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Note

Modification of the filter-inserted tip method for simple and rapid DNA extraction from strawberry

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Abstract In order to perform conveniently DNA extraction from strawberry leaves, we tried to apply the "filter-inserted tip method". This method has been used to extract easily DNA from rice. To examine the utility of the method in strawberry leaves, DNA extraction experiment was carried out, and the yielded DNA samples were used for PCR reactions. PCR reaction was greatly improved with the crushing buffer when 50 mg/ml polyvinylpolypyrrolidone was added. PCR amplicons were obtained for 132–140 out of 144 samples examined, showing high reproducibility of the method. It took approximately 70 min to conduct DNA extractions from 24 samples. It was about 40% of the operation time in the case of using a commercially available DNA extraction kit. The results suggest that this method is suitable for rapid DNA extractions from a large number of samples for PCR reactions, such as marker-assisted selection with simple manipulations, low cost, and without using expensive equipment.

Key words: Cultivar identification, DNA extraction, DNA marker, glass fiber filter, marker-assisted selection.

In recent years, DNA discrimination techniques have been applied to various aspects of plant breeding such as marker-assisted selection using DNA markers, identifications of the cultivars for the protection of the breeding rights, prevention from the contamination of undesirable cultivars, and investigation of genetic distances between closely-related cultivars. These DNA discrimination techniques are based on the detection of polymorphisms in nucleotide sequences in genomic or organelle DNAs. In many cases, genotypic difference is identified by detecting the polymorphisms in various ways after amplifying specific regions of genomic DNA with the PCR method.

There are some important points for the extraction of template DNA used for the PCR method. First, it is not necessary to extract large size DNA molecules. Thus, we do not need to worry too much about the DNA cleavages. Next, polysaccharides and polyphenols contained in plant tissues inhibit reaction process of PCR. This means that it is essential to remove these substances as much as possible. It is especial notable in strawberry. This point is a great influence on the reproducibility of the experiment. Also, a large number of samples have to be handled in many cases, such as line selection in plant breeding. Consequently, it is necessary to develop a new method which enables operational simplification and cost reduction.

Therefore, various methods have been developed for the purpose of high quality DNA extraction by eliminating polysaccharide or polyphenol groups contained in plant tissues, such as the method using diatomaceous earth (Tanaka and Ikeda 2002) and glass fiber filters (Muramoto 2005). Also some other simple and easy DNA extraction methods have also been published (Ikeda et al. 2000; Liu et al. 1995; Mori et al. 2003; Zhu et al. 1993). These methods were expected to enable simple and low cost DNA extraction. Furthermore, a novel DNA extraction method based on that of Muramoto (2005) has been developed (Fukami et al. 2008). The method used glass fiber filters inserted into micropipette tips. It was conducted by pipetting the fluid using a multichannel pipette. This allowed us to manipulate multiple samples at the same time for genomic DNA adsorption, washing and elution (referred to as "the filter-inserted tip method" thereafter).

We tried to DNA extracted from strawberry leaves using the methods described above. However, PCR amplicons were not observed with the resulting DNA solutions (data not shown). Therefore, we improved extraction method by mainly adding the polyvinylpolypyrrolidone (PVPP) in the crushing solution used for the extraction. In this paper, we report a simple DNA extraction method from the leaves of the strawberry.

Following the same procedures to the method of Fukami et al. (2008), glass fiber filters (GF/A, Whatman Co., Ltd.) were soaked in sodium sulfite solution (20 mM Tris-HCl (pH 8.0), 2 mM EDTA-Na2, 10% (w/v) sodium

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sulfite) and dried at room temperature. The dried filters were cut into small pieces and inserted into 200 μl micropipette tips (No. 739296 Greiner Bio-One Co., Ltd., Figure 1). In addition, in order to perform the loading and unloading of liquid smoothly, the tip of the tips was cut off for approximately 3 mm.

