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Detection of the Ribosomal RNA Gene in Pear (*Pyrus* spp.) using Fluorescence *in situ* Hybridization

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The locations of the 18S-5.8S-25S ribosomal RNA gene (rDNA) in the seedlings of Japanese pear [*Pyrus pyrifolia* (Burm. F.) Nakai] 'Osa Gold' and European pear (*P. communis* L.) 'La France' and the 18S ribosomal rDNA in 'Osa Gold' were determined using fluorescence *in situ* hybridization (FISH). 18S-5.8S-25S and 18S rDNA probes were labeled with biotin-16-dUTP. The probes were detected using a fluorescein isothiocyanate (FITC)-avidin conjugate with chromosomes counterstained with propidium iodide (PI). The 18S-5.8S-25S and 18S rDNA sites of 'Osa Gold' and the former of 'La France' were detected in telomeric positions of six chromosomes. The number and positions of rDNA sites were stable.

Key Words: chromosome, FISH, karyotype, rDNA.

Introduction

Chromosome analysis is important for genetic and biotechnological studies including breeding and genome analysis. In pears (*Pyrus* spp.), one of the most important fruit species cultivated in temperate regions, these studies are essential for genetic improvement; therefore, we performed chromosome analysis of pears. We showed the effectiveness of the combination of enzymatic maceration and staining with fluorochrome for karyological analysis of pears (Yamamoto et al., 2010).

Fluorescence *in situ* hybridization (FISH), for use in the physical mapping of genes, is an important technique for chromosome analysis. Detection of ribosomal RNA gene (rDNA) sites using FISH offers molecular cytogenetic information and is essential for chromosome identification and determination of phylogenic relationships among species (Fukui et al., 1994; Nakamura et al., 2001). In fruit trees, rDNA sites have already been detected in *Malus* (Schuster et al., 1997), *Prunus* (Yamamoto et al., 1999), *Diospyros* (Choi et al., 2003a), and *Citrus* (Matsuyama et al., 1996); however, to our knowledge, FISH of rDNA in pears has not been conducted.

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Recently, genome mapping of pears has progressed markedly (Dondini et al., 2004; Yamamoto et al., 2007). The loci of many useful genes associated with scab resistance and black spot disease susceptibility were genetically mapped (Terakami et al., 2006, 2007), and it became necessary to localize genes physically on chromosomes. Thus, there is an urgent need to establish methods of FISH in pears. In this study, we first demonstrated the appropriate conditions for the enzymatic maceration methods for FISH and subsequently examined the number and location of rDNA sites in two commercially important species, Japanese pear [*Pyrus pyrifolia* (Burm. F.) Nakai] and European pear (*P. communis* L.), using FISH.

Materials and Methods

Plant materials and chromosome preparation

Japanese pear [*Pyrus pyrifolia* (Burm. F.) Nakai] cultivar 'Osa Gold' and European pear (*P. communis* L.) cultivar 'La France' were used. The materials used in the present study were obtained from the National Institute of Fruit Tree Science, Japan.

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

(fixative), and stored at –20°C.

Enzymatic maceration and air drying were performed as described by Fukui (1996) and Ohmido and Fukui (1996) with minor modifications. The root tips were washed in distilled water overnight at 4°C to remove the fixative. The meristematic portion of the root tips was cut on a glass slide and placed in a 1.5 mL microtube with the enzyme mixture.

For enzymatic maceration, five combinations of enzyme mixture and duration were attempted: 1 and 2) 4% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult, Japan) and 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd, Japan) and 1 mM EDTA, pH 4.2 (Fukui, 1996) of 60 and 80 min at 37°C and 3, 4, and 5) 4% Cellulase Onozuka RS and 1% Pectolyase Y-23, and 1 mM EDTA, pH 4.2 (Fukui, 2006) of 60, 120, and 180 min at 37°C.

After incubation, the macerated root tips were placed in distilled water for 20 min to remove the enzyme solution. The root tips were placed on glass slides and water was removed from the root tips using a piece of filter paper. Fixative was added and the root tips were tapped with fine forceps until the tissue became almost invisible. After the slides had been air-dried for more than 2 h and 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, chromosomes were observed with a microscope (Nikon ECLIPSE 80i, Japan). After confirmation of each chromosome's position on the slide glass, the chromosomes were destained with 70% methanol.

