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Digoxigenin (DIG)-Based AFLP Analysis of Three Citrus Cultivars

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Abstract

A digoxigenin (DIG)-based amplified fragment length polymorphism (AFLP) method was developed to distinguish three closely related citrus cultivars, 'Kishu', 'Mukaku-Kishu' and 'Hira-Kishu'. In particular, 'Mukaku-Kishu' characterized by seedless fruit is very important for breeding purposes. Initial attempts at using the DIG-based RAPD procedure failed to detect 'Mukaku-Kishu'-specific bands. However, the DIG-based AFLP method enabled to detect such bands, indicating that this method is more sensitive than the DIG-based RAPD procedure. The detailed protocol for the DIG-based AFLP methods is presented in this paper.

Discipline: Biotechnology
Additional key words: PCR, RAPD, fingerprinting

Introduction

Amplified fragment length polymorphism (AFLP), a DNA fingerprinting technique[7] is a very powerful tool for distinguishing closely related cultivars, and has been applied for molecular genetic analysis of peach[5]. However, conventional AFLP methods are rather expensive and require radioactive or fluorescent dye labels and a DNA sequencer.

We had previously developed a DIG-based random amplified polymorphic DNA (RAPD) method[6] and used it for the detection of DNA bands that are absent during somatic embryogenesis in rice plants[8]. We had also applied the DIG-based RAPD methods to analyze genetic relationships among sweet pea cultivars[2]. This procedure was very useful for the detection of minor differences in chromosomal DNAs.

The three *Citrus kinokuni* cultivars, 'Hira-Kishu', 'Mukaku-Kishu' and 'Kishu' are closely related genetically. 'Mukaku-Kishu' is unique in that it is female-sterile and is seedless. DNA markers specific for this trait should be useful for screening seedless lines at the early stage of breeding.

We initially attempted to identify genetic differences among these three closely related cultivars using the DIG-based RAPD method. However, it was impossible to detect a 'Mukaku-Kishu'-specific DNA band using this method. This could be attributed to the fact that the DIG-based RAPD was not sensitive enough to detect small differences among the chromosomal DNAs of the three cultivars.

It was thus necessary to develop a more sensitive method to detect these minor differences. On the other hand, it was also necessary to develop a procedure simpler than the conventional AFLP methods, to detect such fine polymorphism. To satisfy these two requirements, we developed a DIG-based AFLP method which could be used to identify a 'Mukaku-Kishu'-specific DNA band. We report here the detailed protocol for this method.

Materials and methods

1. Plant materials and DNA extraction

Three citrus cultivars, 'Kishu', 'Mukaku-Kishu' and 'Hira-Kishu', were grown at Wakayama Prefectural Fruit

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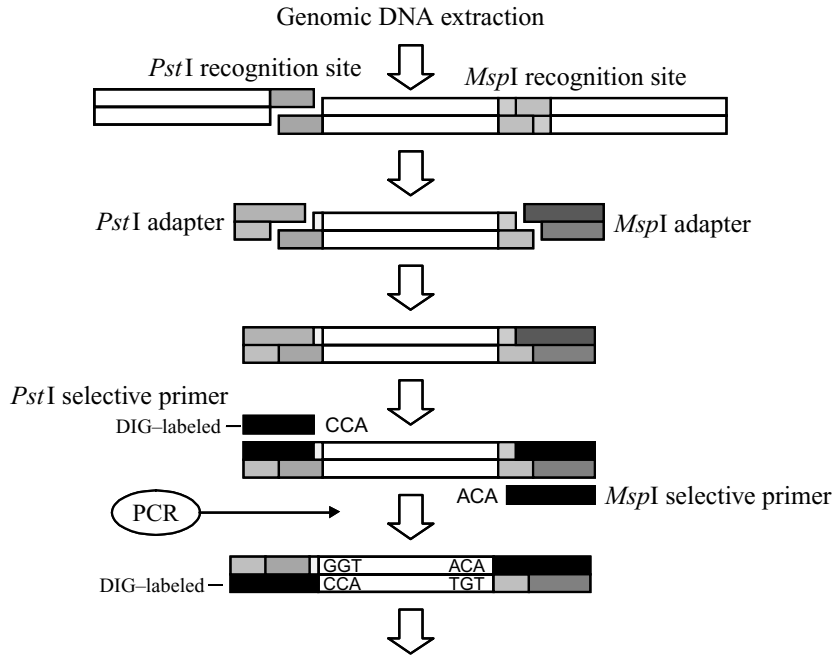

Fig. 1. Schematic representation of a DIG-based AFLP protocol

Genomic DNA was extracted by a CTAB method, and digested with appropriate restriction enzymes (e.g. *Pst*I and *Msp*I). Adapters were ligated. A DIG-labeled primer and another primer were used for PCR. After polyacrylamide gel electrophoresis and blotting onto nylon membrane, DIG immunostaining was performed. Only DIG-labeled DNA fragments were detected by this method.

