with FISH. For example, in the BC_1 plant 95SK11 a D32-13 hybridization site was detected on chromosome 1P that was similar in size to the D32-13 site on the short arm of 2P (Figure 4.1e, arrows), indicating a translocation between chromosome 1P and 2P.

In the BC_1 plant 96SK402 an additional D32-13 hybridization site was detected on one of the chromosomes 7P (arrowhead Figure 4.3g). The morphology of this chromosome was altered from metacentric to sub-metacentric, indicating a duplication of a chromosome segment. Another interesting recombinant chromosome was present in the BC_1 plant 96SK403. According to the hybridization sites of the species-specific probes, this chromosome was the result of an interstitial crossover between the homoeologous chromosomes 5A and 3P (Figure 4.2c, 4.2d, 4.3d). However, the large interstitial A001-I hybridization site of 5A was detected in the distal region of the recombinant chromosome. This could be explained by the assumption that chiasma formation in an inverted region of the two homoeologues resulted in bridge formation at anaphase I, followed by chromosome breakage. Therefore, this chromosome might be an

indication for an ancient inversion of an interstitial segment in the chromosomes 5A and 3P. Bridges at anaphase I and II were frequently observed in the parental hybrid *A. aurea* x *A. inodora*.

Genome constitution of the BC_1 plants

Triploid BC_1 plants

As all four triploid BC_1 plants received 2*n*-gametes from the interspecific hybrid (Kamstra *et al.* 1999a), sixteen chromosomes in each of these BC_1 plants were derived from the hybrid *A. aurea* x *A. inodora*. The other eight chromosomes were derived from the recurrent backcross parent *A. inodora*. GISH showed that in all triploid BC_1 plants eight chromosomes with *A. aurea* centromeres and sixteen with *A. inodora* centromeres were present. Since eight chromosomes with *A. inodora* centromeres originated from the recurrent backcross parent, all other chromosomes (eight of *A. aurea* and eight of *A. inodora*) were derived from the hybrid. The equal distribution of the centromeres indicates that in all cases functional FDR 2*n*-gametes were produced by the hybrid. In the pairs of homoeologous chromosomes in the BC_1 plants, some chromosomes were observed which contained reciprocal crossovers (see later). These reciprocal crossovers are a further confirmation that indeed FDR gametes were involved, because reciprocal crossover products can not be found in gametes that were formed by a second division restitution (SDR) mechanism.

Although all triploid BC_1 plants were obtained from the functioning of FDR gametes, differences in their genome constitution were observed. The number of recombinant chromosomes ranged from 4 to 10 (Table 4.1), the total number of crossover points ranged from 4 to 20 (Figure 4.3). Despite these differences, all BC_1 plants still contained almost one complete *A. aurea* genome.

Aneuploid BC_1 plants

Both aneuploid BC_1 plants ($2n=2x+1=17$) received probably 9 chromosomes from the hybrid A1P2-2, instead of the expected 8 from a haploid gamete. Using GISH and FISH the extra chromosomes were identified. The BC_1 plants 95SK11 and 95SK18 were trisomics for chromosome 7 (two 7P and one 7A) and for the homoeologous chromosomes 3A (one) and 4P (two), respectively. In both plants a more or less equal distribution of *A. aurea* and *A. inodora* segments was observed.

Table 4.4 The expected and observed frequencies of crossover points per chromosome arm as a result of 0-3 chiasmata in all BC_1 plants.

	Number of chiasmata per chromosome arm	Percentage of chromosomes with *n* crossover points			
		n=0	*n*=1	*n*=2	*n*=3
Expected frequencies[a]	0	100			
	1	50	50		
	2	25	50	25	
	3	12.5	37.5	37.5	12.5
	total	46.87	34.37	15.62	3.12
Observed in the BC_1 plants derived from *n* gametes[b]		51.9	44.4	0	3.7
Observed in the BC_1 plants derived from 2*n* gametes[c]		70.83	15.62	7.29	6.25

a) Assuming a random chiasma formation per chromosome arm and random involvement of chromatids at each chiasma and no chiasma interference.

b) Observed in 27 chromosome arms in the two aneuploid BC_1 plants. These 27 chromosome arms, instead of the expected 24 (4 (sub-) metacentrics and 4 acrocentric chromosomes), are the result of the additional chromosomes (one acrocentric and one submetacentric respectively) in the two aneuploid BC_1 plants.

c) Observed in 96 chromosome arms in four BC_1 plants. Due to the restitution mechanism (FDR) of the 2*n*-gametes some of the crossover points in homoeologous chromosome pairs will be the result of the same crossover, giving an overestimation of the crossover frequencies.

Recombination frequencies between the *A. aurea* and *A. inodora* chromosomes

The recombinant chromosomes present in all of the BC_1 plants were the result of homoeologous recombination in the parental hybrid. To estimate the recombination frequency between the *A. aurea* and *A. inodora* chromosomes, the frequency of chromosome arms with 0 to 3 crossover points in the BC_1 plants was calculated. These frequencies were calculated separately for the triploid BC_1 plants (2*n*-gametes) and the aneuploid plants (*n*-gametes), to compare the chiasmata frequency in both types of gametes (Table 4.4). The expected frequencies were calculated for 0-3 chiasmata per bivalent (Table 4.4), assuming random chiasma formation per chromosome arm, random involvement of chromatids at each chiasma and no chiasma interference. The number of breakpoints per chromosome arm observed in the 2*n*-gametes is clearly less than observed in the *n*-gametes. Still the plant numbers are too small to allow a meaningful statistical analysis, but it indicates that prior to the formation of the

2*n*-gametes less chiasmata were formed. However, in both types of gametes up to 3 crossovers per arm were observed (Table 4.4).

DISCUSSION

In this study we have shown that sequential GISH and FISH analysis is very useful to accurately analyse plant genomes. In the six BC_1 plants analysed, GISH provided a clear distinction between the parental genomes of *A. aurea* and *A. inodora*, allowing identification of both genomes and consequently of recombinant chromosomes. In addition, FISH, using two species-specific and two rDNA probes, enabled identification of all individual chromosomes, except some of the recombinant chromosomes. The use of GISH increased the number of recombinant chromosomes that were detected in the BC_1 plants compared to those detected in a previous study using the species-specific probes only (Kamstra *et al.* 1999a).This was expected since the absence of identifiable hybridization sites (for example on chromosome 1) prevented detection of some of the recombinant chromosomes. In addition, multicolour FISH with species-specific probes proved useful to detect additional chromosome rearrangements in the genomes of BC_1 plants, which were not detected through GISH, such as small deletions, duplications and inversions (Figure 4.3).

Chromosome constitution of the functional gametes of the hybrid *A. aurea* x *A. inodora*

Since the BC_1 plants received one complete genome (= 1 haploid set of 8 chromosomes) from the backcross parent *A. inodora*, all other chromosomes were transmitted by the interspecific hybrid *A. aurea* x *A. inodora*. This allowed a detailed study of some of the few viable gametes that were produced by this highly sterile hybrid. As the hybrid produced two different types of viable gametes, e.g., *n*-gametes and 2*n*-gametes (Kamstra *et al.* 1999a), the chromosome constitution of both types of gametes could be studied.

The combined GISH and FISH analysis revealed that the triploid BC_1 plants were derived from FDR 2*n*-gametes. Firstly, because all the plants contained one complete set of *A. aurea* chromosomes (at least the centromeric regions) and two sets of *A. inodora* chromosomes, excluding other mechanisms such as SDR and post-meiotic doubling, which would have resulted in an unequal distribution of the parental chromosomes. Secondly, the presence of recombinant chromosomes in the BC_1 plants, indicated that meiotic recombination occurred between homoeologous chromosomes, excluding restitution mechanisms such as pre-meiotic

chromosome doubling. In addition, the presence of both reciprocal crossover products in the same BC_1 plant is only possible when an FDR mechanism has occurred.

The recombinant chromosomes in all BC_1 plants indicated that high frequencies of meiotic recombination occurred between homoeologous chromosomes of *A. aurea* and *A. inodora* (Figure 4.3; Table 4.4). Although the sample of recombined chromosomes analysed is small, differences were observed between the diploid and triploid BC_1 plants regarding the number of recombinant chromosome arms (Table 4.4) (in the triploids less recombined chromosome arms were observed), indicating reduced chromosome pairing/chiasma formation prior to the formation of these four 2*n*-gametes. However, in both types of gametes the maximum number of crossover points per chromosome arm is the same (Table 4.1), suggesting random chiasma formation in both types of gametes. Here, it needs to be mentioned that, because the 2*n*-gametes were produced by an FDR mechanism, some of the crossovers observed in two homoeologous chromosomes in the BC_1 plants could have resulted from the same crossover (Mendiburu and Peloquin, 1979, Bastiaanssen *et al.* 1998). The absence of the first meiotic division, followed by a segregation of the chromatids in the restitutional meiotic division, results in the incorporation of two non-sister chromatids in the same 2*n*-gamete. In the case of one crossover per bivalent, the chance that both recombined chromatids end up in the same gamete is 25%.

Several crossover points in the pairs of homoeologous chromosomes in the BC_1 plants are probably the result of the same crossover events. Although the triple crossover points in 2A/2P and 2P/2A in the BC_1 plant 95SK04 (Figure 4.3f) and the double crossover points in 6A/6P and 6P/6A in the BC_1 plant 96SK402 (Figures 4.2b and 4.3g), suggest that they are the result of the same crossovers, it is not possible to confirm this experimentally. Obviously, the observed recombination frequency in the 2*n*-gametes is an overestimation of the actual recombination (Table 4.4), which indicates that the observed difference in recombination frequencies between *n*- and 2*n*-gametes is even larger.

The two aneuploid BC_1 plants received *n*-gametes from the hybrid. The *A. aurea* and *A. inodora* chromosomes and chromosome segments are present in approximately equal amounts, indicating random chiasma formation and chromosome segregation during meiosis of the F_1 hybrid. The extra chromosomes which were observed in both BC_1 plants are probably the result of non-disjunction or lagging chromosomes. The fact that no crossovers were observed in these chromosomes indicates that the absence of chiasmata has influenced their meiotic segregation.

Identification of homoeologous chromosomes.

As most of the recombinant chromosomes were identified through sequential FISH analysis, they could be used to identify the homoeologous chromosomes which actually paired and recombined during meiosis of the parental hybrid. Previously, the chromosome numbering of the species *A. aurea* and *A. inodora* was assigned on the basis of their average length and morphology e.g., the position of the centromere (Buitendijk *et al.* 1996, Kamstra *et al.* 1997). However, by this method it was not possible to establish the actual homoeologies of chromosomes. The recombinant chromosomes in the BC_1 plants provided clear evidence on which chromosomes of both parental species are actually homoeologous (Table 4.2). The order of some of the acrocentric chromosomes was different from the order that was assigned on the basis of the chromosome length and morphology: 3A being homoeologous to 4P, 4A to 5P and 5A to 3P. For all other chromosomes the order was as expected.

