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Identification of alien chromosomes in a series of *Allium fistulosum – A. cepa* monosomic addition lines by means of genomic in situ hybridization

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Genomic in situ hybridization (GISH) was used to identify alien chromosomes in a series of eight different *Allium fistulosum – A. cepa* monosomic addition lines. Biotin-labeled total genomic DNA from shallot (*A. cepa* L. Aggregatum group) was used as a probe together with an excess amount of unlabeled blocking DNA from the recipient plant, Japanese bunching onion (*A. fistulosum* L.). Probe hybridization sites were detected by FITC-conjugated avidin and anti-avidin antibody using an epifluorescence microscope. In each mitotic metaphase cell of all the eight types of monosomic addition lines, the alien chromosomes were successfully discriminated from other 16 *A. fistulosum* chromosomes. Furthermore, no clear exchanges of chromosome segments between *A. cepa* and *A. fistulosum* were observed. This finding indicates that in each addition line an entire (unrecombined) *A. cepa* chromosome is present in an integral diploid background of *A. fistulosum*.

INTRODUCTION

Alien monosomic addition lines are very valuable to study genome organization (Singh, 1993). For *Allium*, a complete set of eight alien monosomic addition lines with an extra chromosome from shallot (*Allium cepa* L. Aggregatum group) in a diploid background of Japanese bunching onion (*A. fistulosum* L.) has already been constructed in a previous study (Shigyo et al., 1996). The application of the C-banding technique as proposed by Kalkman (1984) to identify shallot alien chromosomes proved to be difficult as few intercalary chromosome bands were present in shallot. Therefore, an alien chromosome was mainly identified by its length and the position of the centromere. Eventually, statistical analyses with the help of isozyme and rDNA analyses were needed to complete the set. As this identification method is very time consuming it was investigated if genomic in situ hybridization (GISH) could be used for a quick and reliable identification of shallot alien chromosomes in a diploid Japanese bunching onion background.

In situ hybridization has recently become a powerful tool for plant molecular cytogenetics (Jiang and Gill, 1994). In *Allium*, GISH was used successfully by Hizume (1994) and Khrustaleva and Kik (1998) to discriminate between the chromosome complements of *A. cepa* and *A. fistulosum* present in the interspecific hybrid between both species. Furthermore, the technique was used amongst others by Keller et al. (1996), and Friesen et al. (1997) to confirm the hybrid origin of presumed species hybrids in *Allium*.

In the present study, the GISH technique was applied to identify the complete set of eight different alien chromosomes in a series of *A. fistulosum – A. cepa* monosomic addition lines.

MATERIALS AND METHODS

Plant material. The following plants were used: Japanese bunching onion (*A. fistulosum* L., 'Kujyo', 2n = 2X = 16, genomes FF), shallot (*A. cepa* L. Aggregatum group, an accession from Thailand, 2n = 2X = 16, AA), and a series of *A. fistulosum – A. cepa* monosomic addition lines (2n = 2X + 1 = 17, FF + 1A – FF + 8A).

Chromosome preparation. Root tips of the monosomic addition lines were collected and pretreated with 0.05% (w/v) colchicine for 3 h at 20°C. After fixation in the mixture of acetic acid and ethylalcohol (1 : 3; v/v), the root tips were treated with 2% (w/v) cellulase-onozuka RS (Yakult Pharmaceutical Co., Ltd., Tokyo) and 0.5% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo) in 0.1 M citrate buffer, pH 4.5, containing 5 mM EDTA at 37°C for

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five to 10 min, washed in distilled water, and squashed in 45% (v/v) acetic acid. After removal of the coverslips by the dry-ice method the preparations were treated with 45% (v/v) acetic acid to dehydrate the chromosomes, and subsequently the preparations were air-dried overnight.

Preparation of total genomic DNA probes. Total genomic DNA was extracted from young leaves of shallot and *A. fistulosum* using a DNA extraction kit, according to the supplier's protocol (Sanko Junyaku Co., Ltd., Tokyo). The extracted DNA of shallot was labeled with biotin-16-dUTP via nick translation according to the instructions of the supplier (Boehringer Mannheim). The labeled DNA was separated from unincorporated nucleotides using Quick Spin Columns (G-50 Sephadex columns for biotinylated DNA purification, Boehringer Mannheim). The extracted DNA of *A. fistulosum* was used as a blocking DNA.

Genomic in situ hybridization. The chromosome preparations were denatured in 70% (v/v) formamide – 2 × SSC at 70°C for 3 min, dehydrated through a prefreezed ethanol series of 70, 90, 100% for 5 min each, and air-dried. The methods of in situ hybridization and posthybridization wash were according to Mukai and Gill (1991) with minor modifications. The hybridization mixture for 100 µl (total volume) consisted of 50% (v/v) formamide, 2 × SSC, 10% (w/v) dextran sulfate, 1 µg of biotinylated shallot total genomic DNA, and 10 µg of unlabeled *A. fistulosum* total genomic DNA. The mixture (10 µl) was applied to each slide and covered with a plastic coverslip.

