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Full Length Research Paper

A rapid mini-prep DNA extraction method in rice (*Oryza sativa*)

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DNA extraction is an important step in molecular assays and plays a vital role in obtaining high-resolution results in gel-based systems, particularly in the case of cereals with high content of interfering components in the early steps of DNA extraction. Here a rapid mini-prep DNA extraction method, optimized for rice, which was achieved via some modifications in present DNA extraction methods, especially in first step of cell wall lyses and the use of cheap and frequent chemicals found in every laboratory is presented. Normal quality and quantity was obtained by the method. The PCR based assays also revealed the efficiency of the method. This method is applicable with both dry and fresh samples, does not require liquid nitrogen, and is easy, rapid and applicable in every laboratory.

Key words: PCR, DNA extraction, miniprep, rice.

INTRODUCTION

High quality DNA is required for molecular biological studies of plants. Several DNA extraction procedures for isolating genomic DNA from various plant sources have been described, including the salt extraction method and the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) and its modifications (Huang et al., 2000; Rogers and Bendich, 1985). The need for a rapid and simple procedure is urgent, especially when hundreds of samples need to be analyzed (Kang and Yang, 2004).

Most methods require the use of liquid nitrogen (Sharma et al., 2003) or freeze-drying (lyophilization) (Chang et al., 1993; Sperisen et al., 2000) of tissue for the initial grinding, and these processes are unavailable in many laboratories. In the protocols provided by Maniatis et al. (1982), after grinding the tissues in various extraction buffers, DNA is extracted with phenol-chloroform, or the extract is dialyzed against EDTA and a buffered Tris-HCl solution. After extraction, the aqueous phase is con-centrated, either by ethanol or isopropanol preci-pitation (Aljanabi and Martinez, 1997; Fang et al., 1992), or with microconcentrators (e.g., the Wizard genomic DNA purifi-cation system; Promega, USA). However, these methods are not time efficient for consistently obtaining PCR-quality DNA from cereal plants, since they require that the tissues be ground in liquid nitrogen, followed by precipitation of the DNA pellet in ethanol, washing and drying the pellet, etc.

However, in the protocols provided by Kang et al. (2004) the DNA concentrations from cereal crops (rice and maize) were relatively low. They suggested that this may be because homogenization using a hand-operated homogenizer with a plastic tip is incomplete, since the leaves of these plants are stronger than the leaves of tobacco, potato, cabbage, lettuce, and Siberian ginseng. Most laboratories desire a simple and fast procedure for obtaining plant genomic DNA for PCR, and good-quality DNA for complete enzyme digestion. Therefore, a protocol for extracting genomic DNA from young or old, fresh or dry rice leaves that is applicable to a variety of plants, regardless of the complexity of their genomes is presented. This procedure for extracting genomic DNA for PCR from a small amount (~0.5 cm^2) of leaf tissue is rapid and reliable.

MATERIAL AND METHODS

Plant material

The plant material were from rice (*Oryza sativa*) lines [CMS Neda-A (P1), IR24 (P2), IR28 (P3), Amol2 (P4), IR36 (P5), IR60966 (P6) and Amol1 (P7)] and F_2 progenies from crosses of Neda-A × Amol1. The plants used for genomic DNA extraction were grown in a culture room or greenhouse. The seeds were grown in a con-trolled environment at 25°C on a 16/8 h light/darkness photoperiod

Table 1. Primer sequences used in the study.

Primer name	Primer sequences (5`-3`)	Comments
RM1	F: gcgttggttggacctgac	SSR on Chr.1
	R: gcgaaaacacaatgcaaaaa	
RM171	F: acgagatacgtacgcctttg	SSR on Chr.10
	R: aacgcgaggacacgtacttac	
OPA01	caggcccttc	RAPD
OPC02	gtgaggcgtc	RAPD
OPC05	gatgaccgcc	RAPD
SPRF03	F: gaattcaaatccatcaaacataggttct	STS; in A110443
	R: gaattctattggtgtgaagcccaatgtc	
SPRF04	F: gaattccgtataagacaaactgcgttgc	STS; in AB110443
	R: gatccctcctctaataggactgtaggaga	

or were grown in a green house for genomic DNA extraction.