It is expected that, since the genomes of both parental species of the hybrid and the BC_1 plants have diverged a long time ago, alterations, such as ancient inversions or translocations between the chromosomes of *A. aurea* and *A. inodora* are present. By comparative mapping of other plant genomes many instances of translocations and inversions have been found, for example in tomato-potato (Tanksley *et al.* 1992) and wild sunflower species (Rieseberg *et al.* 1995). In our study, at least three instances of ancient chromosomal rearrangements in the two *Alstroemeria* species were detected. Clear evidence was found for an ancient translocation in either chromosome 2P or 2A. Apparently, the distal segment of the long arm of 2P and the distal segment of the short arm of 2A are homoeologous. The analysis of the BC_1 plants as well as the meiotic pairing configuration of these chromosomes confirmed this. In BC_1 plant 95SK11 a chromosome was found in which the distal segment of the short arm of 2P was detected on a recombinant chromosome arm of 1P. It is unclear whether this recombination is the result of meiotic recombination between two non-homologous chromosomes of *A. inodora* or a translocation in mitotic cells. The latter mechanism is more likely because these plants initially went through a callus phase and were propagated for approximately one year *in vitro*. Previously, we have shown that translocation between non-homologous chromosomes can occur during *in vitro* multiplication (Kamstra *et al.* 1999a). A possible inversion of an interstitial segment in one of the homoeologous chromosomes 5A and 3P was indicated by the recombinant chromosome 3P/5A in BC_1 plant 96SK403, in which an interstitial A001-I site was

detected at the distal end of this chromosome. This recombinant chromosome is probably the result of chiasma formation in the inverted region, resulting in a bridge at anaphase I and breakage in this bridge between both interstitial A001-I sites. In the parental hybrid *A. aurea* x *A. inodora* bridges at anaphase I and anaphase II are frequently observed (Kamstra unpubl. obs.), suggesting the presence of one or more inverted regions in the genomes of these *Alstroemeria* species.

Homoeologous recombination between *A. aurea* and *A. inodora* chromosomes

In the BC_1 progeny of the wide hybrid *Gasteria lutzii* x *Aloe aristata* Takahashi *et al.* (1997) found many recombinant chromosomes. In these plants up to 3 crossovers per chromosome arm were observed, like we found in *Alstroemeria*. Although the actual number of BC_1 plants analysed was low, their data suggested that the recombination frequency is higher than would be expected from the number of observed chiasmata at metaphase I. Also other authors recently raised doubt about the 1:1 ratio between crossovers and chiasmata (for review see Sybenga, 1996). Therefore, it would be interesting to study the number of chiasmata at metaphase I of the interspecific hybrid *A. aurea* x *A. inodora* and compare with the number of observed break-points in the BC_1 progeny presented in this study. In our plant material we observed close double- and even close triple-crossovers frequently. These crossovers would probably not be seen as individual chiasmata at metaphase I, which might explain to some extent an underesti-mation of the actual recombination frequency on basis of the number of chiasmata.

In a previous study, we have shown that indeed all chromosomes are able to find a homoeologous pairing partner during meiosis in the hybrid (Kamstra *et al.* 1999a). The finding of recombinant chromosomes for all homoeologous chromosome pairs in the present study indicates that meiotic recombination is not restricted to certain chromosome pairs. However, differences in recombination frequency among the different homoeologous chromosome pairs were observed. Although the sample of BC_1 plants is too small (six plants) to allow meaningful statistical analysis, the larger chromosomes were found to contain more crossover points than the smaller ones. On a total of 61 crossover points in all chromosomes, 4 were observed in the two smallest homoeologous chromosome pairs 7 and 8, whereas 26 crossover points were observed in the large homoeologous pairs 1 and 2, suggesting that the size of the chromosome determines the chance for crossover events. In wheat-rye chromosome pairing it was also shown that the longer chromosome arms possess relatively more chiasmata than the smaller

chromosomes (Bernardo *et al.* 1988). Considering that the total length of chromosomes 1 and 2 of *A. aurea* and *A. inodora* is approximately twice the length of chromosomes 7 and 8, not only the length of the chromosomes seems to influence the occurrence of recombination. In addition, the chromosomes 7 and 8 of *A. aurea* possess, in contrast to the chromosomes 1 and 2, relatively large blocks of constitutive heterochromatin, which might also influence chiasma formation. Among the acrocentric chromosomes differences were observed regarding the number of crossovers per chromosome. Especially, in the homoeologous chromosomes 5A and 3P a large number of crossovers was observed compared to the other acrocentrics. Interestingly, recombinant chromosomes 5A or 3P were detected in all six BC_1 plants, indicating a preferential recombination/transmission of these chromosomes. This was not observed for any of the other chromosomes. It is possible that the length of the two chromosomes influences the occurrence of recombination, because 3P is the largest acrocentric chromosome of *A. inodora* and 5A one of the largest in *A. aurea*. An other explanation for the preferential recombination/transmission of these chromosomes might be selection of specific genotypes.

Recombination was never observed within the large heterochromatic regions of the *A. aurea* genome, according to our study using GISH and FISH. In between the interstitial A001-I bands, which correspond to the heterochromatic regions of the acrocentric chromosomes (Buitendijk *et al.* 1996; Kamstra *et al.* 1997), crossovers were observed frequently (Figures 4.2b and 4.3). In contrast, crossover points were also observed frequently within the D32-13 hybridization sites, supporting the assumption that the chromosomal organisation of the A001-I and D32-13 repeats is very different (Kamstra *et al.* 1997). The D32-13 repeats in the *A. inodora* genome seem to be composed of small blocks of the repeat interspersed with other sequences (Kamstra *et al.* 1997; Kuipers *et al.* 1999), suggesting that the observed crossovers occurred between the small blocks of repeats.

As measured on somatic metaphase chromosomes, the size of the exchanged segments in the recombinant chromosomes ranged from small (less than 1 μm) to large (approximately 10 μm). Based on the genome sizes of both species (Buitendijk *et al.* 1997) and the total length of their DAPI stained chromosomes (Kamstra *et al.* 1997), it was estimated that a chromosome segment of 1 μm contains approximately 100 Mb of DNA. The minimum length between double crossovers was approximately 0.8 μm, indicating that still relatively large stretches of DNA are involved in these exchanges. Therefore, it is possible that other (smaller) double exchanges remain undetected in the BC_1 plants.

5 Meiotic behaviour of individual chromosomes in allotriploid *Alstroemeria* hybrids

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Submitted

ABSTRACT

Chromosome association and chiasma formation was studied in pollen mother cells at metaphase I of four BC_1 plants ($2n$=$3x$=24) obtained from the backcross of the hybrid *A. aurea* x *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 four BC_1 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. In addition, pairing configurations of specific chromosomes indicated the existence of structural rearrangements (inversions, translocations) between the

genomes of *A. inodora* and *A. aurea*. The implications of the absence of homoeologous chromosome pairing on gamete constitution and prospects for introgression in *Alstroemeria* are discussed.

INTRODUCTION

Allotriploid (AAB) plants contain two genomes of one species and one genome from a different one. Generally they are obtained from crossing an allotetraploid (e.g. AABB) with one of the diploid parents (e.g. AA). Such backcross derivatives, better 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 sesquidiploids have been used to produce monosomic additions in several crops, such as *Nicotiana* (Suen *et al.* 1997), *Brassica* (McGrath and Quiros 1990;Chen *et al.* 1997), *Beta* (Mesbah *et al.* 1997), *Allium* (Peffley *et al.* 1985, Shigyo *et al.* 1996), and *Lolium* (Morgan, 1991).

To produce addition lines in *Alstroemeria* and to introgress genes and chromosome segments into the crop species, the interspecific diploid ($2n=2x=16$) hybrid of *Alstroemeria aurea x A. inodora* was backcrossed with *A. inodora* (Kamstra *et al.* 1999a, b). In the BC_1 progeny aneuploids as well as sesquidiploids were observed. The sesquidiploids resulted from unreduced (2n) gametes produced by the F_1 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 BC_1 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 numerous recombinant chromosomes, ranging from 4 to 10 in the different BC_1 plants (Kamstra *et al.* 1999a, b). The number, size and position of the exchanged segments in these chromosomes varied between the plants as well. In addition, structurally altered chromosomes were detected in some of the BC_1 plants (Kamstra *et al.* 1999b). These chromosomes contained hybridization sites for species-specific probes at unexpected locations, which were not found in both 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 analysis of four BC_1 plants using FISH and GISH sequentially applied to spread pollen mother cells at metaphase I to telophase II. To use the sesquidiploids 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: (i) To study the meiotic associations of each individual chromosome in the sesquidiploids and to estimate pairing frequencies. (ii) To confirm the previously surmised structural rearrangements between the chromosomes of both parental species on the basis of the 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

The four allotriploid plants BC_1-1 (95SK04), BC_1-2 (95SK17), BC_1-3 (96SK402) and BC_1-4 (96SK403) were derived from the backcross between the hybrid *A. aurea* x *A. inodora* (AP) with its parent *A. inodora* (P) (Kamstra *et al.* 1999a). We used a combination of FISH and GISH to assess the karyotypes of these sesquidiploids (PPA) with two *A. inodora* (P) genomes and one *A. aurea* (A) genome (Kamstra *et al.* 1999b; Figure 5.1). Chromosomes were classified according to Kamstra *et al.* 1999b.

Meiotic preparations of the BC_1 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% cytohelicase in 0.1 M citrate buffer, pH 4.5) for 2-3 h at 37°C. The softened anthers were squashed in freshly prepared 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 to most slides.

Genomic *in situ* hybridization (GISH)

Probe preparation and GISH was 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 hours at 58°C, hybridized overnight to denatured slides, and detected using Cy3- (Jackson