Biotin-labeled DNA was detected with FITC-conjugated avidin. Each slide was treated with 50 ml of a blocking buffer (4 × SSC containing 10 mM $MgSO_4$, 0.1% [v/v] Tween 20, 3% [w/v] bovine serum albumin) for 15 min at 37°C, and incubated with 50 ml of 20 mg/ml fluorescein avidin DN (Vector Lab., Burlingame) in the blocking buffer for 45 min at 37°C. After washing in a washing buffer (4 × SSC containing 10 mM $MgSO_4$, 0.1% [v/v] Tween 20) five times, 3 min each, at room temperature, the signals from biotin were amplified. Each slide was incubated with 50 ml of 20 mg/ml FITC-conjugated anti-avidin D (Vector Lab., Burlingame) in the blocking buffer for 15 min at 37°C and then washed as described above. Chromosomes were counterstained with 1 µg/ml, propidium iodide. Slides were mounted with antifade (Vectashield, Vector Lab., Burlingame) and inspected using a Zeiss epifluorescence microscope with filter set 09. Images were captured with the microscope using a CCD camera (Hamamatu Photonics Co., Ltd., Hamamatu). At least five good metaphases were examined for each hybridization. The digital images were optimized for contrast and brightness using the program Adobe Photoshop.

RESULTS AND DISCUSSION

Metaphase chromosomes of each monosomic addition line are presented in Figure 1. Probe hybridization sites were detected by FITC-conjugated avidin and anti-avidin antibody that fluorescences green under blue light excitation. *A. fistulosum* chromosomes showed very little probe hybridization. Chromosomes were counterstained with propidium iodide and fluorescent orange-red with the same excitation wavelength. As the strongly labeled shallot chromosomes were also counterstained, the green FITC label and orange-red counterstain interacted to give a yellow to yellow-green fluorescence. In a monosomic addition line with the chromosome 1A of shallot (FF + 1A), one yellow-green fluorescent alien chromosome and 16 orange-red fluorescent chromosomes were observed each in five metaphase cells examined. The alien chromosome was metacentric and much larger than any other *A. fistulosum* chromosome. This result is in good agreement with the conventional karyotype analysis conducted for the identification of alien chromosome 1A in FF + 1A (Shigyo et al., 1996). In the same way, one yellow-green alien chromosome and 16 orange-red chromosomes were observed in the remaining seven types of the monosomic addition line (FF + 2A – FF + 8A). Sizes and morphologies of the alien chromosomes (2A – 8A) identified by GISH corresponded well to those recognized by the conventional karyotype analysis. Consequently, the alien chromosomes were successfully discriminated from the chromosome complement of *A. fistulosum* and identified as respective shallot chromosomes (1A – 8A), by means of GISH.

No clear exchanges of chromosome segments between *A. fistulosum* and shallot were observed. This result shows that in each addition line an entire (unrecombined) *A. cepa* (shallot) chromosome is present in an integral diploid background of *A. fistulosum*. Since the frequency of homoeologous pairing and recombination is quite high in the meiosis of F_1 hybrids between these two species (Emsweller and Jones, 1935; Maeda, 1937; Levan, 1941; Cochran, 1950; Tashiro, 1984; Peffley, 1986), this implies a considerable degree of selection for entire chromosomes in the process of constructing the monosomic addition lines. Our monosomic addition lines were selected from the backcross progenies of interspecific triploids (2 × *A. fistulosum* + 1 × shallot). At metaphase-I (MI) of meiosis, almost all pollen mother cells (PMCs) of the triploids formed eight bivalents and eight univalents (Tashiro et al., unpublished data). This result suggests preferential pairing is regularly occurring among *A. fistulosum* homologues, leaving shallot univalents. Furthermore, all PMCs of the monosomic addition lines used in this study formed eight bivalents and one univalent, and no clear trace for chromosomal exchanges between shallot and *A. fistulosum* were observed on meiotic chromosomes of the monosomic addition lines (Fig. 2; also refer to Fig. 12 in Shigyo, 1997).