DNA extraction

Two different variations of the rice genomic DNA extraction procedure were tested. About 30 mg of finely powdered leaf samples (for the case of dry samples) or ~0.5 cm^2 of culture room- or greenhouse-grown plant leaves were placed in a 1.5-ml microfuge tube. The leaf tissue was homogenized in 100 µl DNA extraction buffer (for dry leaf samples: 2% CTAB, 1.5 M NaCl, 100 mM Tris-HCl pH 8., and 20 mM EDTA pH 8.0; for fresh leaf samples: 1% CTAB, 700 mM NaCl, 10 mM Tris-HCl pH 8., and 50 mM EDTA pH 8.0), using a hand-operated homogenizer with a plastic pestle, for 30-40 s. After an initial homogenization, 350 µl of DNA extraction buffer (pre-warmed up to 65°C and addition of 38 mg/ml sodium bi-sulphite just before the use) were added and vortexed for 60 s. The samples were incubated at 65°C for 20 min for cell lysis. Protocol 1: 0.7 volume of chloroform/isoamyl alcohol (24:1) was added to the samples, mixed by hand for 5 min, and then centrifuged at 14,000 g for 5 min at 4°C. The supernatant was transferred to a fresh tube and extracted another time with 0.7 volume of cold isopropanol alchol. The pellet was dried, and re-suspended in sterile dH_2O containing 20 µg/ml RNase A. Protocol 2: 500 µl of 70% ethanol was added to the pellet obtained at final stage of protocol 1, micro-centrifuged for 1 min and the pellet was dried, and re-suspended in sterile dH_2O containing 20 µg/ml RNase A. The concentration and purity were determined on 1% agarose gels. Five micrograms of each genomic DNA sample were incubated at 37°C for 3 h for complete digestion with 20 U of *Eco*RI (Sibenzyme, Moscow) in a total volume of 50 µl and analyzed on 1.0% agarose gels using 10 µl aliquots of the reaction mixture.

PCR amplifications

By using the genomic DNA isolated from the fresh young leaves (14-day old) or old fresh and dry leaves (at flowering stage) of rice lines or F_2 progenies from cross of Neda-A × Amol1, PCR amplifications were performed in a total volume of 20 µl containing 1× PCR buffer, 0.2 mM dNTP, 10 pmol of each primer (primers in Table 1, exception for RAPD primers), 50 ng template DNA from plants, and 0.25 U Taq DNA polymerase (Sibenzyme, Moscow) using the following profile: a 1 min denaturation at 94°C and 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and a 2 min extension at 72°C, followed by a final extension at 72°C for 7 min. The PCR products were resolved by electrophoresis in 1.5% agarose gels.

In RAPD analysis PCR was performed in 20 µl volumes containing 2 µmol/l of primer, 200 µmol/l dNTPs, 50 mmol/l KCl, 10 mmol/l Tris-HCl, 1.5 mmol/$MgCl_2$, and 1.5 unit of Taq DNA polymerase. The PCR profile was 94°C for 2 min (denaturation), followed by 35 cycles of 94°C for 30 s, 36°C for 45 s, 72°C for 1 min, and finally 72°C for 7 min in the final extension. The products from PCR reaction were resolved by electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bromide.

RESULTS

Here is described a simple and reproducible procedure for PCR amplification of rice DNA, that also is applicable for other plant genomes. Two different variations of the genomic DNA extraction protocol for PCR analysis were compared. The primary mechanical break down via simple homogenization of fresh or dry plant leaf samples was carried out with DNA extraction buffer using a hand-operated homogenizer instead of liquid nitrogen. Then, genomic DNA was extracted as mentioned in “Material and Methods” by protocol 1 and 2.

Genomic DNA from different lines was electrophoresed on 1% agarose gels, and high-molecular-weight DNA was obtained by two protocols (Figure 1A; lanes 2-4 for protocol 1, and lanes 5-8 for protocol 2). When the genomic DNA was digested with *Eco*RI, the DNA extracted by two protocols 1 and 2 was completely digested (Figure 1B), and hence could be used for Southern blot analysis. Therefore, the purity and quality of the genomic DNA is sufficient for enzyme digestion.