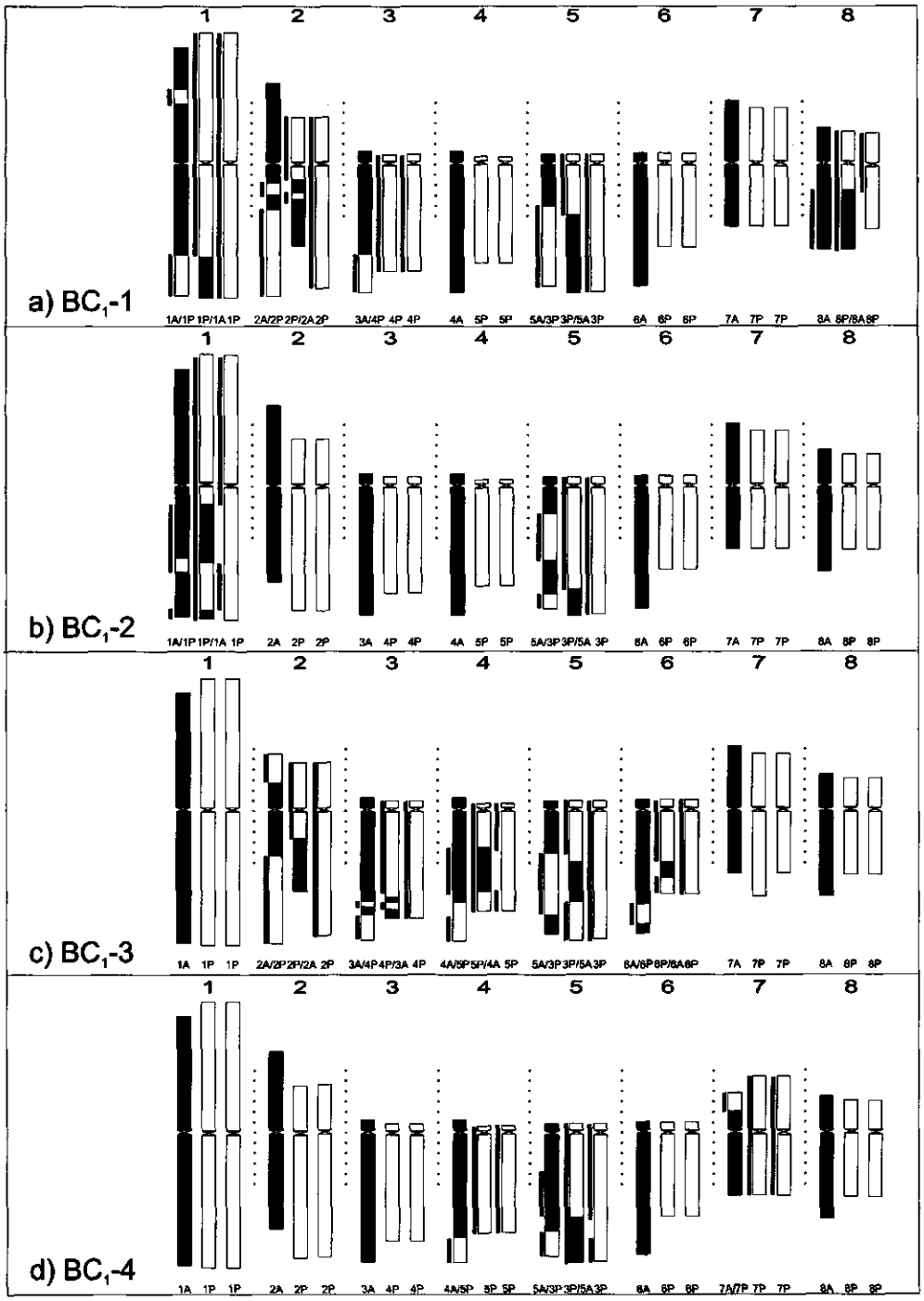
1 2 3 4 5 6 7 8
a) BC1-1
1A/1P 1P/1A 1P 2A/2P 2P/2A 2P 3A/4P 4P 4P 4A 5P 5P 5A/3P 3P/5A 3P 6A 6P 6P 7A 7P 7P 8A 8P/8A 8P
b) BC1-2
1A/1P 1P/1A 1P 2A 2P 2P 3A 4P 4P 4A 5P 5P 5A/3P 3P/5A 3P 6A 6P 6P 7A 7P 7P 8A 8P 8P
c) BC1-3
1A 1P 1P 2A/2P 2P/2A 2P 3A/4P 4P/3A 4P 4A/5P 5P/4A 5P 5A/3P 3P/5A 3P 6A/6P 6P/8A 6P 7A 7P 7P 8A 8P 8P
d) BC1-4
1A 1P 1P 2A 2P 2P 3A 4P 4P 4A/5P 5P 5P 5A/3P 3P/5A 3P 6A 6P 6P 7A/7P 7P 7P 8A 8P 8P

Figure 5.1: Karyotypes of the four sesquidiploids (a-d). *A. aurea* and *A. inodora* chromatin is indicated in 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.

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; 1999b). The biotin- and digoxigenin- labelled probes were detected using Cy3- (Jackson ImmunoResearch Laboratories) and FITC/Alexa 488-conjugated antibodies (Molecular Probes Inc).

RESULTS

All four sesquidiploids BC_1-1, BC_1-2, BC_1-3 and BC_1-4 ($2n=3x=24$) 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 sesquidiploids 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 BC_1 plants

We first analysed the number of univalents (I), bivalents (II) and trivalents (III) in PMCs at metaphase I (Figures 5.2a-b, 5.2k and 5.3a-b). Unequivocal identification of individual chromosomes based on sequential GISH and FISH patterns (Figure 5.3a-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 5.1; Figure 5.4). Bivalent associations were mainly confined to the two homologous *A. inodora* chromosomes, whereas their *A. aurea* homoeologue was present as a univalent (Figure 5.2b as an example). 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 (Figures 5.2a and 5.3a). The frequency ranged from 40.3% to 100% per set of chromosomes and from 80.6% to 100% (=average for recombinant sets) per plant. The average frequency per recombinant chromosome set was 86%. Trivalents for the chromosomes 6 in BC_1-3 were observed in only 40.3% of the MI cells, which was significantly lower than observed for all other recombinant sets. In contrast, in BC_1-2 both recombinant sets (1 and 5) were only associated as trivalents (Table 5.1), which was not observed in any of the other three plants. For all recombinant sets, GISH clearly revealed that the chromosome associations in the trivalents were restricted to homologous segments (Figures 5.2a, 5.3a and 5.3c), i.e. no associations between homoeologous parts were detected. In general, the *A. aurea* segments in these trivalents were unpaired (Figures 5.2a and 5.3a), except for the recombinant sets in which two homologous *A. aurea* segments were present such as set 8 of BC_1-1 or set 4 of BC_1-3 (Figure 5.3a, trivalent of 4A/5P+5P+5P/4A).

In the case of non-recombinant 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 (Figures 5.2a-b, 5.2k and 5.3a-b). The frequency of bivalent + univalent ranged from 42.1% to 97% (Table 5.1). The occurrence of trivalents, which ranged from 3% to 57.9% per set and from 4.4% to 22.1% per plant (Table 5.1), indicated

Table 5.1: Observed frequency (%) of metaphase I associations (trivalent (III) or bivalent+univalent (II+I)) per non-recombinant 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

a) It was not possible to distinguish these two sets in all MI cells and therefore the data of these two sets were combined.
*) Indicates sets containing recombinant chromosomes (see Figure 5.1 for the sizes and locations of exchanged segments).

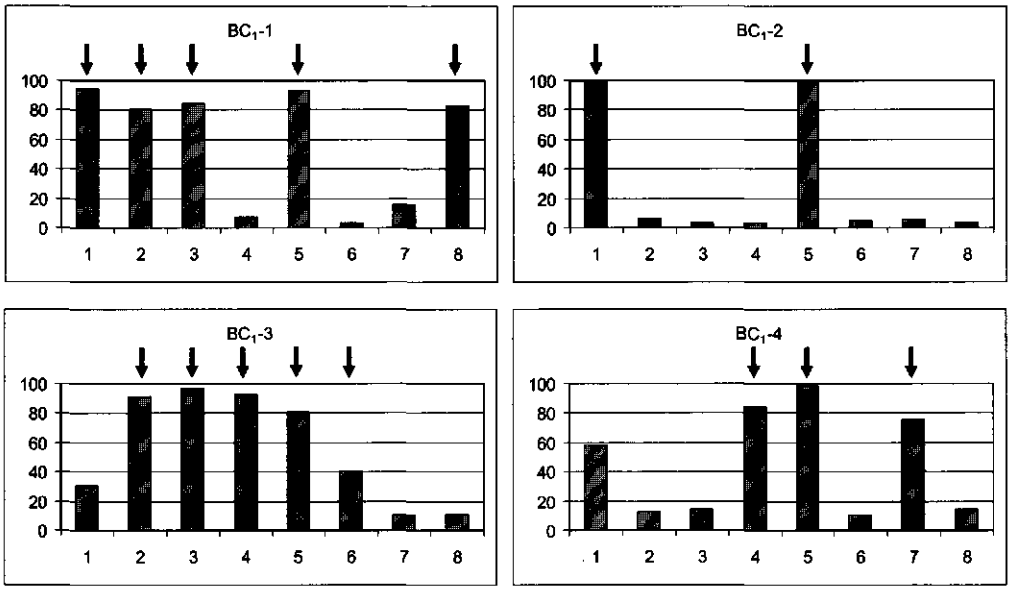

Figure 5.4: Trivalent frequencies observed per set of three homo(eo)logous chromosomes in the four BC_1 plants. Notice the high trivalent frequencies for all recombinant sets (arrows), in contrast to the non-recombinant sets.

pairing and recombination between the homoeologous *A. inodora* and *A. aurea* chromosomes. (see Figure 5.2c). The overall homoeologous chromosome association in all plants was 13.2%. Compared to all other non-recombinant sets of chromosomes, the sets of chromosomes 1 of BC_1-4 and BC_1-3 formed significantly (χ^2; $P < 0.05$) more trivalents (Table 5.1; Figure 5.4).

Metaphase I configurations of the structurally altered chromosomes

In the BC_1-3 a pseudo-iso-chromosome 2A/2P was present, containing two homologous segments of the 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 $5.3d_4$). To confirm the nature of the pseuso-iso-chromosome, we analysed chromosome configurations at metaphase I of this chromosome with its homo(eo)logs. The types of observed chromosome configurations are shown in Figures $5.3c_{1,2,3}$ and $5.3d_{1,2,3}$. In the trivalents the pseudo-iso-chromosome was frequently present as a ring (Figures $5.3c_2$ and $5.3d_2$). At low frequencies (2.2%), this chromosome was observed as a ring univalent (Figures $5.3c_3$ and $5.3d_3$). These observation are in agreement with the assumed chromosome morphology.

In a previous study (Kamstra *et al.* 1999b), we showed that the two homoeologous chromosomes 3P and 5A differed with respect to a reversed interstitial segment. This was revealed by the appearance of an unexpected recombinant chromosome 3P/5A in BC_1-4. In this chromosome a typical interstitial A001-I hybridization site of the parental 5A chromosome was observed at a distal position. Probably a chiasma between a reversed segment of two homoeologous chromosomes had occurred in the parental hybrid, causing bridge formation at anaphase I and consequently chromosome breakage and incorporation of the fragments into the gametes, resulting in this particular recombinant chromosome in BC_1-4. The metaphase I associations of this recombinant chromosome with its homoeologues confirmed the presence of a reversed chromosome segment. BC_1-4 contained one non-recombinant 3P chromosome, one recombinant chromosome 3P (with a distal inverted *A. aurea* segment containing the distal A001-I hybridization site), and one recombinant chromosome 5A containing a distal *A. inodora* segment of 3P (Figure 5.1, Figure 5.2k insert). Because in all BC_1 plants mainly associations between homologous chromosome segments were observed, the association of the two *A. aurea* segments in 5A/3P and 3P/5A could only occur if these were homologous. Trivalents were frequently observed in which both *A. aurea* segments were associated and the orientation of these segments showed that the orientation of the distal segment in 3P/5A was reversed as compared to the homologous segment in 5A/3P (Figure 5.2k insert).

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

At anaphase I all non-recombinant *A. inodora* chromosomes, and most of the recombinant *A. inodora* and *A. aurea* chromosomes segregated regularly to both poles in all BC_1 plants (Figures 5.2d-h and 5.3e-i). In contrast, the non-recombinant *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 5.2d-h and 5.3e-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 (Figures 5.2g and 5.3j). In some telophase I cells the metacentric chromatids were divided in the centromeric regions (Figure 5.3j, 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 (Figure 5.2i-j), thus confirming the unequal distribution of the *A. aurea* chromosomes in the gametes.

Many of the gametes possessed 1 to 3 micronuclei, in which most of the *A. aurea* chromosomes were present (Figure 5.2i-j).