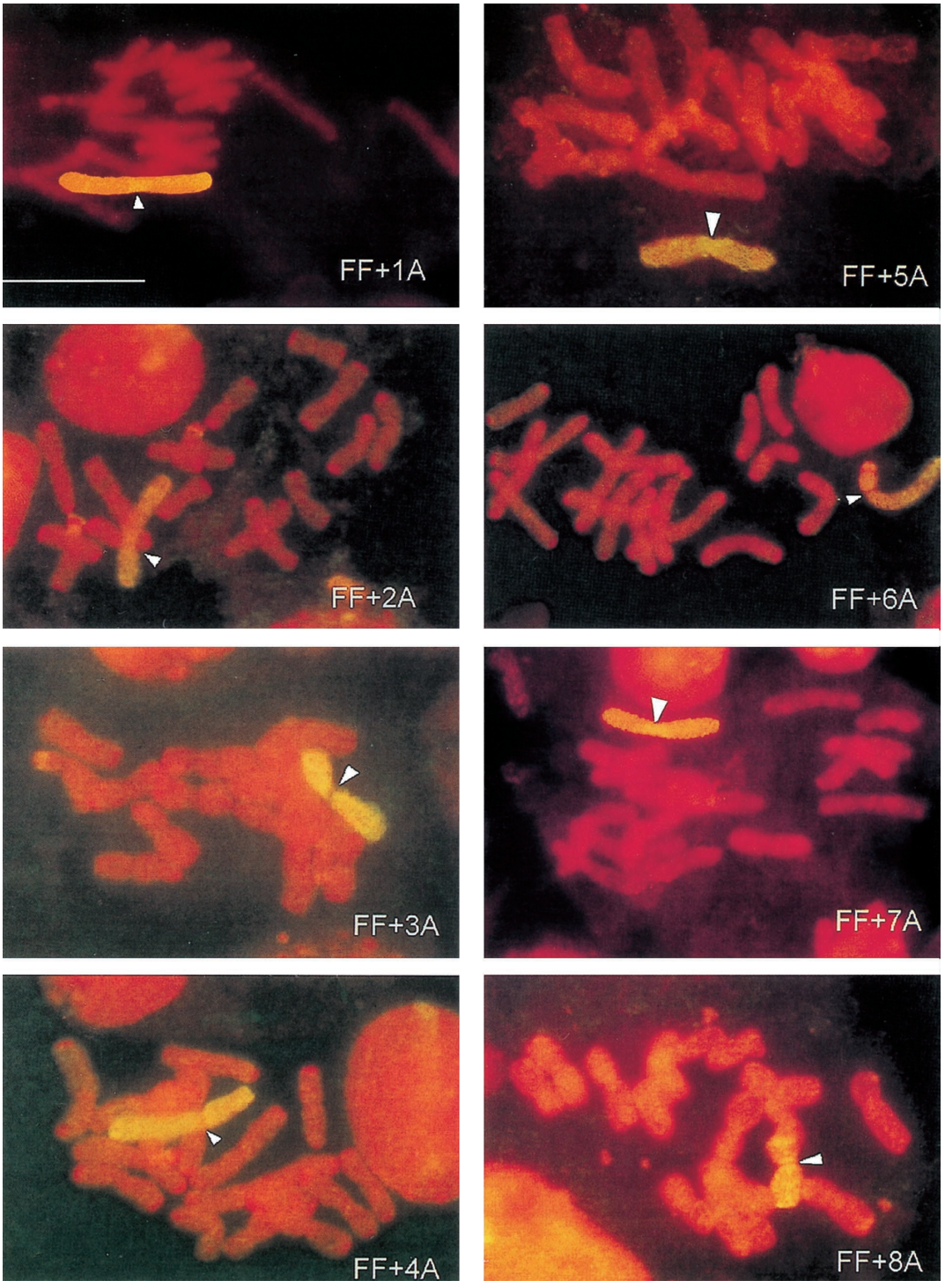

Fig. 1. Somatic metaphase cells of a series of *A. fistulosum* – *A. cepa* monosomic addition lines (FF + 1A – FF + 8A) after genomic in situ hybridization. Each cell possesses one yellow-green alien chromosome of shallot (*A. cepa* Aggregatum group) and 16 orange-red stained chromosomes of *A. fistulosum*. A large-sized metacentric alien chromosome, namely 1A, is observed in FF + 1A; a large-sized sub-metacentric alien chromosome, i.e., 2A, in FF + 2A; a middle-sized sub-metacentric alien chromosome, i.e., 3A, in FF + 3A; a middle-sized sub-metacentric alien chromosome, i.e., 4A, in FF + 4A; a middle-sized metacentric alien chromosome, i.e., 5A, in FF + 5A; a middle-sized sub-telocentric alien chromosome, i.e., 6A, in FF + 6A; a small-sized metacentric alien chromosome, i.e., 7A, in FF + 7A; and a small-sized sub-metacentric alien chromosome, i.e., 8A, in FF + 8A. Each arrowhead indicates the centromere of the alien chromosome.

