Enzymatic maceration/air-drying method for chromosome observations in the young leaf of pear (Pyrus spp.)

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Abstract

A chromosome preparation method using young leaves of pear (Pyrus spp.) was developed. Young leaves, 1-2 cm long, of grafted Japanese pear ‘Kosui’ (Pyrus pyrifolia Nakai) were used as materials. The leaves were cut into approximately 2 mm² for enzymatic maceration/air-drying (EMA). For EMA, enzyme mixture containing 4% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), and 1 mM EDTA, pH 4.2, at 37°C for 60-75 min was optimum for chromosome preparation because a large number of good preparations, with all 34 chromosomes relatively extended and well spread without cytoplasm, were observed. The 18S-5.8S-25S ribosomal RNA gene (rDNA) site was detected in telomeric positions of six chromosomes in fluorescent in situ hybridization (FISH). The number and positions of rDNA sites were the same as in the results using root tips as materials (Yamamoto et al. 2010, 2012). The method developed in the present study is considered to be promising for further cytogenetic studies in pear since true-to-type chromosome samples are obtained from young leaf.

Keywords: chromosome, EMA, FISH, pear, Pyrus

Introduction

Chromosome information is important for genetic and biotechnological studies including breeding and genome analysis. In pear (Pyrus spp.), one of the most important fruit species cultivated in temperate regions, these studies are essential for genetic improvement. Therefore, we have been conducted the following studies. First, an enzymatic maceration/air-drying (EMA) method (Fukui 1996), preparing good chromosome samples with small chromosomes of pears, was developed using root-cells of the seedlings (Yamamoto et al. 2010). Next, fluorescent in situ hybridization (FISH), for detection of the location of a given gene on a chromosome, was also developed and the number and location of 5S and 18S-5.8S-25S ribosomal RNA gene (rDNA) sites were visualized (Yamamoto et al. 2010, 2012).

Although these results of previous studies (Yamamoto et al. 2010, 2012) were very important for the progress of cytogenetic studies in pear, there is a problem. All chromosome samples in these studies on EMA and FISH were derived from the root tips of seedlings (Yamamoto et al. 2010, 2012). However, the genotype of root tips derived from seedlings is not identical to that of the original (mother) plant in fruit trees because of their heterozygosity, although root tips are conventional materials for preparing chromosome samples in higher plants (Fukui 1996). Moreover, roots of a given cultivar are difficult to obtain because almost all scion cultivars are grafted on rootstocks in pear. Plant tissue showing a true-to-type genotype is desirable for materials for chromosome samples to perform accurate cytogenetic studies. Thus, vegetative tissue such as a leaf is desirable material for accurate cytological studies in fruit trees. For this reason, EMA methods using shoot apical meristem and young leaf of persimmon (Diospyros kaki Thunb.) (Zhuang et al. 1990) and citrus (Citrus spp.) (Kitajima et al. 2001) have been developed, respectively. The chromosome samples prepared by this method could be applied to in situ hybridization study in citrus (Kitajima et al. 2007).

It is important to establish EMA and FISH using vegetative tissue as a material in pear as in persimmon and citrus. Thus, in the present study, 1) a chromosome preparation method using young leaves of pear was developed and 2) FISH of 18S-5.8S-25S rDNA was performed using the chromosome samples obtained by the method of <1>.
Materials and methods

Plant materials

Japanese pear ‘Kosui’ (*Pyrus pyrifolia* Nakai) was used. The potted trees were preserved at the Faculty of Agriculture, Kagoshima University (Kagoshima, Japan) and grafted on *Pyrus calleryana* Decne. Young leaves, about 1-2 cm long, at the bud burst stage were used as materials. Fresh young leaf samples were 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.

Enzyme maceration/air drying (EMA) and Giemsa staining

In enzymatic maceration and air drying (EMA), leaf samples were categorized into three types: approximately 1-cm-long leaf (short leaf), upper half of an approximately 2-cm-long leaf (upper long leaf), and lower half of an approximately 2-cm-long leaf (lower long leaf). The leaves were cut into approximately 2 mm² for EMA. EMA was performed as described by Fukui (1996) with minor modifications (Yamamoto et al. 2010). The young leaves 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), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), and 1 mM EDTA, pH 4.2, at 37°C. Four durations of enzyme treatment (45, 60, 75, and 90 min) were tested. Thus, there were 12 kinds of enzyme treatment (three kinds of leaf sample × four kinds of duration of enzyme treatment). Three cut leaf samples were used in each treatment. After incubation, the macerated leaves were placed in distilled water for 20 min to remove the enzyme solution. The leaves were placed on glass slides and water was removed from them using a piece of filter paper. Fixative was added and the leaves were tapped with forceps until the tissue spread. Slides were air-dried for at least for 3 h.

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 under a microscope (Nikon Eclipse 80i, Japan), the chromosomes were destained with 70% methanol.

The root tips of ‘Osa Gold’ (*Pyrus pyrifolia*) seedlings were cut into approximately 2 cm, at the bud burst stage and 4% Cellulase Onozuka RS, 1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Japan), and 1 mM EDTA, pH 4.2, at 37°C for 3 h (Yamamoto et al. 2010). All other treatments were the same as for the leaf samples.