At anaphase I of the BC_1-2 and BC_1-3 plants, bridges were frequently observed, especially in the latter (in 75% of all AI cells). Usually the bridges, which occurred in the non-recombinant chromosomes 8P in BC_1-3 (Figure 5.3e-f) and 1P in BC_1-2 (Figure 5.2e-f), were accompanied by an acentric fragment (Figures 5.2e-f and 5.3h-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 BC_1 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 (Figures 5.2e-f and 5.3h-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 BC_1-3 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 5.1). 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.

DISCUSSION

The sequential GISH/FISH analysis revealed additional information compared to the analysis of meiosis based on GISH alone (cf. Parokonny *et al.* 1994; 1997; Benavente *et al.* 1996; King *et al.* 1994), since it enabled us to identify and study the pairing behaviour of all individual chromosomes. In general, preferential chromosome pairing of homologous chromosome segments was observed, as shown by the high frequencies of bivalent + univalent configurations for the non-recombinant chromosome sets and the high frequencies of trivalent configurations for the sets containing recombinant chromosomes (Table 5.1; Figure 5.4). In addition, in the trivalents of recombinant sets only the homologous chromosome segments were associated.

Homoeologous pairing between *A. aurea* and *A. inodora* chromosomes was observed in low frequencies for most chromosomes. The strong preferential pairing and the low frequencies

of homoeologous pairing in the BC_1 plants are in contrast to what was observed in the diploid parental hybrid, in which high frequencies of homoeologous chromosome pairing (80% heteromorphic bivalents) and recombination were observed (Kamstra *et al.* 1999a, b). Thus, in the BC_1s the presence of homologous *A. inodora* chromosomes leads to preferential pairing and the alien homoeologue is left out as a univalent.

The strong tendency of the recombinant sets of three homo(oe)logous chromosomes to form trivalents (86%), in contrast to non-recombinant sets (13.2%), can be explained by the presence or absence, respectively, of homologous chromosome segments among the three chromosomes in a set. GISH clearly revealed that the homologous chromosome segments in the recombinant sets were generally associated at metaphase I, in contrast to the homoeologous segments. The large number of trivalents observed for these chromosome sets indicates that, even if the homologous segments are relatively small, chiasmata are formed in high frequencies. In most of the recombinant sets, the sizes of the homologous segments in some of the chromosomes ranged from approximately 10-50% of the chromosome arms (Figure 5.1, black bars; Kamstra *et al.* 1999b), whereas the trivalent frequencies were similar. Therefore, the size of the exchanged segments is not correlated with the trivalent frequency. Only the recombinant set of chromosomes 6 in BC_1-3 showed a reduced trivalent formation (40.3%) compared to all other recombinant sets (Table 1; Figure 5.4). The exchanged interstitial segment in chromosome 6A/6P is small (Figures 5.1 and 5.3a) and, in addition, a large cluster of the tandem repetitive sequence D32-13 is located on this segment (Figure 5.3b). The presence of this cluster might reduce pairing in this chromosome segment. Still, the 40.3% of trivalents indicates that even in this small chromosome segment chiasmata are formed frequently. Remarkable is the pairing behaviour of both recombinant sets in BC_1-2, which invariably formed two trivalents in all cells (Table 1; Figures 5.2a-b and 5.4). This was not observed for any of the other recombinant chromosome sets, although the frequencies are on average high. Both recombinant chromosome sets contain relatively large homologous segments in all chromosomes (Figure 5.1), which might explain to some extent the pairing behaviour, which results in 100% trivalents for these two chromosomes.

The actual chromosomal position of homologous segments among a set of three homo(eo)logous chromosomes does not seem to influence the pairing behaviour. The recombinant chromosomes possessed recombined segments that were homologous to other segments at distal, interstitial or proximal position. Differences in pairing behaviour of these different

segments were not observed, which is in agreement with previous data suggesting that chiasmata can occur along the entire chromosome length (Kamstra *et al.* 1999b). In addition, the observed chromosome configurations in the trivalents (for example frying pans) indicate that pairing partner switches occur regularly between these chromosomes. Therefore, several pairing initiation sites seem to be present along these chromosomes.

Homoeologous chromosome pairing was observed at low frequencies in all plants. Variation was observed among the individual chromosomes and per plant. The average frequency was rather low (13%), however, both non-recombinant *A. aurea* chromosomes 1 in BC_1-3 and BC_1-4 were frequently associated with their homoeologous *A. inodora* chromosomes 1 (30.6% and 57.9%, respectively). In both *A. aurea* and *A. inodora*, the chromosomes 1 are the largest compared to the other chromosomes (Buitendijk *et al.* 1997), therefore chromosome size seems to influence the pairing frequency, as was also shown in wheat (Bernardo *et al.* 1988, Cuardrado *et al.* 1997).

The fate of individual chromosomes

The non-recombinant *A. inodora* chromosomes and the recombinant *A. aurea* and *A. inodora* chromosomes disjoined regularly at anaphase I and II, which corresponded to their pairing behaviour at metaphase I. For example, the recombinant *A. aurea* chromosomes, which possessed *A. inodora* segments, paired with their *A. inodora* counterparts at metaphase I at high frequencies. As a result, these chromosomes recombined and segregated regularly at anaphase I and II, and were randomly distributed into the gametes.

The non-recombinant *A. aurea* chromosomes behaved differently. As these were usually not associated at metaphase I, these chromosomes remained lagging in the middle of the equatorial plane, and segregated into chromatids during the first meiotic division. As a consequence, these chromosomes were randomly distributed into the gametes. Probably most of these chromosomes get lost, as was indicated by the presence of one or more micronuclei in the gametes, in which *A. aurea* chromatin was usually detected. However, homoeologous pairing between non-recombinant *A. aurea* chromosomes and their homoeologues occurred at low frequencies, resulting in trivalents (Table 5.1). All chromosomes involved in these trivalents seemed to segregate regularly during meiosis (for example chromosomes 2 in Figure 5.2e), which made clear that at least parts of these non-recombinant chromosomes are at low frequencies transmitted to the gametes.

The bridges, which were frequently observed in BC_1-2 and BC_1-3 between two non-recombinant *A. inodora* chromosomes, suggested the presence of paracentric inversions. These chromosome aberrations were probably caused during the prolonged *in vitro* multiplication as shown previously for *Alstroemeria* (Kamstra *et al.* 1999a). In addition, the absence of bridges during meiosis of the (backcross) parent *A. inodora*, indicated that these aberrations did not exist previously in this species.

Implications for introgression of alien chromosomes

The low frequencies of homoeologous chromosome pairing in the four sesquidiploids showed that most homoeologous recombination between the *A. aurea* and *A. inodora* genomes/chromosomes had already occurred during the meiosis of the parental hybrid. This has implications for further breeding. The low frequencies of homoeologous recombination in the sesquidiploids imply that the chromosome and chromosome segments of *A. aurea* that are present in the sesquidiploids, are either transmitted completely to the next generation or these are lost. No further segmentation of the *A. aurea* chromosomes will cause problems when the desired characteristics are linked to undesired ones.

The random segregation of non-recombinant *A. aurea* chromosomes at anaphase II and telophase II suggested that in BC_2 plants a random number of *A. aurea* chromosomes can be present, including monosomic addition lines, as was found in other plant species (Suen *et al.* 1997, Chen *et al.* 1997,Mesbah *et al.* 1997, Peffley *et al.* 1985, Shigyo *et al.* 1996, Morgan, 1991; and others). As mentioned, many gametes of the BC_1 plants contained one to three micronuclei containing *A. aurea* chromosomes, which failed to be directly incorporated into the nucleus. The question remains whether these micronuclei might hamper fertility of the actual gametes of the BC_1 plants and whether the chromosomes in the micronuclei can be transmitted to the progeny. The amount of stainable pollen per BC_1 plant varied from 30-60% (Kamstra *et al.* 1999a, unpbl. obs). However, after extensive backcrossing a small number of BC_2 plants was obtained, indicating that probably additional factors reduce the actual viability of the gametes of the BC_1 plants. The genome constitution of the BC_2 plants will be analysed in the future and will reveal the type of viable gametes that are produced in the sesquidiploids.

6 General Discussion

Introgression of alien chromosomes has been used in many crops to transfer desired characteristics from one species into another one. To facilitate this transfer distant hybrids are made and backcrossed with the recipient parent to produce an offspring in which certain chromosomes of the donor species are retained and others are eliminated. Homoeologous recombination between the donor and recipient genome is required to further fragmentize the chromosomes in the offspring and facilitate stable introgression of alien chromosome fragments with desired traits.

The possibility to accurately identify the genome constitution of distant hybrids and their backcross progeny by using molecular cytogenetics greatly enhances the monitoring of the introgression process. It allows the selection for genotypes with the desired genome or chromosome constitution in an early stage, thus speeding up the process. In addition, the analysis of the genome constitution of the offspring from a distant hybrid allows a detailed study of the viable gametes that are produced in the hybrid itself. This reveals basic information on the types of viable gametes that are produced in distant hybrids, which is important knowledge for (introgression) breeding. The power of molecular cytogenetic analysis for introgression breeding is clearly demonstrated for an interspecific hybrid between *A. aurea* and *A. inodora* (Chapter 2) and its backcross progeny (Chapters 3 and 4). Besides this, molecular cytogenetics can also be applied for the analysis of important processes such as meiotic chromosome pairing and recombination, as was shown in chapters 3 and 4 for the interspecific hybrid and in chapter 5 for the backcross progeny.

Molecular cytogenetic analysis in *Alstroemeria*.

Alstroemeria clearly possesses several characteristics that enable to study introgression with molecular cytogenetics: a small basic number (x=8) of large chromosomes that show morphological variation, large genomes that are (highly) differentiated between species (Kuipers *et al.* 1997; Chapters 3, 4 and 5), presence of specific tandem repeats at specific sites on the chromosomes (Chapter 2), high frequencies of homoeologous chromosome pairing and consequently recombination in interspecific hybrids (Chapters 3 and 4). The identification of individual chromosomes is essential to monitor introgression of particular chromosomes. Unequivocal identification of all individual chromosomes was only attained by using a molecular cytogenetic

analysis with multiple probes (Chapter 2). Four highly repetitive DNA families were mapped on mitotic chromosomes. The huge variation between the two species, *A. aurea* and *A. inodora*, regarding the presence of particular blocks of repetitive DNA at distinct locations on the chromosomes was used to identify all individual chromosomes (Chapter 2). The possibility to identify individual chromosomes was exploited for the analysis of the backcross plant material.

FISH with the species specific repeats A001-I and D32-13 could be used to clearly show the presence of particular genomes or chromosomes (Chapters 2, 3, 4 and 5), and revealed whether plants were true hybrids (Chapter 2) or successful backcrosses (Chapters 3 and 4). In addition, the possibility to apply these probes to meiotic chromosomes clearly enabled a more accurate investigation of meiotic chromosome association in hybrids and their offspring (Chapters 3 and 5). In combination with GISH, which was first used in *Alstroemeria* by Kuipers *et al.* (1997), these repetitive DNA families proved very useful to investigate the genome constitution of the obtained plant material (Chapter 4). The possibility to hybridize different probes sequentially to the same chromosome preparation enabled comparison of the obtained hybridization patterns with each other. This was exploited in chapters 2 and 4 to construct detailed karyotypes of the two species and the backcross plants. Although this type of analysis is time consuming and requires accuracy and experience, it is worthwhile to perform. In all, the molecular cytogenetic analysis in *Alstroemeria* has revealed valuable data about the chromosome organisation of the different species, the constitution of gametes in wide hybrids, meiotic behaviour of chromosomes in hybrids as well as in their progeny, extent and position of homoeologous recombination and prospects for introgression of alien chromatin.

