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Technical Note

Rapid and simple DNA extraction method from rice using a glass-fiber filter inserted pipette tip

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Abstract A novel and simple DNA extraction method was developed, which was called "the filter-inserted tip method". This method used glass fiber filters inserted within micropipette tips. It was conducted by drawing and expelling fluid by pipetting using a multichannel pipette. This allowed us to manipulate a large number of samples at the same time for DNA adsorption, fixing, washing and extraction. The 3 mm×3 mm size of glass fiber filter was suitable for this method. To examine the reproducibility of the method, DNA extraction experiment was carried out using rice leaves, and the yielded DNA samples were used for PCR reactions. As a result, PCR amplifications were recognized for 234 out of 236 samples examined, showing high reproducibility of the method. It took approximately 20 min to conduct DNA extractions from 16 samples. These 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-assist selection.

In recent years, DNA discrimination techniques have been applied to various aspects of plant breeding such as marker-assisted selection using DNA markers, cultivar identifications of the cultivars for the protection of the breeding rights and for the prevention from the contamination of undesirable cultivars, and the examination of the relationships between closely-related cultivar lines. 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 a part of the 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 act inhibitorily for the PCR reactions. This means that it is essential to remove these substances as much as possible. 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.

Monna et al. (2002) employed a simple-rapid DNA extraction method with alcohol precipitation for crushed liquid samples to determine a genotype of DNA marker for sd-1 gene in rice. This method was adopted, with some modifications, to determine DNA markers for rice blast resistance genes at the Piz locus (Hayashi et al. 2004). In addition, 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 lkeda 2002) and glass fiber filters (Muramoto 2005) http://www6.ipdl.inpit.go.jp/Tokujitu/tjbansakuk.ipdl. Also some other simple-and-easy DNA extraction methods have also been published (Zhu et al. 1993; Liu et al. 1995; Ikeda et al. 2000; Mori et al. 2003). These methods were expected to enable simple and low cost DNA extraction. In the present study, we also developed a new DNA extraction method based on that of Muramoto et al. (2003) (referred to as "the original method" thereafter). Our 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 a large number of samples at the same time for genomic DNA adsorption, washing and elution (referred to as "the filter-inserted tip method" thereafter). Using this method, some manipulations required in "the original method" such as opening and closing of the tube lids, and centrifugal separation could be omitted. Furthermore, many samples

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could be processed at the same time by using a multichannel pipette, that enables easy and rapid DNA extractions.

Following the same procedures to "the original method", glass fiber filters (GF/A, Whatman Co., Ltd.) were soaked in sodium sulfite solution (20 mM Tris-HCl (pH 8.0), 2 mM EDTA-Na_2, 10% sodium 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.). In addition, the tip of the tips was cut off for approximately 3 mm to prevent from blockage by plant tissue fragments involved in the crude sap of the samples.

Since this study is aiming at handling a large number of samples, the fixing solution, washing solution, 70% ethanol and 1/10 TE were initially dispensed into 96-well flat bottom micro plate (BM Equipment Co., Ltd.). Multiple sample solutions were then applied to the wells at once using a multichannel pipette (N59727, Finnpipette, LabSystems). These samples were subsequently treated with repeated pipetting for the adsorptions to the glass fiber, fixing, washing and elution of DNAs.

After the preparation of the tips (referred to as "the filter-inserted tip" thereafter) described above, the following manipulations were carried out. Approximately 20 mg of rice leaf segment was crushed with 100 μl of crushing solution (20 mM Tris-HCl (pH 8.0), 2 mM EDTA-Na_2) using a multi-sample homogenizer (Multibeads shocker, model MB301, Yasui Kikai Co., Ltd) at 2,000 rpm for 6 s. Then the samples were centrifuged at 5,000 rpm for 20 s, in order to collect the sap liquid at the bottom of the tube. The collected sap was sucked with "the filter-inserted tip" to adsorb DNA into the inserted glass fiber filter. One hundred microliter of fixing solution (100 mM Tris-HCl (pH 8.0), 10 mM EDTA-Na_2, 7 M guanidine hydrochloride) was pipetted 3 times using the pipette with the filter-inserted tip. The tip was then kept at room temperature for 5 min or 1 min.