After the preparation of the tips (hereafter filter-inserted tip) described above, the following manipulations were carried out. Since this study is aiming at handling a large number of samples, the fixing solution, washing solution, 70% (v/v) ethanol and 1/10 TE were initially dispensed into 96-well flat bottom micro plate (BM Equipment Co., Ltd.). Approximately 20 mg of strawberry leaf segment was crushed with 200 μl of crushing solution (200 mM Tris-HCl (pH 7.5), 25 mM EDTA-Na2, 250 mM NaCl, 0.5% (w/v) SDS and 10% (w/v) sodium sulfite) using a multi-sample homogenizer (Shake Master ver. 1.2A, BioMedical Science Co., Ltd., Tokyo, Japan) for 45 s. Then the samples were incubated at 65°C for 10 min. and centrifuged at 12,000 rpm for 3 min. Supernatant 7 μl was sucked up with a micropipette. The tip of the pipette was inserted into the "filter-inserted tip" from the side of mounting the pipette, the supernatant was adhered to the glass filter paper. After pipetting one time 100 μl of fixing solution (100 mM Tris-HCl (pH 8.0), 10 mM EDTA-Na2, 7 M guanidine hydrochloride) with the "filter-inserted tips" attached a multichannel micropipette (N59727, Finnpipette, LabSystems), the tips were kept at room temperature for 1 min. The tips were then subjected to washing with 200 μl of washing solution (50 mM Tris-HCl (pH 8.0), 5 mM EDTA-Na2, 200 mM sodium chloride, 60% (v/v) ethanol) by repeating the pipetting 10 times. After repeating this washing manipulation four times again with fresh washing solution, the washing manipulation was conducted with 200 μl of 70% (v/v) ethanol solution. To elute DNA in the glass fiber filter, 100 μl of 1/10 TE (1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)) was pipetted by repeating suction and discharge for 10 times. The solution yielded was used as a DNA template solution for PCR reactions (Figure 2).

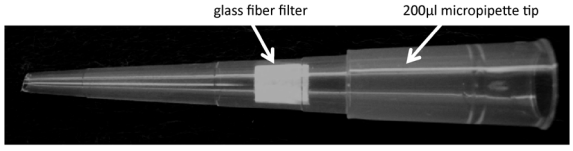

Figure 1. The "filter-inserted tip" used in this study.

Based on the manipulations described above, the effect of the addition of PVPP to the crushing solution

1) Dispense each solutions into 96-well micro plates prior to DNA extraction: fixing solution (100µl/well), washing solution (200µl/well x 5), 70% (v/v) ethanol (100µl/well) and 1/10 TE (100µl/well) per a sample.

↓

2) Crush 20mg of strawberry leaf with 200µl of crushing solution using a multi-sample homogenizer for 45 s.

↓

3) Incubate the samples at 65°C for 10min. Then, centrifuge at 12,000 rpm for 3min.

↓

4) Pipette 7µl of supernatant with a 200µl micropipette tip. Insert the tip into the "filter-inserted tip" from the side of mounting the pipette, and adsorb the supernatant to the glass filter paper.

↓

5) Attach the "filter-inserted tips" to a multichannel micropipette.

↓

6) Pipette the fixing solution in the 96-well micro plate with the "filter-inserted tips" and eject the solution into the well again. Keep the tips at room temperature for one min.

↓

7) Pipette the washing solution 10 times in a well for washing. Repeat the set of washing manipulation four times more with fresh washing solution.

↓

8) Pipette 70% (v/v) ethanol solution 10 times in a well.

↓

9) Pipette 1/10 TE 10 times in a well to elute DNA on the glass fiber filter. Use the solution yielded as DNA template for PCR reactions.

Figure 2. The flow chart of the protocol.

on PCR amplifications were evaluated using the primers PGP-FwA, PGP-RvA(N), CYT-Fw(N) and CYT-Rv. PCR experiments were performed according to the published manual (NARO Institute of Vegetable and Tea Science 2007) by using GeneAmp PCR System 9700 (PE Applied Biosystems, Calif., USA) . Electrophoresis was performed with 3% (w/v) agarose gels.

The results obtained by the method developed in the present study were compared with those obtained by the method using a commercially available kit (DNeasy Plant Mini Kit, Qiagen, Valencia, CA, USA, hereafter "DNeasy") in terms of success ratio of amplification, the time frame required for the manipulations, and easiness of the manipulations. In the "filter-inserted tip method", 50 mg PVPP was added to 1 ml crushing solution. The conditions for PCR reactions were the same as described above.

To evaluate the reproducibility of the method developed in the present study, the success ratio of amplification was evaluated with DNAs extracted from 144 strawberry leaf samples. Condition of PCR was same as described above. Two primer pairs for strawberry simple sequence repeat (SSR) marker FVES1038 and FVES2619 (Isobe et al. 2013) were tested along with the two primer pairs described above. The Reaction mixture (reaction volume: 15 μl) contained 3 μl DNA solution, 0.11 μl Paq5000 DNA polymerase (Agilent Technologies, Tokyo, Japan), 1.5 μl 10X PCR buffer, 1.2 μl 2.5 mM dNTPs, and 0.6 μl each of primers (10 μM). PCR was performed with the following program: 94°C for 10 min; 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 7 min.