Chromosome organisation of the species *A. aurea* and *A. inodora*

The physical localisation of the highly repetitive DNA sequences revealed valuable insights into the chromosome organisation of the species *A. aurea* and *A. inodora*. species-specific tandem repeats that were isolated from either species, were physically localised on the chromosomes. Despite their different sequence origin, the

physical localisation of these repeats revealed analogous hybridization patterns on the chromosomes of both species (Chapter 2). The presence of different species-specific tandem repeats on similar chromosomal sites indicated that the accumulation of particular repetitive DNA sequences on the chromosomes is not random, but that some kind of mechanism may be active which accumulates specific sequences at specific locations in the genomes of both species. In

A. aurea the A001-I repetitive DNA blocks co-localised with the heterochromatin bands (C-bands), which were previously described by Buitendijk and Ramanna (1996). The non-random distribution of heterochromatin and A001-I bands in the chromosomes of *A. aurea*, suggest that a mechanism for heterochromatin dispersion as described by Schweizer and Loidl (1987) is responsible for this non-random distribution. According to this model, initiation of heterochromatin amplification and C-band formation takes place at telomeres, from which transposition to equilocal intercalary regions in non-homologous chromosome arms is assumed to occur, followed by coevolution of heterochromatin/c-band patterns in chromosome arms of similar size. For example, the equilocal distribution of bands, similar sizes and base composition of bands, the presence of distal bands on shorter chromosome arms, whereas longer arms show interstitial bands, is similar to what was found in grasshoppers and liliaceous plants (for references see Schweizer and Loidl 1987). Furthermore the presence in all heterochromatin bands of the ribosomal 18S-25S rDNA in combination with the A001-I repeat indicated that all bands have a comparable composition and probably a common origin. In *A. inodora*, a species without conspicuous C-bands, the D32-13 sequences were present as small arrays of tandem repeats in contrast to the large blocks of A001-I repeats in *A. aurea* (Chapter 2; Kuipers *et al.* 1999). However, the distribution of the species-specific repeats among the chromosomes of *A. inodora* and *A. aurea*, respectively, showed remarkable similarities. Probably the arrays of D32-13 repeats are too small to be stained by C-banding as heterochromatin or alternatively the *A. inodora* repeats lack the DNA elements that bind proteins or other molecules to produce the heterochromatic C-band-like structure (Gill and Jiang, 1998).

Fluorescent *in situ* hybridization with the species-specific probes revealed that the composition of the A001-I bands was different from that of the D32-13 bands (Chapter 2). No recombination was observed within the A001-I bands in the BC_1 plants, whereas in D32-13 bands recombination occurred frequently (Chapter 4). Pachytene stages in the interspecific hybrid *A. aurea* x *A. inodora* confirmed the different pairing behaviour of these regions. At pachytene the homoeologous chromosomes of *A. aurea* and *A. inodora* are paired over almost their entire lengths, but the A001-I bands clearly form loops in most cases (Figure 6.1). Kuipers *et al.* (1998) showed that in the A001-I sites in *A. aurea* no Ty1-copia retrotransposable elements were present, whereas these were found in (between) the D32-13 sites of *A. inodora*, supporting the idea that these regions are composed of clearly different sequences.

Another striking feature was the large number of ribosomal sites in the *Alstroemeria* genomes (Chapter 2). In most plant species, the rDNA sites are usually confined to one or a few pairs of chromosomes (Schmidt *et al.* 1994; Galasso *et al.* 1995; Fominaya *et al.* 1995; Xu and Earle 1994; among many others), whereas in *Alstroemeria*, one to four sites are present on almost all chromosomes. Generally, in plants the number of ribosomal sites is correlated with the genome size. A high degree of variation in the number of rDNA sites is occasionally observed between species of the genus Aegilops (Badeava *et al.* 1997). Similar variation in the number of sites per species was observed among *Alstroemeria* species (Chapter 2; unpl. obs.). In addition, the physical position on the chromosomes of the rDNA sites varied greatly between different species (Kamstra *et al.* 1997; unpl. obs.), which is in contrast to the more or less conserved chromosome morphology (Buitendijk and Ramanna, 1996) and homoeology (Chapter 4) in the *Alstroemeria* species.

Most of the rDNA sites in *A. aurea* correspond to the heterochromatin blocks, and therefore, the rDNA sequence might have been distributed among the chromosomes in combination with the A001-I sequence as a result of a distribution mechanism as described by Schweizer and Loidl (1987). Interestingly, in *A. inodora*, which possessed an equal amount of rDNA sites, the rDNA sites did not correspond to the D32-13 sites, but were located at in proximal regions. Apparently, in *A. inodora* the rDNA sites were not distributed in combination with the D32-13 repeats.

Comparative analysis of the chromosomes of *A. aurea* and *A. inodora*

The identification of the homoeologous chromosomes in the species *A. aurea* and *A. inodora* (Chapter 4), which was established through analysis of the recombinant chromosomes in the BC_1 plants, was essential for the construction of the detailed karyotypes of all BC_1 plants (Chapter 4). The identification of homoeologous chromosomes was confirmed by analysis of chromosome associations at metaphase I of the interspecific hybrid. In addition, the strict bivalent formation in diploid hybrids indicated the absence of large structural changes among the chromosomes. Analysis of recombinant chromosomes revealed that the basic chromosome organisation in both species is almost identical. This was confirmed by using GISH on pachytene stages of the interspecific hybrid which revealed almost completely paired homoeologous chromosomes (Figure 6.1), indicating that sufficient homology to enable chromosome pairing is present between the chromosomes of both species. This homology seems to

be present along the entire length of chromosomes, except for the heterochromatic regions in *A. aurea* and some other unidentifiable regions, which are not paired at pachytene (see above). Some differences in the chromosome organisation between the two species were detected, such as the locations of rDNA sites (Chapter 2) and the location of some homoeologous chromosome segments (Chapters 4 and 5), as was shown in chromosomes 2P and 2A, and in chromosomes 5A and 3P. Probably not all structural differences (inversions, translocations) between the two genomes were detected by our analysis. Firstly, because the analysis, which revealed the structural differences, was only performed on a small population of six BC_1 plants. In addition, meiotic configurations of homoeologous chromosomes in the hybrid itself might be used to reveal more structural changes and confirm assumed changes, as was shown for chromosomes 2A and 2P (Chapter 4). Secondly, the resolution of the used techniques will only reveal large structural differences. Thirdly, structural differences between the genomes of both species will have an effect on gamete formation and viability and, therefore, the use of BC_1 plants that are the products of viable gametes might not reveal all differences. We often observed bridges at anaphase I and II of the hybrid A1P2, some of which were probably the result of chiasma formation in the reversed interstitial chromosome segment between 5A and 3P (Chapter 4).

Meiosis in distant hybrids

FISH offers a means to analyse meiosis in distant hybrids and their derivatives in more detail than was previously possible using conventional cytological techniques. Especially, critical observations can be made on homoeologous chromosome pairing, recombination, behaviour, disjunction, structural alterations as well as the products of meiosis (King *et al.* 1994; 1998; Miller *et al.* 1994; Parokonny *et al.* 1994; 1997; Jacobsen *et al.* 1995; Takahasi *et al.* 1997). Generally, in plants chromosome pairing analysis is confined to metaphase I associations and in most cases the extent of pairing is underestimated (Sybenga 1974). GISH and FISH techniques provide ways to accurately visualise associated chromosomes, and therefore to estimate the extent of pairing more precisely. Because the recombinant chromosomes can be identified through GISH and FISH at late metaphase I or anaphase I and II stages, the extent of recombination can be assessed indirectly. Except in the rare cases of the presence of chromosome markers, such as the knobs in maize, physical identification of recombinant chromosomes was not possible through traditional cytology. As is evident in the case of *Alstroemeria*, recombi-

nant chromosomes can be routinely identified both in mitosis and meiosis by molecular cytogenetic analysis (Chapters 3, 4 and 5). In addition, the distribution of crossovers along the chromosomes can be accurately visualised. Besides this, the estimation of the degree of interference was confined in the past to genetic linkage analysis. As has been demonstrated in chapter 4, the number of crossovers per chromosome can be directly assessed. However, this type of analysis requires large and well differentiated chromosomes.

Meiotic chromosome associations in allotriploids (sesquidiploids) has been used to estimate the degree of genome differentiation between species. Pairing in allotriploids has been used as model system (see Chapman and Kimber, 1992). A drawback in such analysis is that the presence, or absence, of recombinant segments in the alien genomes has not been taken into account because it was not possible to detect these by classical cytology. The analysis of sesquidiploids with two genomes of *A. inodora* and one of *A. aurea* clearly demonstrated the influence of recombinant segments on trivalent frequencies (Chapter 5). This illustrates the importance of GISH and FISH as a tool for establishing genome relationships among species.

The viable gametes in distant hybrids of *Alstroemeria*

The highly sterile interspecific hybrid *A. aurea* x *A. inodora* produced a few viable gametes that could be used for further backcrossing. By analysing the backcross progeny plants it was possible to determine the kind of viable gametes that were produced by the hybrid. First of all, the occurrence of two different ploidy levels among the progeny, indicated that viable *n*- and 2*n*-gametes were produced (Chapters 3 and 4). The phenomenon of 2*n*-gamete formation has been found to occur in many plant species. For interspecific hybrids unreduced gamete formation is often the only way to produce viable gametes, leading additionally to sexual polyploidization of many crop plants (Harlan and DeWet, 1975). 2*n*-gamete formation in interspecific *Alstroemeria* hybrids is the main reason that all current cultivars are polyploids (Ramanna 1991).

2*n*-gametes can be formed by several types of nuclear restitution mechanism of which first division restitution (FDR) and second division restitution (SDR) are the most common (Mok and Peloquin, 1975). FDR 2*n*-gametes basically contain all parental chromosomes, since they originate from one equational division of the entire chromosome complement as a result of modified meiotic division. In this case, the non-sister chromatids are included in each restitution nucleus. Genetically, FDR gametes therefore result in homogeneous populations of

gametes. In the case of SDR, the products of the reduction division are restituted and the sister chromatids are included in the same restitution nucleus. Because the individual pairs of homoeologous chromosomes assort during disjunction, the SDR gametes result in heterogeneous populations. In short, FDR gametes contain the two parental genomes nearly intact, whereas in SDR gametes the genomes are disrupted.

The potential of GISH as a tool to determine the restitution mechanism of $2n$-gametes in distant hybrids was demonstrated in chapter 4. Differential staining of chromatin of the parental genomes can easily be used to discriminate between an equal (FDR) or an unequal distribution of parental chromosomes (SDR). The accurate identification of chromosomes, by using for example FISH with repetitive DNA probes, can provide further information about the restitution mechanism, because SDR gametes contain two homologues for each chromosome, whereas FDR gametes contain two homoeologues. Analysis of the genome constitution in the triploid BC_1 plants revealed the presence of two homoeologues for each chromosome pair and, therefore, confirmed the FDR mechanism. In addition, the presence of reciprocal crossover products in the same $2n$-gamete can only occur in the case of FDR. Pairs of homoeologous chromosomes showing reciprocal crossovers were detected in several triploid BC_1 plants confirming the FDR mechanism.