DNA samples prepared using the two different extraction procedures (lanes 2-8 in Figure 1A) were subjected to PCR amplification using different primers: RM171 on chromosome 10 and RM1 on chromosome 1 (both SSR rice markers). All the genomic DNA samples produced a clear, sharp, and reproducible PCR product when the primers were used for PCR amplification (Figure 2A). Although two variations of the DNA extraction procedure

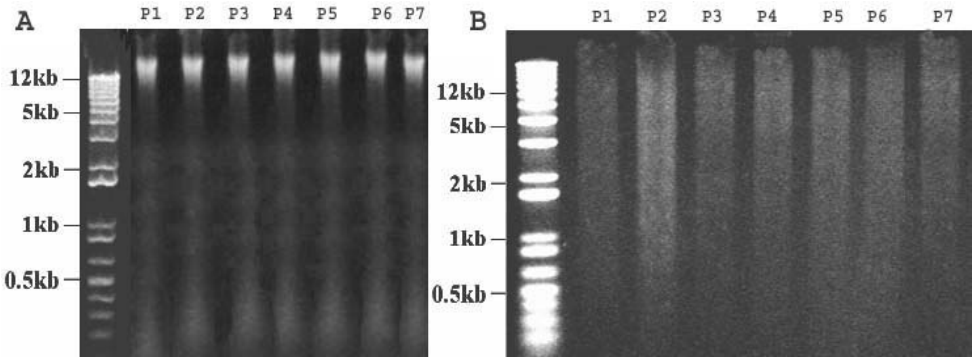

Figure 1. Total genomic DNA extracted by protocol 1 (lanes 2-4) and by protocol 2 (lanes 5-8) from seven rice lines (A) and then digested with *Eco*RI (B). P1: CMS Neda-A, P2: IR24, P3: IR28, P4: Amol2, P5: IR36, P6: IR60966 and P7: Amol1.

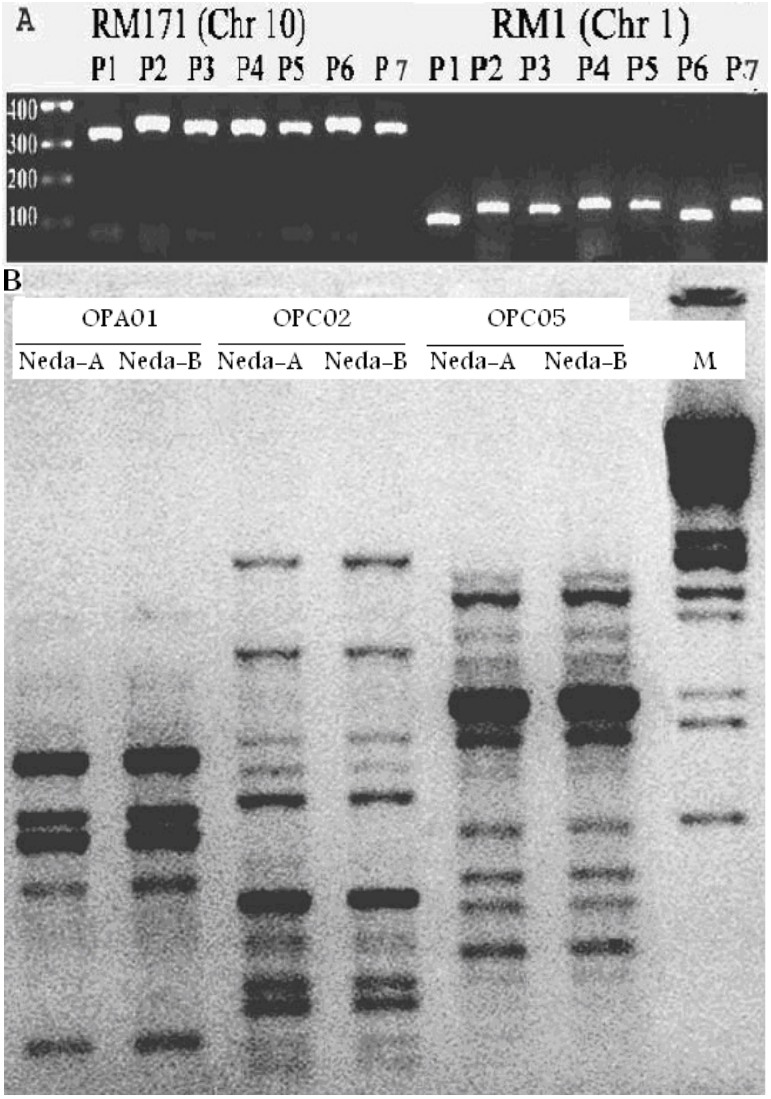

Figure 2. (**A**) PCR amplification of total genomic DNA extracted by protocol 1 by RM171 on chromosome 10 and RM1 on chromosome 1. P1: CMS Neda-A, P2: IR24, P3: IR28, P4: Amol2, P5: IR36, P6: IR60966 and P7: Amol1; (**B**) PCR amplification of total genomic DNA extracted by protocol 1 using RAPD primers, OPA01, OPC02 and OPC05.

