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Fluorescent Staining Analysis of Chromosomes in Pear (*Pyrus* spp.)

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Fluorescent banding patterns of pear chromosomes were determined from samples taken from root tips of open-pollinated seedlings of six cultivars from three species [*Pyrus pyrifolia* (Burm. F) Nakai (Japanese pear), *P. communis* L. (European pear), and *P. bretschneideri* Rehder (Chinese pear)]. Root tips were pretreated in 2 mM 8-hydroxyquinoline at 10°C for 4 h; the chromosome samples were prepared by the enzymatic maceration and air-drying method. All cultivars used in this study had 2n = 34 chromosomes. Chromomycin A_3 (CMA) positive (+) bands were observed in telomeric positions of four chromosomes. In some samples, these CMA + bands were observed at satellite positions. 4'-6-diamidino-2-phenylindole (DAPI) negative bands (–) were seen to correspond with CMA + bands. Thirty chromosomes had no CMA +/DAPI – bands. No propidium iodide (PI) bands were observed in any chromosomes. The number and positions of CMA + bands were stable, and there was no difference among the three species.

Key Words: chromomycin A_3, CMA, DAPI, karyotype, PI, propidium iodide.

Introduction

Chromosome analysis is important for genetic and biotechnological studies including breeding and genome analysis. In the pear (*Pyrus* spp.), one of the most important fruit trees cultivated in temperate regions, these studies are essential for genetic improvement. However, to our knowledge, these kinds of studies have never been conducted, although chromosome counting has been reported (Ito and Fukushima, 1934, 1937).

Chromosome analysis of some fruit trees has progressed by application of the enzymatic maceration method and/or fluorescent staining (De Melo et al., 2001; Guerra, 1993; Kitajima et al., 2001; Schuster, 1996; Yamamoto et al., 1999; Zhuang et al., 1990). Clearly defined chromosome samples have been obtained by the enzymatic maceration method (Kurata and Omura, 1978). Some morphologically similar chromosomes have been identified by banding methods using the base-specific binding fluorochrome, guanine-cytosine (GC)-specific chromomycin A_3 (CMA), and adenine-thymine (AT)-specific 4'-6-diamidino-2-phenylindole (DAPI) (Schweizer, 1976). Moreover, all chromosomes could be identified by means of genomic *in situ* hybridization or by using a haploid plant as the source of material in the case of citrus (Kitajima et al., 2007; Yamamoto and Tominaga, 2004).

In the pear, similar studies are necessary for future development of genome analysis (Terakami et al., 2007; Yamamoto et al., 2007) and ploidy manipulation (Kadota and Niimi, 2004). Thus, there is an urgent need to perform chromosome analysis for karyological and molecular cytogenetic investigation of the pear. In this study, three commercially important species were used: Japanese pear [*Pyrus pyrifolia* (Burm. F.)], European pear (*P. communis* L.), and Chinese pear (*P. bretschneideri* Rehder). Chromosome samples from these species were prepared by enzymatic maceration and air drying, and stained with fluorochrome to determine banding patterns.

Materials and Methods

Japanese pears 'Osa Gold' and 'Niitaka', European pears 'La France' and 'Max Red Bartlett', and Chinese pears 'Enli' and 'Yali' were used (Table 1). The materials used in this study were obtained from the National Institute of Fruit Tree Science, Tsukuba, Ibaraki, Japan.

Roots of young seedlings from open-pollinated fruits were the source of the material used in this study. Seeds were germinated in Petri dishes at 15°C in the dark.

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Root tips of approximately 1 cm in length were excised, immersed in 2 mM 8-hydroxyquinoline at 10°C for 4 h in the dark, fixed in methanol-acetic acid (3 : 1), and stored at −20°C.

Enzymatic maceration and air drying were performed as described by Fukui (1996) with minor modifications. The root tips were washed in distilled water to remove the fixative and macerated in an enzyme mixture containing 4% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult, Japan), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Japan), and 1 mM EDTA, pH 4.2, at 37°C for 50–70 min.