Homoeologous recombination in the hybrid

Metaphase I associations in the distant hybrid *A. aurea* x *A. inodora* were analysed to determine the frequency of homoeologous chromosome pairing. The use of FISH with the species-specific probes D32-13 and A001-I revealed accurately whether chromosomes were associated or not at metaphase I (Chapter 3). Rather high frequencies of associated chromosomes were detected, indicating that meiotic recombination between homoeologous *A. aurea* and *A. inodora* chromosomes does occur. This was confirmed by the analysis of the BC_1 plants. FISH showed the presence of two different species-specific probes on the same chromosome in the BC_1 plants, which was most likely the result of homoeologous recombination (Chapter 3). However, the GISH analysis revealed in much more detail the presence of recombinant chromosomes, which were clearly the result of crossing-over between homoeologous chromosomes (Chapter 4). Moreover, using GISH it was possible to detect recombinations between all *A. aurea* and *A. inodora* chromosomes, which was not possible by using FISH with the species-specific repetitive probes, due to the absence of FISH markers for certain chromo-

somes. Nevertheless, it might be possible that small exchanged segments as a result from double crossovers remained unnoticed, because the smallest exchanged segment which could be visualised using GISH still contained approximately 100 Mbp (Chapter 4).

The potential of GISH to unravel the extent and position of crossover events

GISH can be used to differentially stain chromatin belonging to the parental genomes, and therefore, to determine the number of crossover events per chromosome in distant hybrids, either through an analysis at anaphase I or anaphase II or in the progeny of the hybrid. Such an analysis gives an estimation of the actual number of chiasmata that are formed. Due to technical difficulties to separate all chromosomes at anaphase I and II in a reasonable number of cells, the analysis of progeny plants was a good alternative, since in these plants the gametes of the distant hybrid are 'fixed'. Because it is possible to distinguish the single genome contributed by the backcross parent in the BC_1 plants, the composition of genome(s) derived from the hybrid could be determined accurately.

Takahashi *et al.* (1997) showed in their analysis of two backcross progeny plants from an *Gasteria* x *Aloe* hybrid that the number of crossovers per chromosome ranged from 1 to 3. In the hybrid *A. aurea* x *A. inodora* the number of crossovers ranged from at least 1 to 4 per chromosome (Chapter 4). In the progeny of the Gasteria x *Aloe* hybrid the number of observed crossovers was higher than expected from the number of chiasmata at metaphase I, raising doubts about the assumed 1:1 ratio of chiasmata and crossovers (Takahasi *et al.* 1997). One of their explanations was that probably some chiasmata remained undetected, probably because they occurred in very narrow segments. Our analysis in *Alstroemeria* showed several chromosomes with multiple crossovers in very narrow chromosome segments. It is likely that the corresponding chiasmata can not be resolved and thus would be scored as a single chiasma. High resolution genetic maps also suggest much higher overall recombination frequencies than corresponding chiasma scores (Nilsson *et al.* 1993, Gill *et al.* 1995, Hall *et al.* 1997).

In addition, using GISH it was possible to determine the physical position of crossover events. In *Alstroemeria*, crossover events can occur along the entire length of the chromosomes; from highly proximal to distal (Figure 6.2). Using FISH with the specific repeats we were able to accurately identify the recombinant chromosomes in the BC_1 plants which enabled to determine per set of homoeologous chromosomes where and in what frequency crossovers had occurred. As shown in Figure 6.2, different recombination frequencies per set of

homoeologous chromosomes were observed. Although the number of plants in which crossovers were analysed is small, there seems to be a positive correlation between the physical size of the chromosome and the number of recombinations. In addition, chiasma counts per individual chromosome at metaphase I in the hybrid confirmed preferential pairing and chiasma formation in the larger chromosomes (unpl. obs.). Similar preferences were detected in pairing behaviour of homoeologous chromosomes in the BC_1 plant (Chapter 5). Size differences between the chromosomes might explain the different pairing behaviour to some extent; the larger the chromosomes are the higher chance for chiasma formation. However, other factors might influence chromosome recombination as well, such as the presence of heterochromatin. The heterochromatic blocks in *A. aurea*, for example, completely lack recombination. Interestingly, the larger *A. aurea* chromosomes contain no or small blocks of heterochromatin as compared to the smaller chromosomes.

The prospects of introgression in *Alstroemeria*

Although laborious, it is possible to backcross distant *Alstroemeria* hybrids with their parents as we showed in this study for the hybrid *A. aurea* x *A. inodora*. The low success rates of these crosses indicate the distant relatedness of both parental species *A. aurea* and *A. inodora* (Chapter 3). However, the BC_1 and BC_2 plants that were obtained clearly showed that it is feasible to introgress alien chromosomes in *Alstroemeria*. One bottleneck is the time it takes to produce the successive backcross generations, which in our study was approximately 2 years.

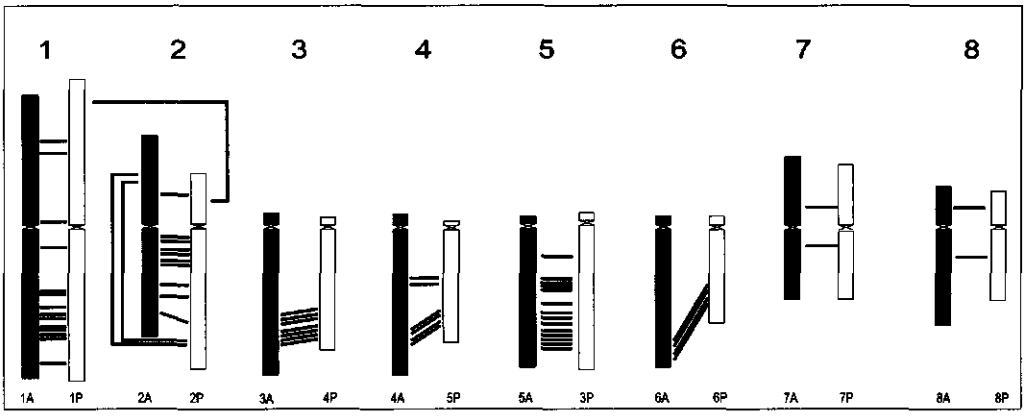

Figure 6.2: Physical position of crossovers detected in the recombinant chromosomes in 6 BC_1 plants. The lines between the homoeologous chromosomes indicate the observed position of the breakpoints in the recombinant chromosomes. The presence of reciprocal crossover products in FDR gametes might give an overestimation of the number of crossovers in certain chromosomes (see Chapter 4). Note the variation in the number of crossovers which were detected between the individual chromosomes.

The detailed analysis using GISH and FISH clearly revealed which chromosomes were present and which were absent in the backcross plants. This is important for introgression, because this information can be used for further breeding to select the genotypes which contain particular chromosomes, and can be used to produce, for example, addition lines.

The presence of recombinant chromosomes in all BC_1 plants indicated that meiotic recombination between the chromosomes of *A. aurea* and *A. inodora* occurs in a rather high frequency. Therefore, it should be feasible to transfer small chromosome segments from one species into the other. This can be important to eliminate linkage with undesired genes. Furthermore, meiotic recombination between homoeologous chromosomes is believed to be one of the prerequisites for stable introgression of chromosome segments (Islam and Shepherd, 1992; Jiang *et al.* 1994). However, it needs to be mentioned that because *Alstroemeria* chromosomes are very large the observed exchanged chromosome segments were considerably large (Chapter 4), which can cause problems in the case of tight linkage (linkage drag). The smallest detected recombinant segment in *Alstroemeria* resulting from a double crossover (Chapter 4), as revealed from GISH, approximately equals the size of the entire *Arabidopsis* genome (Bennett and Leitch, 1997).

The interspecific hybrid produced two different types of gametes (Chapter 3). Both types, theoretically, have specific advantages for introgression breeding. In Figure 6.3 it is indicated how our knowledge of chromosome behaviour in successive backcross generations of *Alstroemeria* could be exploided for further breeding to produce addition and introgression lines. The *n*-gametes indicate regular meiosis in the hybrid prior to the formation of these gametes, resulting in a random distribution of parental chromosomes of the hybrid into the gametes. This was observed in the diploid BC_1 plants (Chapter 4) derived from *n*-gametes, in which approximately half of the transmitted chromosomes or chromosome segments were from *A. aurea* and the other half from *A. inodora*. However, although potentially useful for introgression of *A. aurea* chromosomes into *A. inodora*, these diploid BC_1 plants did not flower, and therefore, could not be used for further research.

The triploid BC_1 plants on the other hand produced abundant flowers and could be used for further breeding. These plants received $2n$-gametes from the hybrid, which were produced by an FDR mechanism. Therefore, basically all chromosomes of the parental genomes were transferred to the backcross plants, resulting in sesquidiploid BC_1 plants (=allotriploids with genome constitution PPA; P=*A. inodora* , A=*A. aurea*). Sesquidiploids have been used in many

plant species as a basis to produce alien addition lines (Suen et al. 1997; McGrath and Quiros 1990; Chen et al. 1997; Mesbah et al. 1997; Peffley et al. 1985; Shigyo et al. 1996; Morgan, 1991), since a random distribution of alien chromosomes can be expected in the progeny of these plants. In this study, the sesquidiploid BC_1 plants showed as expected preferential pairing between the *A. inodora* chromosomes and lagging of *A. aurea* chromosomes, resulting in a random distribution of *A. aurea* chromosomes into the gametes (Chapter 5). Most of the pollen of these plants was stainable (Chapter 3), and thus apparently viable. However, germination of the pollen was rather poor (unpl. obs.), resulting in only a few BC_2 plants. Not all of the BC_2 plants were fully analysed, therefore, it was not possible to determine whether the *A. aurea* chromosomes were transmitted randomly. However, one of the analysed plants surprisingly showed a genome constitution similar to that of its triploid BC_1 parent, containing almost all *A. aurea* chromosomes, whereas another plant contained no detectable *A. aurea* chromosomes (unpl. obs.).

In the parental hybrid *A. aurea* x *A. inodora* high frequencies of homoeologous recombination were observed. As expected, homoeologous recombination occurred in much lower frequencies in the triploid BC1 plants (Chapter 5). In these plants mainly preferential pairing between homologous chromosomes or chromosome segments was observed, which was clearly demonstrated by GISH analysis of the meiotic configurations of the recombinant chromosomes. In these configurations usually the single *A. aurea* chromosome segments were not associated indicating that the entire segment could be either lost or transmitted to the next generation (see Figure 6.3). However, it needs to be mentioned that because the recombinant chromosomes were usually associated at metaphase I, these chromosomes disjoin regularly and thus are incorporated into the gametes, in contrast to the non-recombinant *A. aurea* chromosomes.

Still, the non-recombinant *A. aurea* chromosomes were associated with their homoeologues in low frequencies (Chapter 5). Therefore, it might be possible that this also occurs in the recombinant chromosomes. Analysis of the BC_2 plants can elucidate this more accurately than the MI analysis of the BC_1 plants.