The filter-inserted tips with adsorbed DNA were then attached to a multichannel pipette, and subjected to washing with 150 μl of washing solution (50 mM Tris-HCl (pH 8.0), 5 mM EDTA-Na_2, 200 mM sodium chloride, 60% ethanol) by repeating the pipetting 10 times. After repeating this washing manipulation once again with fresh washing solution, the same manipulation was conducted with 150 μl of 70% ethanol solution. The tip was then treated with 100 μl of 1/10 TE (1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)) by repeated pipetting for 10 times to elute DNA from the glass fiber filter. The solution yielded was used as a DNA template solution for PCR reactions.

Based on the manipulations described above, the effects of the fixing time and the size of glass fiber filter on PCR amplifications were evaluated using the DNA marker G144 (RGP; http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html). For PCR, each reaction mixture (reaction volume: 15 μl) contained 3 μl DNA solution, 0.5 U of Hot Start Taq polymerase (Takara, Osaka, Japan), 1.5 μl 10× PCR buffer, 1.2 μl 2.5 mM dNTPs, and 0.38 μl each of primers (10 μM). Three primers, G144S (5′-TGCATGCATGCAATCCCCGC-3′), G144R (5′-ATCATTACCGCCAAAAAACG-3′) and G144Fow (5′-TCGATCGCCTTTTCTACGCA-3′), were used to amplify the DNA fragment. The amplification scheme consisted of 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. The PCR reactions were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems, Calif., USA) and PCR products were visualized by gel electrophoresis on 2% (w/v) agarose.

To evaluate the effect of the length of fixing time on the PCR amplifications, the period at room temperature after soaking the filter in the fixing solution was defined either for 5 min or 1 min. The experiment was repeated 4 times.

For the experiment to evaluate the effect of the size of glass fiber paper on the PCR amplifications, two different size, 3 mm×3 mm and 9 mm×6 mm, of inserting filters were prepared with the fixing time of 1 min. The experiment was repeated 4 times.

The results obtained by the method developed in the present study were compared with those obtained by the method of Monna et al. (2002), with modifications by Hayashi et al. (2004) (referred to as "the Monna method" thereafter) with special reference to the frequency of adequate PCR amplifications, the timeframe required for the manipulations, and easiness of the manipulations. In "the filter-inserted tip method", the fixing time was adjusted to 1 min and the size of the glass fiber filter used was 3 mm×3 mm. The conditions for PCR reactions were the same as described above.

With respect to the fixing manipulation, no differences were recognized in the PCR amplification between 1 and 5 min of fixing time (Figure 1). Thus, the fixing time was set at 1 min in the following experiments to shorten the time.

The extract obtained from the 3 mm×3 mm filter had less coloration than that obtained from 9 mm×6mm filter, suggesting that the extract by the 3 mm×3 mm filter had lower impurities (data not shown). The PCR reactions with the use of this extract produced stable, excellent amplifications (Figure 2). On the other hand, the extract obtained from the 9 mm×6 mm filter had light-green coloration and sometimes gave no PCR products, although the frequency of the failure was low. This indicated that the PCR failure was caused by the impurities derived from plant tissues (lane 4 in Figure 2). To avoid such problem, it is necessary to prevent the impurities even if it might sacrifice the yield of

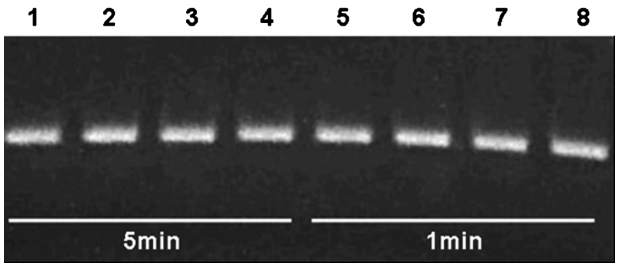

Figure 1. Effect of the length of fixing time for DNA extraction on PCR amplification using the extracted DNA as a template. DNA extraction procedure was conducted with the fixing time of either 5 min (lanes 1-4) or 1 min (lanes 5–8).