Experimental Station. Young leaves from each cultivar were subjected to DNA extraction by the cetyltrimethylammonium bromide (CTAB) method[4]. Total DNA was digested with a 6-base cutter *Pst*I or *Eco*RI, followed by a 4-base cutter *Msp*I.

2. Sequence of adapter and PCR primers

The six adapter sequences were as follows:
*Pst*I-adapter-1, 5'-CTCGTAGACTGCCGTACATGCA-3',
*Pst*I-adapter-2, 5'-CATCTGACGCATGT-3',
*Eco*RI-adapter-1, 5'-CTCGTAGACTGCGTACC-3',
*Eco*RI-adapter-2, 5'-CTGACGCATGGTTAA-3',
*Msp*I-adapter-1, 5'-GACGATGAGTCCTGAC-3',
*Msp*I-adapter-2, 5'-TACTCAGGACTCGC-3',
PCR primers for *Pst*I and *Eco*RI, which were labeled with digoxigenin at the 5'-terminal, were obtained from Nissinbo Inc. (Tokyo). Their sequences were as follows:
*Pst*I-DIG primer-1, 5'-GACTGCGTACATGCAGCCA-3',
*Eco*RI-DIG primer-1, 5'-GACTGCGTACCAATTCAAG-3'.
The sequence of the *Msp*I primer-1 was 5'-GATGAGTCCTGAGCGGAAC-3'.

3. Gel electrophoresis and DIG-detection

PCR products were electrophoresed on an 8% (acrylamide:BIS acrylamide = 39:1) non-denaturing polyacrylamide gel (200 mm × 400 mm × 1 mm) in Tris-borate buffer (pH 8.3), blotted onto a nylon membrane (Hybond N+, Amersham) overnight, and stained by an immunochemical method as described previously[6,8]. The filter was treated with anti-DIG-alkaline phosphatase and Fab fragments, and then visualized with the colorimetric substrates (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) provided in the DIG detection kit (Boehringer Mannheim). For DNA standard molecular markers, ϕ×174/*Hae*III digests were labeled with a terminal transferase using the DNA oligonucleotide 3' end labeling kit (Boehringer Mannheim).

Results and discussion

1. Scheme of DIG-based AFLP

Fig. 1 shows the scheme for the DIG-based AFLP method. Results were obtained two days after DNA preparation. The factors to be considered for obtaining good results are described in the following sections.

2. Useful technical information for good results

Citrus genomic DNA was extracted by the method

of Murray and Thompson[4]. In our previous report on DIG-RAPD[2], we used a simultaneous DNA extraction method[1] for the preparation of DNA. However, we found that this method was not suitable for AFLP analysis and that the use of the Murray and Thompson procedure although more time-consuming, was preferable for preparing chromosomal DNA.

In the first report on AFLP[7], *Mse*I was used as a frequent cutter enzyme. In our study, however, we used *Msp*I (recognition sequence: CCGG) as the frequent cutter enzyme in place of *Mse*I (recognition sequence: TTAA), because *Msp*I is more popular and less expensive. As shown in Fig. 2, *Msp*I was as effective as *Mse*I.

3. An example of DIG-based AFLP

One and a half μg of citrus genomic DNA was digested with 12 units of *Msp*I in 20 μL of *Msp*I reaction mixture at 37ºC for 1 h. DNA was recovered by ethanol precipitation, and then digested with 12 units of *Pst*I in 40 μL of a *Pst*I reaction mixture at 37ºC for 1 h. After *Pst*I digestion, DNA was recovered by ethanol precipitation and dissolved in 8 μL of water. Adapter ligation was performed with 20 pmol of *Pst*I adapter, 100 pmol of *Msp*I adapter, and 70 units of T4 DNA ligase in T4 DNA ligase buffer containing 1 μg of BSA at 37ºC for 3 h. Total volume was 10 μL.

Out of 10 μL of ligated DNA, 0.3 μL was used for amplification. The PCR amplification mixture (25 μL) contained ligated DNA, DIG-labeled *Pst*I primer, *Msp*I primer, 0.2 mM of dNTP, and 1 unit of Taq DNA polymerase. The PCR conditions were as follows: 94ºC preheating for 60 s, 30 cycles of heating at 94ºC for 60 s, annealing at 60ºC for 30 s, extension at 72ºC for 120 s, followed by another extension at 72ºC for 300 s.

After PCR amplification, one-tenth of the volume was electrophoresed on an 8% polyacrylamide gel. The gel was blotted onto a nylon membrane filter and the baked filter was immunostained as described in the materials and methods.

4. Cultivar-specific DNA band

According to the scheme shown in Fig. 1, we carried out 24 experiments with combinations of 4 *Msp*I primers and 3 *Pst*I or 3 *Eco*RI DIG-labeled primers. Fig. 2 shows the DIG-based AFLP profile obtained using a *Pst*I DIG-labeled primer and *Msp*I primer combination. A specific band for 'Mukaku-Kishu' was detected in lane 8.