As was pointed out before, it is possible to introgress chromosome segments from one *Alstroemeria* species into another. Furthermore, it should be possible to transfer complete chromosomes from one species into another species resulting in addition lines. The development of addition lines will provide valuable tools for determining the location of important genes. In

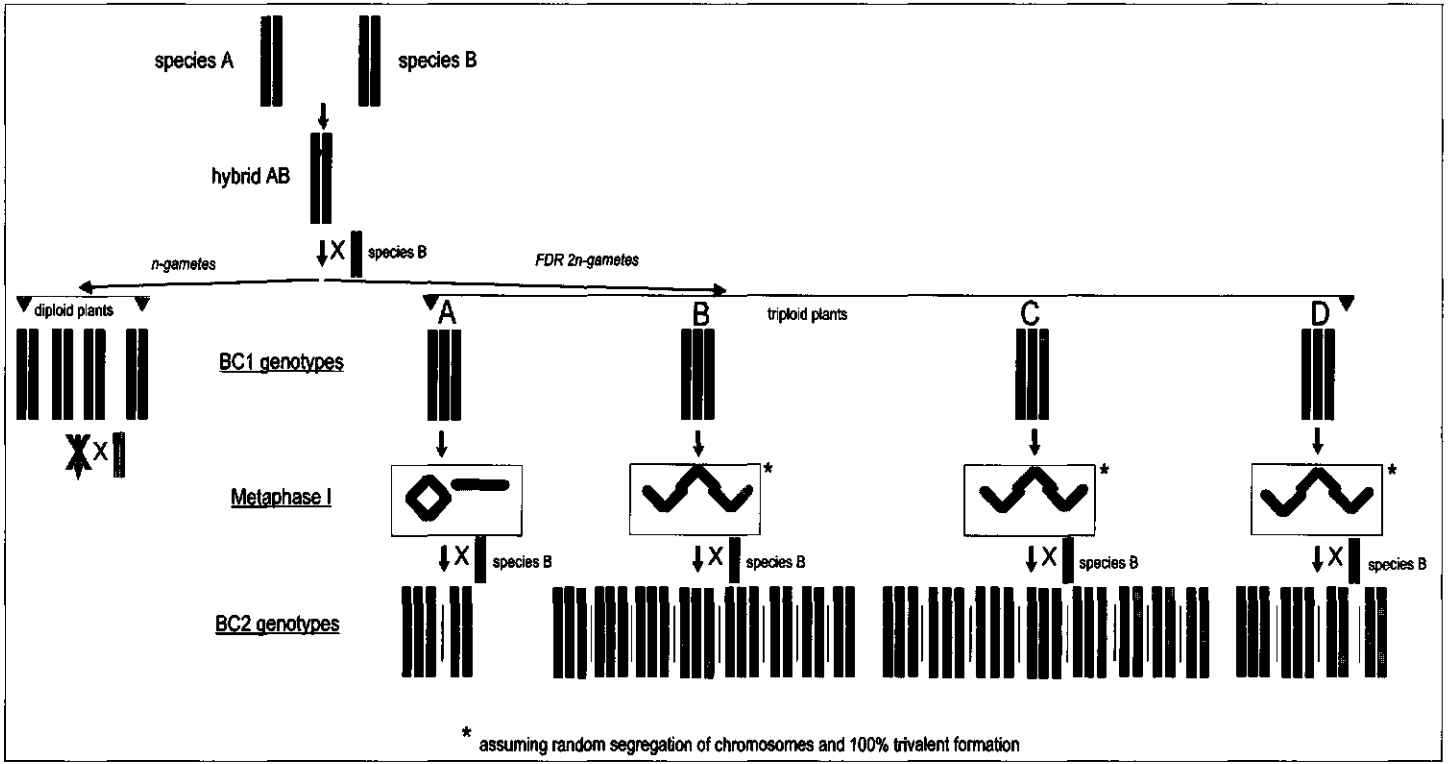

Figure 3. A schematic representation of the consequences for the production of alien addition lines or introgression of small alien segments in *Alstroemeria*, derived from the results discussed in chapters 3, 4 and 5. This is indicated for only one chromosome pair. One interstitial crossover per chromosome during meiosis in the hybrid was assumed. During meiosis in the triploid BC1 plants we observed preferential pairing of homologous chromosome segments. Metaphase I configurations of the expected genotypes (A, B, C and D) in the case of FDR gametes are indicated. We assumed that only chiasmata are formed in homologous chromosome segments, which leads to no further recombination between homoeologous chromosomes. Random segregation of homologous and homoeologous chromosomes was expected to occur during meiosis in the BC_1 plants. The expected BC_2 genotypes are indicated.

Alstroemeria not much is known about the location of characteristics on the chromosomes. Therefore, addition lines will enable to determine which chromosomes carry genes determining particular characteristics. In addition, the observed meiotic recombination between homoeologous chromosomes indicated that it should be possible to produce lines with small introgressed segments from *A. aurea*, which can be used to physically map genes which determine visible traits.

In addition to localising traits on chromosomes, addition lines can also be used to assign molecular markers to specific chromosomes. Currently, an AFLP map is being constructed in *Alstroemeria* (Han *et al.* 1998), but it is not possible to assign the linkage groups to particular chromosomes. The described backcross plants already have the potential to assign a few of the linkage groups to chromosomes. Eventually addition lines might provide means to do this for all linkage groups. Furthermore, the construction of lines that only contain small introgressed *A. aurea* segments might be feasible. If it is possible to construct a series of recombinant chromosomes of one particular chromosome it might provide ways to produce 'introgression maps' of *Alstroemeria* as was recently described for *Festuca-Lolium* hybrids by King *et al.* (1998). The advantage of our plant material is that it is possible to accurately identify all *A. aurea* and *A. inodora* chromosomes using FISH, which can be exploited to select for plant material containing one particular chromosome.

Our molecular cytogenetic analysis has shown the potential of this technique for introgression research with special focus on determination of the fate of particular chromosomes, possibilities for stable introgression through homoeologous recombination and the construction of potentially valuable plant material such as introgression and addition lines. Further backcrossing will enable full exploitation of the potential of this plant material.

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Summary

This thesis describes the results of a molecular cytogenetic investigation of the process of introgression in *Alstroemeria*. The aim of this study was to transfer chromosomes or genes from one *Alstroemeria* species into another. For this, two distantly related species, *A. aurea* and *A. inodora*, were hybridized and the resulting hybrids were further backcrossed with the species *A. inodora*. To monitor the process of introgression accurately it was necessary to identify the individual chromosomes of both parental species.

In addition to classical karyotyping, reliable criteria for unequivocal identification of all individual somatic chromosomes of the species *A. aurea* and *A. inodora* have been developed by applying fluorescence *in situ* hybridization (FISH) with tandemly repeated DNA probes (Chapter 2). Four probes, two species-specific, A001-I (for *A. aurea*) and D32-13 (for *A. inodora*), and two ribosomal rDNA repeats, pTa71 (18S-25S) and pTa794 (5S), revealed specific banding patterns per chromosome, enabling identification of all individual chromosomes. In contrast to most plant species, both *Alstroemeria* species contained a large number of rDNA sites (per haploid genome: 20 in *A. aurea* and 16 in *A. inodora*). Remarkably, the physical location of the rDNA sites varied greatly between the two species; only a few were found at similar positions.

Besides identification of somatic chromosomes, FISH with the species-specific probes was used for the identification of meiotic chromosomes to accurately determine the extent of homoeologous association in the diploid parental hybrid. Multicolour FISH experiments revealed a large number of bivalents per Metaphase I cell (average 6.7 bivalents per MI) of the hybrid *A. aurea* x *A. inodora*, which indicated that homoeologous recombination occurs frequently in the hybrid.

Successful backcrossing of the highly sterile distant hybrid *A. aurea* x *A. inodora* with its parent *A. inodora* resulted in a small number of first generation backcross plants (BC_1). To establish whether and how frequently homoeologous recombination occurred during meiosis in the hybrid, the BC_1 plants were analysed with genomic *in situ* hybridization (GISH). The presence of recombinant chromosomes in all plants provided evidence for homoeologous recombination. All plants were analysed using a combination of FISH with the repetitive probes and GISH that allowed identification of all chromosomes and the construction of detailed karyotypes. In addition, identification of the recombinant chromosomes revealed

which of the chromosomes of *A. aurea* and *A. inodora* were actually homoeologous. The recombinant chromosomes showed a large number of recombination points, indicating that multiple crossovers had occurred. The distribution of the recombination points indicated that chiasmata are formed along the entire length of the chromosomes. Furthermore, the analysis of recombinant chromosomes revealed structural differences (one translocation and one inversion) between two sets of homoeologous chromosomes. These differences were confirmed by analysis of metaphase I configurations of these chromosomes in the hybrid and the BC_1 plants (Chapters 4 and 5).

The detailed analysis of the genome constitution of the plants revealed two different types of plants, near diploids ($2n=2x+1=17$) and triploids ($2n=3x=24$), indicating that two different types of gametes were functional in the hybrid, viz., *n*- and 2*n*-gametes. The GISH and FISH analysis provided evidence that 2*n*-gametes were functional both from male and female parents and had originated in all cases through a mechanism genetically leading to first division restitution (FDR) (Chapter 4).

In order to investigate the fate of the introgressed *A. aurea* segments in *A. inodora*, meiosis was analysed using GISH and FISH in the triploid BC_1 plants. These plants contained two more or less complete genomes of *A. inodora* and one of *A. aurea* as a result of the FDR mechanism (Chapter 4). Metaphase I configurations were studied per set of three homo(eo)logous chromosomes. This analysis revealed, as expected, preferential pairing of the *A. inodora* chromosomes, whereas the *A. aurea* chromosomes usually were unpaired. Only low frequencies of homoeologous chromosome pairing were observed, which is in contrast to what was found in the parental hybrid. Interestingly, the recombinant chromosomes generally formed trivalents with their two homo(eo)logues. These chromosomes were only associated at homologous (recombinant) chromosome segments. At later stages of meiosis, normal distribution of the *A. inodora* chromosomes and the recombinant chromosomes into the gametes was observed, whereas the non-recombinant *A. aurea* chromosomes were randomly distributed among the gametes, pointing towards the perspective for obtaining addition lines or introgression of alien chromosome segments in *Alstroemeria*.

For breeding, the most important observation, which was made in this molecular cytogenetic study of *Alstroemeria*, was the high frequency of pairing and recombination between homoeologous chromosomes in the interspecific hybrid and the almost absence of this phenomenon in the further backcross generations. In these backcrosses, preferential pairing

between homologous chromosomes was observed. For efficient introgression breeding more emphasis should be given to the production of sufficient BC_1 plants and the detection of desired recombinants in this generation.

Samenvatting

In de plantenveredeling wordt teruggegrepen naar wilde verwanten van gecultiveerde soorten als deze wilde verwanten interessante eigenschappen bezitten die niet in de gecultiveerde soort aanwezig zijn. Om deze eigenschappen over te brengen naar de gecultiveerde soorten wordt gebruik gemaakt van een terugkruisingsprogramma. Hiervoor wordt de wilde verwant met de gecultiveerde soort gekruist om de eigenschappen van beide soorten te combineren. De uit deze kruising voortgekomen hybriden bevatten zowel gewenste alsmede vele ongewenste eigenschappen van de wilde verwant. Om de ongewenste eigenschappen te verwijderen worden vervolgens de hybriden teruggekruist met de gecultiveerde soort om nieuwe combinaties te maken van eigenschappen van de wilde soort en de gecultiveerde soort. In het nakomelingschap van opeenvolgende terugkruisingen worden steeds minder eigenschappen van de wilde verwant gevonden. Door te selecteren voor de gewenste eigenschap en verder terug te kruisen met de gecultiveerde soort is het mogelijk om planten te verkrijgen die alle eigenschappen van de gecultiveerde soort en de gewenste eigenschap uit de wilde verwant bevatten.