Chromosomes were stained with 2% Giemsa solution (Merck Co., Germany) in 1/30 M phosphate buffer (pH 6.8) for 15 min, rinsed with distilled water, air dried, and then mounted with xylene. After confirmation of each chromosome's position on the slide glass, the chromosomes were destained with 70% methanol. Chromosomes were also stained with 0.5 $g \cdot L^{-1}$ CMA and 1.0 $\mu g \cdot L^{-1}$ DAPI in accordance with Hizume (1991), and observed under a fluorescence microscope with a BV and UV filter cassette, respectively. Moreover, some chromosomes derived from seedlings of 'Osa Gold' were stained with 12.5 $\mu g \cdot L^{-1}$ propidium iodide (PI), and observed with a G filter cassette. From the preparation derived from seedlings of 'Osa Gold' stained with Giemsa, eight cells were selected for use in determining chromosome length.

Results

The enzymatic maceration and air-drying method yielded a chromosome preparation derived from seedlings of 'Osa Gold' which was suitable for chromosome analysis (Fig. 1). The chromosome number was 2n = 34 and the total length of its chromosomes was 76.7 μm. The relative length of each chromosome ranged from 2.0 to 4.3% of the total (Fig. 2).

When CMA staining was performed, four out of 34 chromosomes exhibited CMA positive (+) bands, and thirty chromosomes had no CMA + or negative (−) bands in 21 out of 22 seedlings of 'Osa Gold'. The four CMA + bands were located in telomeric regions of the four chromosomes. In some preparations, these telomeric CMA + bands were observed at between one and three satellite positions in each chromosome (Fig. 1B, 1E, and Table 1). These four CMA + bands corresponded to DAPI − bands (Fig. 1B, 1C). Two CMA +/DAPI − bands were located on relatively long chromosomes (2.8 and 2.4 μm), and the other two were on short chromosomes (2.1 and 1.9 μm) (Fig. 2). Neither positive nor negative bands were observed in PI-stained chromosomes (Fig. 1D).

All samples had 2n = 34 chromosomes, and almost all chromosome samples derived from seedlings showed four telomeric CMA + bands among six cultivars belonging to three *Pyrus* species (Fig. 3 and Table 1). These four CMA + bands were observed at satellite positions in some preparations derived from seedlings as in 'Osa Gold'. No species or varietal difference was found in the number and location of CMA + bands.

Discussion

This study showed the effectiveness of the enzymatic maceration method for preparation of chromosomes suitable for karyological and molecular cytogenetic analyses. Chromosomes in the samples were relatively extended and well-spread. This result agrees with those of previous reports that the enzymatic maceration method is reliable for observing chromosomes of fruit trees (Kitajima et al., 2001; Schuster, 1996; Yamamoto et al., 1999; Zhuang et al., 1990).

Fluorochrome staining analysis revealed that the pear has four chromosomes with telomeric CMA +/DAPI − bands. Neither PI+ nor − bands were observed. The characteristics of the stains used are as follows: CMA and DAPI are GC- and AT-specific, respectively, and PI is not base specific. The CMA +/DAPI − regions are considered to be GC-rich regions of chromosomes. CMA +/DAPI − bands were located at satellites in some cells. When chromosomes are too condensed, satellites are sometimes not observed even though they are present (Guerra et al., 1997). It is considered that our results concerning CMA +/DAPI − bands located at telomeric regions or satellites were dependent not on species or cultivars but on the conditions of chromosome condensation. Almost all satellites observed were CMA +/DAPI − bands. Two chromosomes with CMA +/DAPI − bands were relatively long and two were short. These chromosomes with CMA +/DAPI − bands could be easily distinguished from other chromosomes without

Table 1. Reproduciblity of four CMA positive (+) bands which appeared in chromosomes derived from open-pollinated seedlings of six pear cultivars.