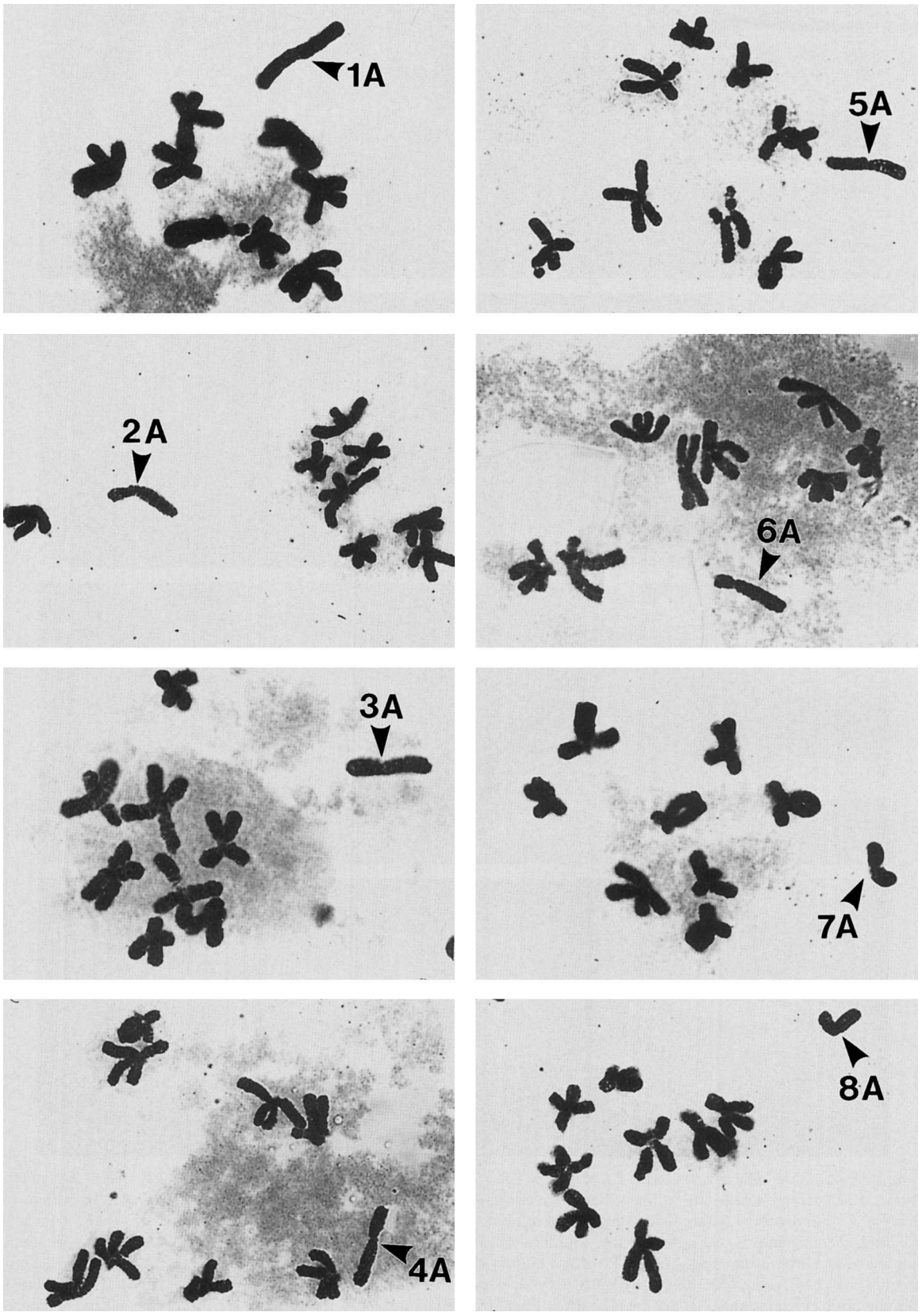

Fig. 2. Chromosomes at meiotic metaphase-I in PMCs of a series of alien monosomic addition lines. Each arrowhead points to extra chromosomes 1A to 8A.

Since the bivalents formed localized chiasmata those are a characteristic of the meiotic chromosomes of *A. fistulosum*, it has to be sure that the solitary univalent is derived from shallot chromosome. The limitation of GISH, in terms of the size of the segment that can be identified, has not yet been explored as Schwarzacher et al. (1992) had described in their paper. It seems that GISH technique has a limitation to detect very small alien segment. However, the interspecific chromosomal exchange seems not to take place to a large extent under the restricted conditions mentioned above. Therefore, all shallot alien chromosomes must have been derived from a non-recombinant chromatid in meiosis.

In conclusion, GISH proved to be a valuable tool to identify alien *A. cepa* chromosomes in a diploid *A. fistulosum* background. The monosomic addition line set has great potential to link the intraspecific RFLP onion recombination map (King et al., 1998) and the interspecific AFLP *A. cepa* × *A. roylei* recombination map (van Heusden et al., unpublished data) to physical chromosomes. The combination of both recombination maps and the monosomic addition line series will result in an integrated *Allium* map which will be very beneficial for applied as well as fundamental research in *Allium*.

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