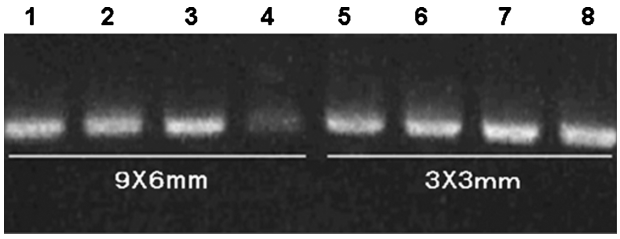

Figure 2. Effect of the size of glass fiber filter for DNA extraction on the PCR amplification using the extracted DNA as a template. The size of glass fiber filter was 9×6 mm (lanes 1–4) or 3×3 mm (lanes 5–8).

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

	Number of samples	Number of samples PCR reactions succeeded	Success rate of PCR (%)
The filter-inserted tip method	236	234	99
The Monna method	236	227	96

DNA since the PCR reaction is highly sensitive. Therefore, the following experiment was performed with the 3 mm×3 mm filter-inserted tip.

For 236 samples of rice leaves, DNA extractions and PCR reactions were conducted with both "the filter-inserted tip method" and "the Monna method". 234 samples processed with the filter-inserted tip and 227 samples processed with "the Monna method" resulted in excellent PCR reactions (Table 1). With these results, it was considered that either method could possibly be performed to give reproducible DNA extraction which was suitable for PCR amplifications.

It required approximately 20 min for treating 16 samples using "the filter-inserted tip method". In contrast, it took over 50 min in "the Monna method", which included the time for the loading/unloading of samples to the centrifuge with 15 min of centrifugation after the mixing of isopropanol, that was recommended by Hayashi et al. (2004).

With respect to the operational efficiency, "the Monna method" 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, "the filter-inserted tip method" did not require the centrifuge operation except for the collection of the crushed sample at the bottom of microtube, suggesting its simplicity. Furthermore, the solution required for the extraction, such as the fixing solution and washing solution, 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 reaction system. The obtained DNA samples also could be used for typing C1186 DNA marker (RGP; http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html) by means of PCR amplification. Therefore, the quality of the obtained DNA samples was thought to be high enough for PCR amplification. In addition, the equipment and necessary small items were inexpensive and easily obtained. The main piece of equipment required was a multi-sample homogenizer, which is, however, replaced by other equipment depending on the situation in the laboratory. Therefore, this method can possibly be performed cost effectively, 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. Actually, our laboratory has adopted this method for the line selection of pest resistance in rice cultivars using DNA markers among 1,000 samples or other larger scale of experiments.

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References

Hayashi K, Hashimoto N, Daigen M, Ashikawa I (2004) Development of PCR-based SNP markers for rice blast resistance genes at the Piz locus. *Theor Appl Genet* 108: 1212–1220

Ikeda N, Yamada T, Kamijima O, Ishii T (2000) Rice wild QTL

analysis. 6. Ultra-simple DNA extraction method for marker-assisted selection using rice microsatellite markers. *Breed Res* 2 (Suppl. 2): 134 (in Japanese)

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J* 8: 457–463

Monna L, Kitazawa N, Yoshino R, Suzuki J, Masuda H, Maehara Y, Tanji M, Sato M, Nasu S, Minobe Y (2002) Positional cloning of rice semidwarfing gene, sd-1: Rice "Green revolution gene" encodes a mutant enzyme involved in gibberellin synthesis. *DNA Res* 9: 11–17

Mori K, Komura K, Hosaka K (2003) DNA marker-assisted selection in potato breeding using an one-min DNA extraction method. *Breed Res* 5 (Suppl. 2): 191 (in Japanese)

Muramoto Y (2005) Nucleic acid isolation method and nucleic acid binding Carrier. *Industrial Property Digital Library* P2005-218321A. 2005-8-18 (in Japanese)

Tanaka J, lkeda S (2002) Rapid and efficient DNA extraction method from various plant species using diatomaceous earth and a spin filter. *Breed Sci* 52: 151–155

Zhu H, Qu F, Zhu LH (1993) Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucl Acids Res* 21: 5279–5280