Species	Cultivar	No. of seedlings examined	No. of seedlings which had four CMA (+) bands
Pyrus pyrifolia (Japanese pear)	'Osa Gold'	22	21
	'Niitaka'	12	10
P. communis (European pear)	'La France'	8	7
	'Max Red Bartlett'	9	7
P. bretschneideri (Chinese pear)	'Enli'	11	11
	'Yali'	9	8

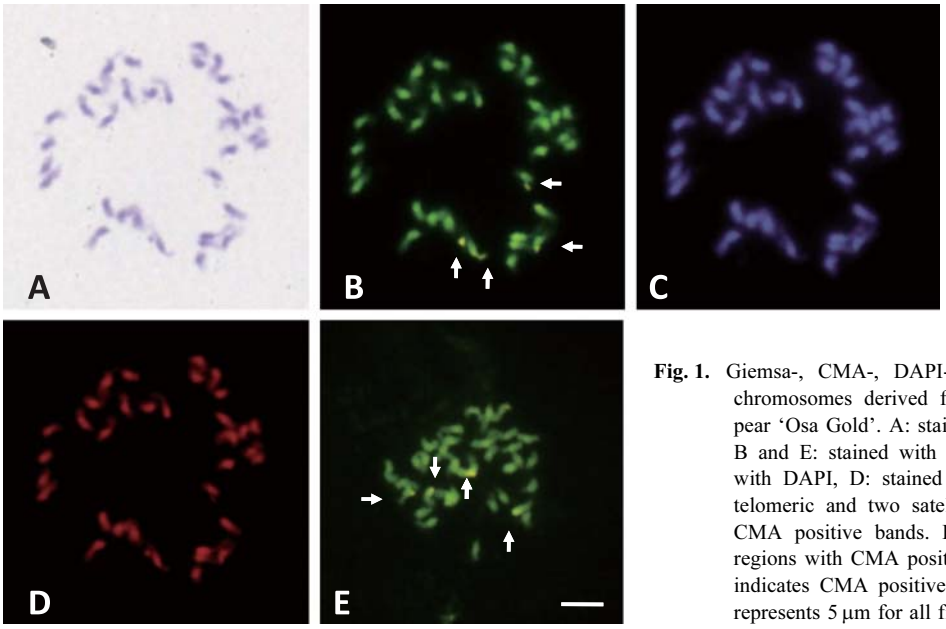

Fig. 1. Giemsa-, CMA-, DAPI-, and PI-stained chromosomes derived from seedlings of pear 'Osa Gold'. A: stained with Giemsa, B and E: stained with CMA, C: stained with DAPI, D: stained with PI. B: two telomeric and two satellite regions with CMA positive bands. E: four telomeric regions with CMA positive bands. Arrow indicates CMA positive bands. Bar in E represents 5 μm for all figures.

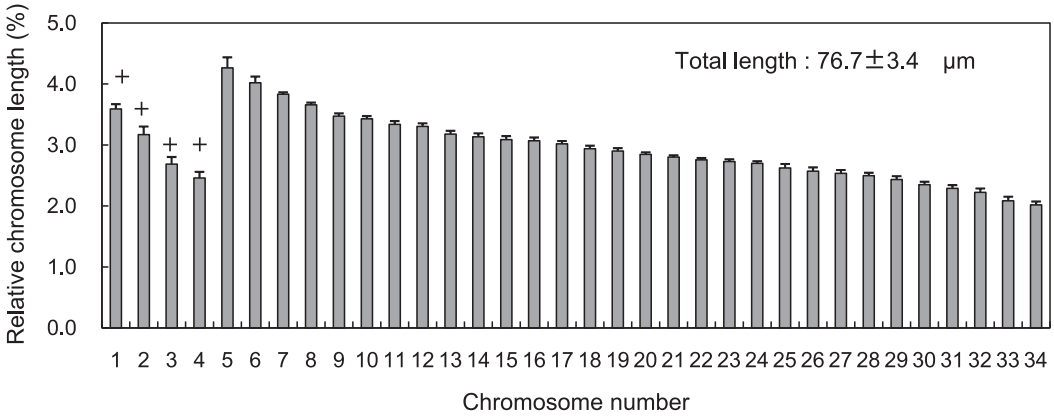

Fig. 2. The relative length (% of the total length) of each of 34 chromosomes derived from seedlings of Japanese pear 'Osa gold'. +: Chromosome with CMA positive band.