Table 1 shows the number of DIG-AFLP fragments observed in three citrus cultivars. The adapter combination of *Msp*I and *Eco*RI gave 270 bands including 54 polymorphic bands, of which 16 bands appeared only in 'Mukaku-Kishu'. In the case of *Pst*I and *Msp*I (example

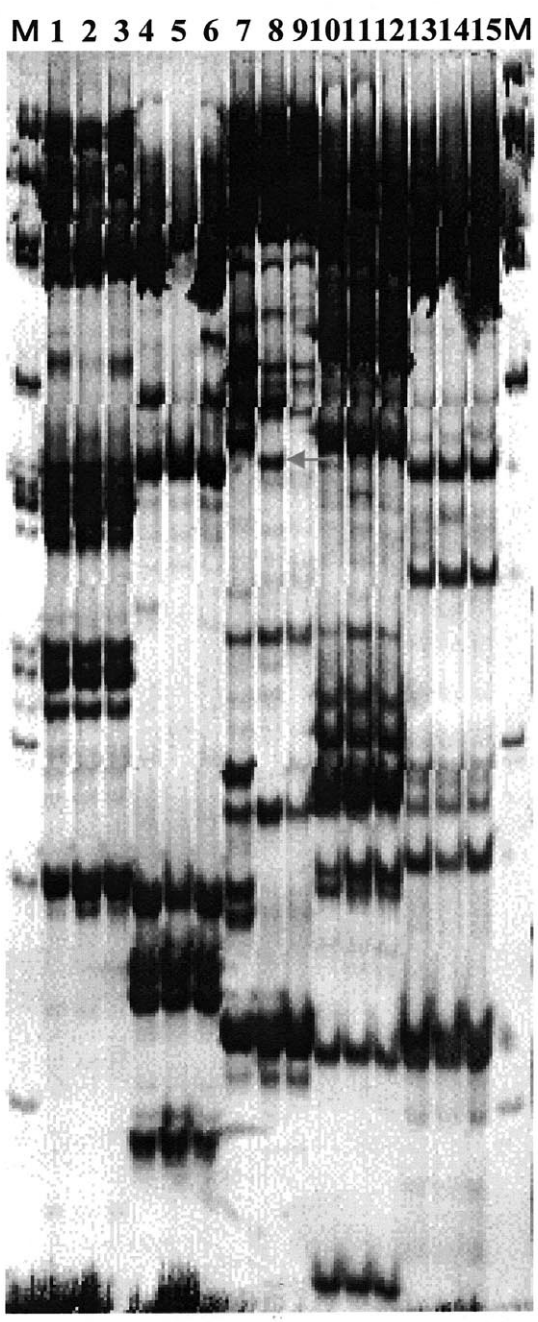

Fig. 2. Profile of DIG-based AFLP analysis of chromosomal DNA from three citrus cultivars
Lanes 1,4,7,10 & 13: 'Hira-Kishu' samples.
Lanes 2,5,8,11 & 14: 'Mukaku-Kishu' samples.
Lanes 3,6,9,12 & 15: 'Kishu' samples.
Lanes 1–3: *Pst*I-1/*Msp*I-1, lanes 4–6: *Pst*I-2/*Msp*I-1, lanes 7–9: *Pst*I-3/*Msp*I-1, lanes 10–12: *Pst*I-1/*Msp*I-2, and lanes 13–15: *Pst*I-2/*Msp*I-2. Lane M: ϕ×174/*Hae*III digests. Arrow indicates a 'Mukaku-Kishu' specific band.

Table 1. Number of DIG-AFLP fragments

Adapter combination	No. of total bands detected	Polymorphic bands	Detected only in 'Mukaku-Kishu'
*Eco*RI/*Msp*I	270	54	16
*Pst*I I/*Msp*I	185	71	15
Total	455	125	31

shown in Fig. 2), 15 bands specific for 'Mukaku-Kishu' were obtained out of 185 DNA bands.

5. Frequency of appearance of DIG-based AFLP bands

As shown in Table 1, the number of visible bands obtained with the adapter combination of *Eco*RI and *Msp*I was higher than that obtained with *Pst*I and *Msp*I. On the other hand, the number of polymorphic bands obtained with *Eco*RI and *Msp*I was lower than that obtained with *Pst*I and *Msp*I, presumably by chance. The probability of detecting 'Mukaku-Kishu'-specific bands by using DIG-based AFLP was found to be much higher than by using DIG-based RAPD.

6. Conclusion

In this report, we described in detail the protocol for a DIG-based AFLP method. The experimental procedure for DIG-based AFLP is more complex than that for DIG-based RAPD[6], but the DIG-based AFLP method enables to distinguish closely related cultivars. Here, we demonstrated that this method could be applied to detect several DNA bands specific for 'Mukaku-Kishu', which could not have been detected using the DIG-based RAPD method. This procedure can be applied in the absence of expensive DNA sequencer system[9] or radioactive chemicals required for RI (radioisotope incorporated)-RAPD[3]. Therefore, the DIG-based AFLP method can be applied even in laboratories with standard equipment.

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