Amplification was observed in all samples when PVPP was added the crushing solution(100 mg/ml), while no amplification was observed without PVPP suggesting PVPP improve greatly the result of PCR (Figure 3).

We furthermore tested the different concentration of PVPP in crushing solution (30, 50 or 100 mg/ml). Result of DNA was most stable with the DNAs extracted crushing solution containing 50 mg/ml PVPP. In the case of using a DNA solution with 30 or 100 mg/ml PVPP, the result of PCR was less stable, sometimes gave no PCR products. Therefore, in the subsequent experiments, we used a crushing solution with the addition of 50 mg/ml PVPP (Figure 4).

For 144 samples of strawberry leaves, DNA extractions and PCR reactions were conducted with the"filter-inserted tip method". The success rate of the PCR reactions were more than 90% in the all tested primer pairs. Therefore, this method was considered to have sufficient reproducibility for use in routine PCR experiments.

For 24 samples of strawberry leaves, DNA extraction and PCR reactions were conducted with both the"filter inserted tip method" and "DNeasy". The success rate of the PCR reactions were equivalent in the both methods (Table 1).

Approximately 70 min was taken for DNA extraction for 24 samples using the "filter inserted tip method", whereas over 180 min was required for "DNeasy", including 60 min for sample grinding with a mortar and pestles.

In terms of operational efficiency, "DNeasy" involved relatively complicated manipulations such as the transfer of liquid between tubes, opening and closing of the tube lids and the loading/unloading of tubes to the centrifuge. In contrast, manipulation of the "filter-inserted tip

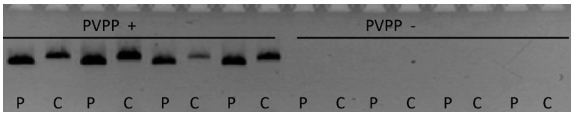

Figure 3. Effect of the addition of the polyvinylpolypyrrolidone to the crushing solution on PCR amplification using the extracted DNA as a template. The polyvinylpolypyrrolidone was added to the crushing solution (lanes 1–8) or not added (lanes 9–16). The primers PGP-FwA and PGP-RvA(N) (lanes 1, 3, 5, 7, 9, 11, 13 and 15), CYT-Fw(N) and CYT-Rv (lanes 2, 4, 6, 8, 10, 12, 14 and 16), were used for evaluation.

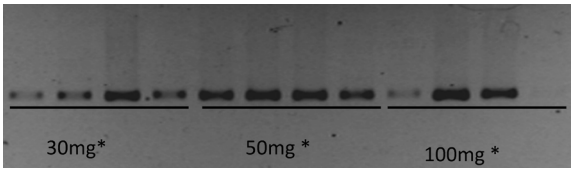

Figure 4. Effect of the addition amount of the polyvinylpolypyrrolidone to the crushing solution on PCR amplification using the extracted DNA as a template. * The numbers indicate the amount of PVPP per crushing solution 1 ml. The primers PGP-FwA and PGP-RvA(N) were used for evaluation.

Table 1. A comparison of the reproducibility in "the filter-inserted tip method" and "DNeasy".

	Number of samples	PGPA-RSAI(N)		CYT-BsaBI(N)		FVES1038		FVES2619	
		Number of samples PCR reactions succeeded	Success rate of PCR (%)	Number of samples PCR reactions succeeded	Success rate of PCR (%)	Number of samples PCR reactions succeeded	Success rate of PCR (%)	Number of samples PCR reactions succeeded	Success rate of PCR (%)
The filter-inserted tip method	144	139	97	140	97	132	92	139	97
The filter-inserted tip method	24	23	96	23	96				
DNeasy	24	23	96	23	96				

method" was simple, did not require the centrifuge operation except for the collection of the crushed samples at the bottom of microtubes. Furthermore, the solutions for the extraction, i.e., fixing and washing solutions, can be prepared in microplates beforehand in the "filter-inserted tip method". This allows us to handle multiple samples at once with a relatively simple manipulation.

With the results described above, the "filter-inserted tip method" ensured faster DNA extractions and simplified manipulations. The obtained DNA samples were revealed to produce excellent PCR amplifications in our PCR condition.

In addition, the necessary equipments and necessary small items were inexpensive and easily obtained. We used a multi-sample homogenizer for grinding samples, however, it can be replaced by another equipment depending on the situation in the laboratory. Therefore, we concluded that this method was cost effective, even in laboratories, which have no expensive equipment. It was considered that this method can be applied to the marker-assisted selection, cultivar identifications and the examination of closely-related relationships between cultivar lines.

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