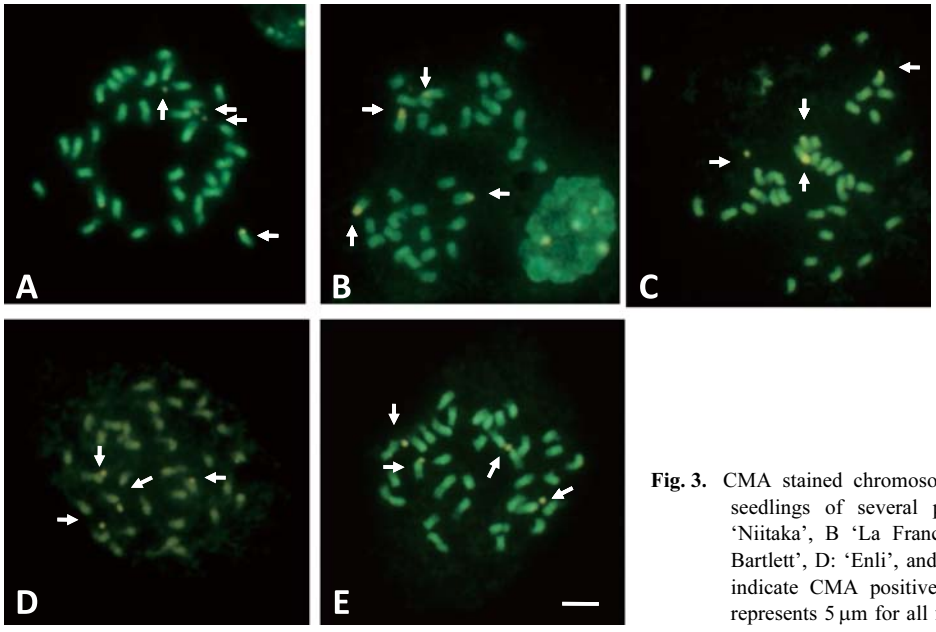

Fig. 3. CMA stained chromosomes derived from seedlings of several pear cultivars. A: 'Niitaka', B 'La France', C: 'Max Red Bartlett', D: 'Enli', and E: 'Yali'. Arrows indicate CMA positive bands. Bar in E represents 5 μm for all figures.

observing CMA +/DAPI – banding patterns on CMA staining.

In this study we used open-pollinated seedlings as samples. These seedlings with the exception of 'Osa Gold' were probably generated by cross-pollination because of the self-incompatibilty of pear. The seedlings from 'Osa Gold' were probably generated by self-pollination because this cultivar is self-compatible (Masuda et al., 1998). Although the genotype of each seedling was not identical to that of the original cultivar, almost all seedlings showed the same CMA + bands. In addition, no species or varietal difference of CMA banding pattern was observed. It could be concluded that the CMA + banding of the pear chromosome is very stable. This result agrees with the results of peach CMA/DAPI banding patterns, which showed no variation among seedlings and cultivars (Yamamoto et al., 1999). However, the results for citrus demonstrated differences in CMA banding patterns among seedlings and species (Yamamoto, 2007; Yang et al., 2002). In the present study, a few seedlings did not exhibit the four CMA + bands. This is not considered to be caused by genetic differences, since genetically identical cells do not always exhibit identical CMA + bands (Befu et al., 2000).

We demonstrated that there are no variants in the CMA +/DAPI – banding pattern among Japanese, European, and Chinese pears. The divergence of chromosome configuration seems to be very low or non-existent in view of the fluorescent banding pattern at least in these three species. However, only a few chromosomes could be identified because of the simple banding pattern. For improved elucidation of divergent chromosome configurations and identification of all chromosomes, we plan to conduct further studies of pears using fluorescent *in situ* hybridization (FISH), which is a powerful tool for chromosome analysis (Fukui et al., 1994).

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