Preparation of probes and their sequencing

For detection of the 18S-5.8S-25S rDNA sites, two kinds of probe were used in this study: 1) a 9.0 kb fragment including a full-length 18S-5.8S-25S rDNA repeat unit of wheat (Barker et al., 1988; Gerlach and Bedbrook, 1979) and 2) an 18S rDNA probe of 310 bp that was amplified from genomic DNA of 'Osa Nijisseiki' by PCR using a pair of primers (NS5: 5'-AACTTAAAGGAATTGACGGAAG-3' and NS6: 5'-GCATCACAGACCTGTTATTGCCTC-3'), which were based on fungal rDNA sequences (White et al., 1990). PCR amplification was performed in a 50 μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dNTP, 25 pmol each forward and reverse primer, 25 ng genomic DNA, and 2.5 units Taq polymerase (Invitrogen, USA). Amplification was conducted under the following conditions: initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, for denaturation, annealing and primer extension, respectively. The 18S-5.8S-25S and 18S rDNA probes obtained were labeled with biotin-16-dUTP (Roche, Germany) by the standard random primed labeling protocol.

The purified DNA fragments were sequenced in both directions in an ABI Prism 3130 xl sequencer (Applied Biosystems, USA) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) according to the manufacturer's instructions. Sequence data of the partial 18S ribosomal RNA gene of pears have been submitted to DDBJ/GenBank/EMBL under the following accession number: AB535217.

Fluorescence in situ hybridization

FISH was performed according to the method of Ohmido and Fukui (1996). Prior to hybridization, chromosome samples were treated with 100 ng·mL^{-1} RNase A (Sigma-Aldrich, Japan) in 2 × SSC (0.3 M NaCl and 30 mM Na-citrate) at 37°C for 60 min, dehydrated through a 70%, 95%, and 99% ethanol series for 5 min each and dried. The hybridization mixture (100 ng biotinylated probe/slide dissolved in a mixture of equal part of 50% formamide and 2 × SSC) was denatured for 10 min at 90°C and then immediately cooled down to 0°C. The chromosome samples were denatured in 50% formamide/2 × SSC for 6 min at 70°C with the hybridization mixture on a thermal cyclar with a flat aluminum bed (Astec PC816, Japan) and then hybridized for 18 h at 37°C. After hybridization, they were washed twice in 2 × SSC, once in 50% formamide/2 × SSC and once in 4 × SSC, each for 10 min at 42°C. Chromosome samples were blocked with 5% bovine serum in BT buffer (0.1 M sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) at 37°C for 5 min. Fluorescein (FITC)-avidin (1%, Vector Laboratories, USA) in 1% bovine serum albumin (BSA) in BT buffer was dropped onto the chromosome samples, which were then incubated at 37°C for 60 min. After the FITC-avidin solution had been rinsed with BT buffer three times at 40°C for 5 min each, samples were blocked with 5% goat serum albumin in BT buffer at 37°C for 5 min. Biotinylated anti-avidin (1%, Vector Laboratories) in 5% goat serum in BT buffer was dropped onto the chromosome samples and they were then incubated at 37°C for 60 min for secondary amplification. After washing with BT buffer three times at 40°C for 5 min each, they were blocked with 5% bovine serum in BT buffer at 37°C for 5 min. Then, 1% FITC-avidin in 1% BSA in BT buffer was dropped onto them, and they were incubated at 37°C for 60 min. After rinsing twice with BT buffer and once with 2 × SSC at 40°C for 5 min each, the chromosome samples were finally counterstained with 0.5 μg·mL^{-1} propidium iodide (PI) in an antifadant solution (Vector Shield, Vector Laboratories).

The chromosome samples were observed with a fluorescence microscope (Nikon ECLIPSE 80i) with a microscope digital camera (Olympus DP71, Japan). FITC signals were visualized using a B filter. Chromosomes counterstained with PI were visualized using a G filter. Signal images were analyzed by imaging software (Olympus DP-BSW Ver. 03.01).

Results and Discussion

In a previous report (Yamamoto et al., 2010), we established enzymatic maceration and fluorescence staining for pear chromosomes. Although preparation by the enzymatic composition and duration of incubation were sufficient for fluorescence staining analysis, a little cytoplasm occasionally remained (Fig. 1A). Since FISH requires preparation without cytoplasm (Fukui et al., 1994; Mukai, 1996), improved enzyme maceration conditions should be investigated. First, we prolonged the duration of enzymatic maceration for 80 min. In this condition, although chromosomes were observed without cytoplasm in the background, chromosomes were sometime lost (Fig. 1B, 1C) due to excessive duration. Next, an enzyme mixture containing 4% Cellulase Onozuka RS and 1% Pectolyase Y-23 for 60, 120 and 180 min was used. With this enzyme composition, cytoplasm remained in the preparation for 60 and 120 min (Fig. 1D, 1E). The best preparation, with all 34 chromosomes relatively extended and well spread without cytoplasm, was 4% Cellulase Onozuka RS and 1% Pectolyase Y-23 for 180 min (Fig. 1F); therefore, all chromosome samples used in subsequent FISH analysis were prepared under this condition.