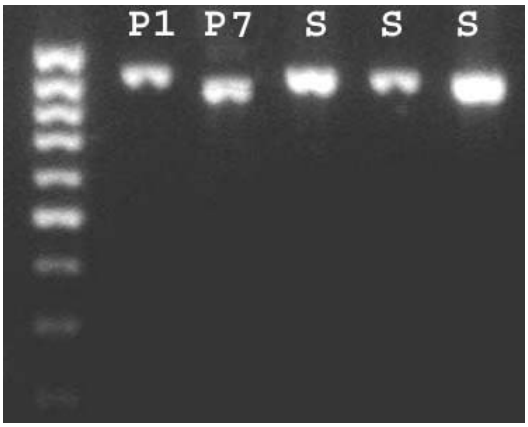

Figure 3. PCR products of primer combination SPRF03-F/SPRF04-R. P1: CMS Neda-A; P7: Amol1; S: F_2 sterile progenies of a cross between Neda A and Amol1.

were used, there was no difference between lanes of two protocols. This result suggests that the pellet after the first chloroform treatment (protocol 1) was sufficiently pure to be used as the DNA template for PCR amplification. Therefore, PCR amplification with another RAPD primers, OPA01, OPC02 and OPC05, was performed using the DNA template extracted using the first protocol (Figure 2B).

The PCR amplification was successful, and the same banding pattern was seen when the PCR amplification (production of a relatively large DNA fragments up to 3000 bp) was repeated. Therefore, the DNA template extracted using the first method was sufficient for amplification of relatively large DNA fragments, and it was used as the DNA template to amplify specific DNA from rice plants.

To examine the presence of *Rf1A* gene (Akagi et al., 2004) in the rice genomic DNA, two different lines (one sterile line, Neda-A, and a fertility restorer line, Amol1) and some their F_2 sterile progenies were screened by PCR analysis (Figure 3). PCR amplification using primer combinations SPRF03-F/SPRF04-R resulted in ~940-, and ~890-bp fragments, respectively. The primer produced fragments containing *Rf1A* gene, which confirmed the occurrence of the gene in the lines used. A polymorphic product was produced using genomic DNA from sterile and fertility restorer plant with primer combination (Figure 3), indicating existence of some differences between CMS and fertile lines in the region. Relatively large DNA fragments were amplified, although liquid nitrogen was not used, but simply used a hand-operated homogenizer with a plastic tip.

DISCUSSION

There are many advantages in using this genomic DNA extraction method to obtain template for PCR amplification. Many different cereal plants could be amplified using the same DNA extraction method and the same PCR protocol. Using this protocol, DNA was successfully amplified repeatedly from all examined lines. Since this method does not require liquid nitrogen, expensive commercial DNA extraction kits, or ethanol precipitation to produce DNA template for PCR, considerable time and expense can be saved. The time required for this DNA extraction method is less than 60 min, which is extraordinary compared with other genomic DNA extraction methods (Doyle and Doyle, 1987; Steiner et al., 1995; Kang et al., 1998). With this procedure, a very small sample is required for DNA extraction. There is no sample waste with this method, whereas much larger samples are required when plant samples are ground in a mortar and pestle with liquid nitrogen and transferred to a tube. Previously reported techniques require several steps (Steiner et al., 1995), use of expensive enzymes such as proteinase K (Kang et al., 1998), or beads and shakers (Dilworth and Frey, 2000). Although in protocol developed by Burr et al. (2001) for one-step plant DNA isolation, if plant material more than 1 mm^2 was used in the extraction, co-extracts (e.g., chlorophyll) were extracted alongside the DNA and inhibited the PCR. On the contrary, this protocol does require appropriate sample size to extract DNA. Warner et al. (2001) also reported a rapid DNA extraction method in barley, which requires NaOH. However, the extracted DNA samples were easily degraded. The DNA samples extracted by this protocol were very stable and could be stored in 4^{o}C for two years without degradation.

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