Fluorescence in situ hybridization (FISH)

The rDNA clone, 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), was labeled with biotin by the standard nick translation protocol (Invitrogen, BioNick Labeling System). FISH was performed according to the method of Ohmido and Fukui (1996) with slight modifications (Yamamoto et al. 2010). The biotinylated probe was hybridized to chromosomal rDNA *in situ* and detected with a fluorescein isothiocyanate (FITC)-avidin conjugate (Vector, California, USA) by fluorescence microscope (Nikon ECLIPSE 80i, Japan). FITC signals were visualized using a B filter. Chromosomes were counterstained with 2.0 mg L⁻¹ DAPI in an antifadant solution (Vector Shield; Vector Laboratories) and visualized using a UV filter. Signal images were analyzed using imaging software (DP-BSW Ver. 03.01, Olympus).

Results and Discussion

Good preparations, with all 34 chromosomes relatively extended and well spread without cytoplasm, were observed for all combinations of leaf samples and durations of enzyme treatment (Fig. 1 A to L and Table 1). The number of chromosome preparations per slide was the highest (12.3) in lower long leaf/45 min and the lowest (4.7) in lower long leaf/90 min. The numbers of observed preparations tended to be high in short leaf, except for 45 min treatment. Although almost the same number of preparations was observed in 45, 60, and 75 min treatments, there were fewer in 90 min treatment. In 90 min treatment, chromosomes were probably lost due to the excessive duration (Fig. 1 O and P). The same result of a loss of chromosomes due to an excessive duration was reported in root tips (Yamamoto et al. 2010). On the other hand, a little cytoplasm occasionally remained in 45 min treatment (Fig. 1 M and N). Thus, 60-70 min treatment in this enzyme composition is sufficient for EMA for pear leaf samples. Approximately 15 preparations per slide were observed in root tip samples (Table 1). Although, compared with this result, the numbers of preparations per slide were low in leaf samples, the EMA method developed in the present study using leaf samples as materials is considered to be practical because the differences of the numbers of preparations were not so large between leaf and root tip samples.

Next, subsequent FISH analysis was conducted in the present study. In *in situ* hybridization with the 18S-5.8S-25S rDNA probe revealed signals on six chromosomes of ‘Kosui’. The six signal sites were located in telomeric regions of the six chromosomes (Fig. 2). FISH using 18S-5.8S-25S rDNA probe has already been performed in pear chromosome samples derived from root tips (Yamamoto et al. 2010, 2012). All seedlings of Japanese pear, European pear (*P. communis* L.), and Toyotomi Nashi (*P. mikiwana* Koidz.) showed six signal sites located on telomeric regions of the six chromosomes. These results are identical to those of the present study. As a consequence, chromosome preparation by EMA using leaf samples is considered to be suitable for *in situ* hybridization study in pear.

In conclusion, chromosome preparations sufficient for *in situ* hybridization study could be obtained from the following materials and EMA conditions: young leaves, about 1-2 cm, at the bud burst stage and 4% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), and 1 mM EDTA, pH 4.2, at 37°C for 60-75 min. EMA methods using young leaf developed in the present study are considered to be promising for further chromosome study and should contribute to the progress of breeding and genome studies in pear. For this purpose, determination of the physical locations of useful gene loci is indispensable for the progress of genome studies. The locations of useful genes on chromosomes have been elucidated in apple.
Yamamoto et al. Figure 1. Example of Giemsa-stained chromosomes prepared using several combinations of leaf samples and durations of enzyme treatment in Japanese pear 'Kosui' (2n=2x=34). Bar in A represents 5 μm for all figures. Leaf samples: A, B, C, D, M, and O: short (approximately 1 cm long) leaf; E, F, G, H, N, and P: upper portion of long (approximately 2 cm long) leaf; and I, J, K, and L: lower portion of long leaf. Duration of enzyme treatment: A, E, I, M, and N: 45 min; B, F, and J: 60 min; C, G, and K: 75 min; and D, H, L, O, and P: 90 min. Enzyme composition: 4% Cellulase Onozuka RS, 1.5% Macerozyme R200 and 0.3% Pectolyase Y-23.

Yamamoto et al. Figure 2. FISH of 18S-5.8S-25S probe on somatic chromosomes of Japanese pear 'Kosui'. Arrowheads indicate rDNA sites. Counterstaining: DAPI. Bar = 5 μm.

Table 1. Effect of type of sample and duration of enzyme treatment on observed number of chromosome preparations per slide derived from young leaf in pear (Pyrus pyrifolia).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Material</th>
<th>Number of chromosome preparations per slide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Duration of enzyme treatment</td>
</tr>
<tr>
<td>Kosui</td>
<td>Leaf, short</td>
<td>10.5 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>Leaf, long, upper half</td>
<td>9.5 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Leaf, long, lower half</td>
<td>12.3 ± 5.7</td>
</tr>
<tr>
<td>Osa Gold</td>
<td>Root tip</td>
<td>n. d.</td>
</tr>
</tbody>
</table>

a) Leaf: 4% Cellulase Onozuka RS, 1.5% Macerozyme R200, 0.3% Pectolyase Y-23, 37°C,
root tip: 4% Cellulase Onozuka RS, 1% Pectolyase Y-23, 37°C
b) Short: young leaf approximately 1 cm long, long: young leaf approximately 2 cm long
c) Not determined
d) Self-pollinated seedlings
EMA method for chromosome observations in the young leaf of pear (Pyrus spp.) and citrus (Mendes et al. 2011). Therefore, physical mapping of several useful genes, such as for disease resistance and self-incompatibility, should be conducted in pear.

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References