The 310 bp sequences of pear amplified using the primer based on the fungal 18S rDNA sequence correspond to the arrangement of 1117–1426 bp sequences of 18S rDNA in wheat (accession number: AJ272181, DNA Data Bank of Japan (DDBJ), http://getentry.ddbj.nig.sc.jp/top-j.html). The sequence identity of both sequences was 299/310 (96.5%). From this result, it can be concluded that the amplified sequence in the present study was partial 18S rDNA.

In situ hybridization with the 18S-5.8S-25S rDNA probe revealed signals on six chromosomes of 'Osa Gold' and 'La France' seedlings. The six signal sites were located in telomeric regions of the six chromosomes (Fig. 2A, 2B). The 18S-5.8S-25S rDNA sites were stable, and no variation among seedlings was detected between the two cultivars (Table 1). These six chromo-somes with rDNA could be easily distinguished from the other 28 chromosomes without observing rDNA sites.

In situ hybridization with the 18S rDNA probe revealed signals on six chromosomes of 'Osa Gold' seedlings. The six signal sites were located in telomeric regions of the six chromosomes, and no variation among seedlings was observed (Fig. 2C and Table 1). The detected number and location of 18S rDNA sites were identical with those of 18S-5.8S-25S rDNA.

In the present study, we used open-pollinated seedlings as samples. The seedlings from 'Osa Gold' were probably generated by self-pollination because this cultivar is self-compatible (Masuda et al., 1998), whereas the seedlings from 'La France' were probably generated by cross-pollination because of its self-incompatibilty. Although the genotype of each seedling was not identical to that of the original cultivar, the number and location of rDNA sites were stable among the seedlings of both cultivars. This result agrees with the observed chromomycin A_3 (CMA)/4'-6-diamidino-2-phenylindole (DAPI) banding patterns, which showed no variation among seedlings and species in pears (Yamamoto et al., 2010). It could be concluded that the divergence of chromosome configuration is very low in view of the rDNA sites and fluorescent banding patterns in pears;

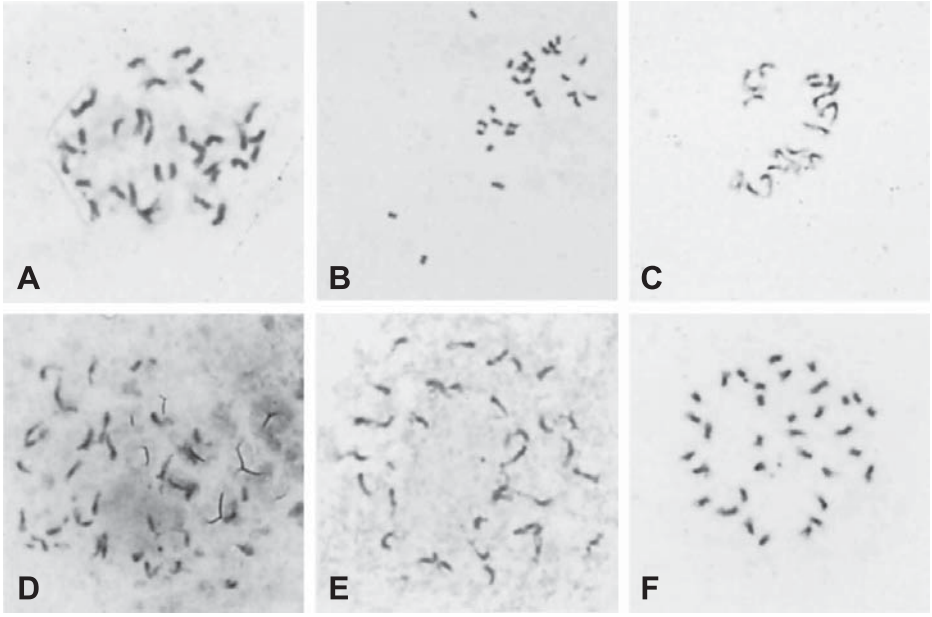

Fig. 1. Example of Giemsa-stained chromosomes prepared using several combinations of enzyme composition and durations of enzyme treatment in 'Osa Gold' seedlings. A, B, and C: 4% Cellulase Onozuka RS, 1.5% Macerozyme R200 and 0.3% Pectolyase Y-23. D, E, and F: 4% Cellulase Onozuka RS and 1% Pectolyase Y-23. A and D: 60 min, B and C: 80 min, E: 120 min and F: 180 min.