Eigenschappen zijn opgeslagen in het erfelijk materiaal (DNA) van een plant. Dit DNA zit in de kern verpakt in een aantal chromosomen. Naarmate plantensoorten minder verwant zijn, zullen er meer verschillen in vorm, grootte en opbouw van de chromosomen tussen de soorten zijn. Van de verschillen kunnen we gebruik maken om de chromosomen van de beide oudersoorten van een hybride in een terugkruisingsprogramma te herkennen. Met behulp van de techniek genomische *in situ* hybridisatie (GISH) is het mogelijk om het totaal aan chromosomen (de genomen) van beide ouders in een hybride verschillend te kleuren en onder een fluorescentiemicroscoop zichtbaar te maken. Met deze techniek is het dus mogelijk om de chromosomen behorende bij een bepaalde soort te identificeren. Hierdoor kunnen we in terugkruisingsnakomelingen van een hybride bepalen hoeveel chromosomen van de wilde verwant nog aanwezig zijn. Aangezien eigenschappen op bepaalde chromosomen liggen is het nodig om te bepalen welke chromosomen van de wilde verwant nog aanwezig zijn. Soms kunnen de individuele chromosomen van een soort worden geïdentificeerd op basis van hun morfologie, maar meestal is dat niet mogelijk. Het is echter wel mogelijk de chromosomen zo te bewerken, bijvoorbeeld met behulp van fluorescentie *in situ* hybridisatie (FISH) met specifieke

DNA sequenties, dat er een uniek banderingspatroon op de chromosomen zichtbaar wordt. Hiermee kunnen dan alle individuele chromosomen wel worden geïdentificeerd.

In dit proefschrift is de *in situ* hybridisatie techniek gebruikt voor het analyseren van terugkruisingsnakomelingen van een interspecifieke *Alstroemeria* hybride. Het doel van deze studie was de mogelijkheden te onderzoeken om chromosomen of genen van één *Alstroemeria* soort naar een andere soort over te brengen. Hiervoor is gebruik gemaakt van twee ver verwante soorten; *Alstroemeria aurea en A. inodora*, respectievelijk een Chileense en een Braziliaanse soort. Deze soorten zijn met veel moeite gekruist en de uit deze kruising voortgekomen interspecieke hybride is vervolgens verder teruggekruist met één van de ouders. Beide *Alstroemeria* soorten bevatten 8 verschillende chromosomen. Het bleek mogelijk om de chromosomen van de beide oudersoorten te kunnen onderscheiden met behulp van genomische *in situ* hybridisatie.

Door middel van fluorescentie *in situ* hybridisatie is getest of alle individuele chromosomen van *A. aurea* en *A. inodora* afzonderlijk te identificeren zijn. Hiervoor zijn enkele repetitieve DNA sequenties op de chromosomen van beide soorten gehybridiseerd. Vervolgens zijn deze sequenties (probes) gedetecteerd met behulp van fluorochromen met verschillende kleuren en bestudeerd onder een fluorescentiemicroscoop. De gebruikte repetitieve DNA probes waren twee soortspecifieke (A001-I voor *A. aurea* en D32-13 voor *A. inodora*) en twee ribosomale sequenties (pTa71 (18S-25S rDNA) en pTa794 (5S rDNA)). Hybridisatie van deze sequenties op mitotische metafase chromosomen bleek voor ieder chromosoom een uniek bandenpatroon op te leveren. Op grond van hun hybridisatiepatroon waren de 8 verschillende chromosomen van de beide *Alstroemeria* soorten individueel identificeerbaar (Hoofdstuk 2). Naast de identificatie van chromosomen zijn met deze studie ook meer inzichten in de opbouw van de chromosomen van de beide *Alstroemeria* soorten verkregen.

In tegenstelling tot de meeste plantensoorten, waar op slechts enkele plaatsen in het genoom ribosomale sequenties voorkomen, bleken deze sequenties in de *A. aurea* en *A. inodora* op respectievelijk 20 en 16 plaatsen in het genoom aanwezig te zijn. Opvallend waren tevens de verschillen tussen de beide soorten met betrekking tot de locatie van de ribosomale sequenties op de chromosomen (Hoofdstuk 2).

Naast het identificeren van chromosomen tijdens de mitotische celdeling, is het ook mogelijk om FISH met de soortspecifieke DNA sequenties te gebruiken voor onderzoek naar paringsgedrag van chromosomen tijdens de meiose. Naarmate de beide oudersoorten van een

hybride minder verwant zijn zullen de chromosomen van de beide soorten (de homoeologen) tijdens de meiose onderling minder goed paren en recombineren dan in de soorten zelf. Recombinatie tussen de chromosomen van beide oudersoorten is gewenst, omdat op deze manier uitwisseling van chromosoomsegmenten tussen de beide soorten optreedt. In de interspecifieke hybride *A. aurea* x *A. inodora* zijn op deze manier veel bivalenten (gepaarde chromosomen) tussen de homoeologe chromosomen van de beide oudersoorten aangetoond. Er werden gemiddeld 6.7 bivalenten per metafase I kern aangetroffen. Dit hoge aantal bivalenten duidde op veelvuldige homoeologe recombinatie tijdens de meiose van de *A. aurea* x *A. inodora* hybride. Homoeologe recombinatie wordt gezien als een sleutelfactor voor het stabiel overbrengen van chromosoomsegmenten van de ene naar de andere soort.

Ondanks de lage fertiliteit van de *A. aurea* x *A. inodora* hybride was het mogelijk deze terug te kruisen met de ouder *A. inodora* (Hoofdstuk 3). Dit heeft in een klein aantal terugkruisingsplanten (BC_1) geresulteerd. Voor het aantonen van recombinatie (overkruisingen) tussen de chromosomen van beide soorten werd gebruik gemaakt van genomische *in situ* hybridisatie (GISH). Voor GISH is het totale DNA van één van de beide oudersoorten op de chromosomen van de hybride gehybridiseerd, waarna de genomen van de beide soorten onderscheiden konden worden. In alle BC1 planten zijn recombinante chromosomen gevonden met chromosoomsegmenten van zowel *A. aurea* als *A. inodora* op hetzelfde chromosoom. De vele recombinante chromosomen in alle BC_1 planten bevestigden dat homoeologe recombinatie veelvuldig optreed. Het aantal recombinatie punten (1 tot 4) per recombinant chromosoom geeft aan dat meerdere overkruisingen per chromosoom veelvuldig voorkomen. Recombinatie blijkt over het hele chromosoom voor te kunnen komen. De genoomsamenstelling van de verschillende BC_1 planten is geanalyseerd m.b.v. GISH en FISH met repetitieve DNA sequenties. In deze planten zijn alle individuele chromosomen geïdentificeerd. Door middel van identificatie van de recombinante chromosomen kon bepaald worden welke chromosomen van *A. aurea* en *A. inodora* homoeoloog zijn. Door middel van analyse van de recombinante chromosomen zijn structurele verschillen tussen bepaalde chromosomen van *A. aurea* en *A. inodora* aangetoond. Deze verschillen werden bevestigd door een analyse te maken van het paringsgedrag van deze chromosomen (Hoofdstukken 4 en 5).

Er zijn zowel diploïde als triploïde BC_1 planten verkregen. Dit is het gevolg van het optreden van twee verschillende typen gameten in de hybride met gereduceerde (8) of ongereduceerde (16) chromosoom aantallen. Gedetailleerde analyse van de genoomsamen-

stelling van de BC_1 planten met GISH en FISH heeft aangetoond dat de ongereduceerde gameten zijn ontstaan door middel van een mechanisme dat genetisch gelijk is aan first division restitution (FDR) (Hoofdstuk 4).

De meiose in de triploïde BC_1 planten is beschreven in hoofdstuk 5. Deze triploïde planten bevatten als gevolg van het FDR mechanisme (Hoofdstuk 4) twee genomen van *A. inodora* en één van *A. aurea*. Door preferentiële paring vormen de twee homologe *A. inodora* chromosomen bivalenten en het homoeologe *A. aurea* chromosoom is zoals verwacht aanwezig als een univalent. Soms werden trivalenten gevonden, wat aangeeft dat in lage frequenties homoeologe paring optreedt in de triploïde BC_1 planten. De in deze BC_1 planten aanwezige recombinante chromosomen vormen bijna altijd trivalenten met hun homo(eo)logen. In deze trivalenten zijn de homologe segmenten bijna altijd gepaard, in tegenstelling tot de homoeologe segmenten. De recombinante chromosomen en de *A. inodora* chromosomen segregeren tijdens de latere stadia van de meiose normaal. De niet-recombinante *A. aurea* chromosomen daarentegen delen zich al tijdens de eerste meiotische deling in twee chromatiden die vervolgens willekeurig over de gameten worden verdeeld. De normale segregatie van de recombinante chromosomen en de willekeurige verdeling van de *A. aurea* chromosomen over de gameten geven aan dat het mogelijk moet zijn om in de volgende terugkruisingsgeneraties additielijnen of introgressies te verkrijgen.

Een voor de veredeling belangrijke observatie is dat er veel recombinatie tussen de *A. aurea* en *A. inodora* chromosomen tijdens de meiose van de interspecifieke hybride werd aangetroffen en nauwelijks in de terugkruisingsnakomelingen. In deze triploïde terugkruisingen treedt vooral preferentiële paring tussen homologe chromosomen op. Voor efficiënte introgressie van eigenschappen is het daarom belangrijk om veel BC_1 planten te produceren en genotypen met veel recombinatie te selecteren als basis voor de verder terugkruisings procedure.

Nawoord

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Silvan

Curriculum vitae

Silvan Adelmar Kamstra werd op 26 december 1968 geboren te Groningen. In 1986 behaalde hij het HAVO- en in 1988 het VWO-diploma aan het Augustinuscollege te Groningen. In 1988 begon hij aan de studie Plantenveredeling aan de Landbouwuniversiteit Wageningen (LUW). De doctoraalstudie omvatte een drietal afstudeervakken. Tijdens het eerste afstudeervak bij de vakgroep Plantenveredeling deed hij onderzoek naar soortskruisingsbarrières in *Alstroemeria*. Het tweede afstudeervak betrof een analyse van de genomen van terugkruisingsmateriaal van aardappel-tomaat fusies bij de vakgroep Erfelijkheidsleer. Hiervoor werd gebruik gemaakt van de 'chromosome painting' techniek. Het derde afstudeervak bij de vakgroep Erfelijkheidsleer werd uitgevoerd bij het John Innes Institute te Norwich (UK) had tot doel specifieke DNA fracties te isoleren voor optimalisatie van de 'chromosome painting' techniek. In 1994 studeerde hij af, waarna hij op 1 januari 1995 begon als Assistent in Opleiding bij het laboratorium voor Plantenveredeling van de LUW. De resultaten van het promotieonderzoek staan beschreven in dit proefschrift.