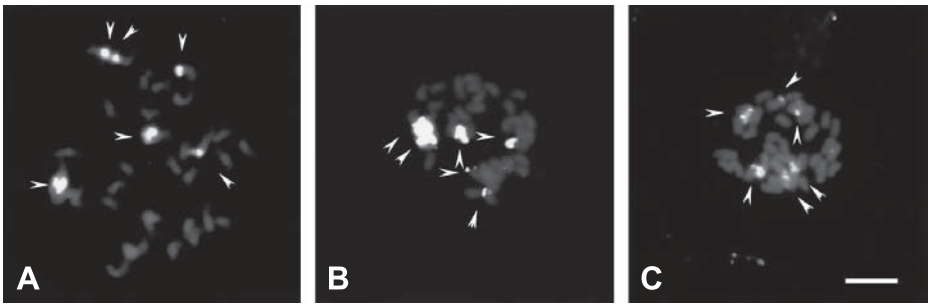

Fig. 2. FISH of 18S-5.8S-25S and 18S rDNA probes on somatic chromosomes of pear seedlings. A and C: 'Osa Gold', B 'La France'. A and B: 18S-5.8S-25S rDNA, C: 18S rDNA. Arrowheads indicate rDNA sites. Counterstaining: PI. Bar in C represents 5 μm for all figures.

Table 1. Reproduciblity of six rDNA sites which appeared in chromosomes derived from open-pollinated seedlings of pear (*Pyrus* spp).

Cultivar	Species	rDNA genes used as a probe	No. of seedlings examined	No. of seedlings with six rDNA sites
Osa Gold	*P. pyrifolia*	18S-5.8S-25S	16	14
Osa Gold	*P. pyrifolia*	18S	4	4
La France	*P. communis*	18S-5.8S-25S	4	4

however in the present study, two out of 16 seedlings did not exhibit the six rDNA sites. The number of observed rDNA sites of these seedlings was less than six. This was not considered to have been caused by genetic differences, since genetically identical cells prepared from young leaves did not always exhibit identical rDNA sites due to the conditions of chromosome preparation (Yamamoto, unpublished data).

This is the first report on the number and location of rDNA sites in pears; however, 18S-25S (same loci as 18S-5.8S-25S) rDNA sites in apple (*Malus* × *domestica* Borkh.) have been reported (Schuster et al., 1997) in another important fruit tree with 2n = 34 chromosomes belonging to the same subfamily Maloideae of family Rosaceae as pears. The rDNA sites of apple were detected in telomeric regions of eight chromosomes. There is some resemblance of the rDNA sites between pears and apples; all six and eight rDNA sites are located at telomeric regions of pears and apples, respectively. The number of chromosomes with rDNA sites is not identical, which seems to indicate some divergence of chromosome configuration between pears and apples.

The results of FISH for 18S rDNA were identical to those of 18S-5.8S-25S rDNA. The 18S rDNA probe used in the present study was a partial 310 bp fragment amplified by PCR based on a fungal 18S rDNA sequence that constitutes a portion of 18S-5.8S-25S (White et al., 1990). Compared with 18S-5.8S-25S rDNA, probe preparation of the amplified 18S rDNA was simple; it required no culture of *E. coli*, and thus no rDNA plasmid or extraction/purification of the plasmid. For 5S rDNA detection, PCR amplified probes have already been used for fruit trees (Carvalho et al., 2005; Choi et al., 2003b). Hence, PCR amplification of 18S rDNA using the primer based on the fungal rDNA sequence is considered to be reliable and effective for FISH in fruit trees, as is that of 5S rDNA.

We identified 18S-5.8S-25S rDNA sites on pear chromosomes. Recently, multi-color FISH using 5S and 18S-5.8S-25S rDNA has been developed for fruit trees (Carvalho et al., 2005; Choi et al., 2003b). The obtained results offer more useful information on chromosome identification and the determination of phylogenic relationships than FISH using a single probe. In addition, detection of the physical location of useful gene loci has progressed in important crops and vegetables (Iwano et al., 1998; Ohmido et al., 1998); therefore, we are planning to conduct multi-color FISH for further chromosome identification and physical mapping of useful genes in pears.

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