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A Simplified Rice DNA Extraction Protocol for PCR Analysis

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Abstract: A simple protocol was established for DNA extraction using etiolated rice seedlings, whereby rice DNA was directly extracted in 0.5 mol/L NaOH solution in a single eppendorf tube. Results of comparative PCR analyses and electrophoresis showed that the DNA extracted using this method was as good and useful as that using standard CTAB method.

Key words: DNA extraction; rice; polymerase chain reaction; molecular marker; simple sequence repeats; transgene

DNA molecular marker technology has been advancing rapidly during past decades and its application perspective seems very brilliant in crop breeding, varietal purity test of crop seeds and germplasm fingerprinting [1]. Among various DNA markers, the PCR-based marker is the most popular and efficient one since it needs small amounts of DNA with relative low quality [2]. Meanwhile, PCR is also an essential approach for detection of target genes in transgenic breeding and genetic modified (GM) food detection. Since the DNA extraction method was first established by Marmur in 1961 using SDS and chloroform, scientists have been devoting themselves to the improvement and simplification of DNA extraction methods and many kits for DNA isolation are now commercially available on the market [3-5]. However, commercial DNA kits, though they are very reliable and user-friendly, are too expensive to be the choice of researchers working on applied sciences, e.g., plant breeding in developing countries. On the other hand, most improved or simplified DNA protocols are still very tedious and time-consuming when applied to large populations in rice breeding programs. Therefore, DNA extraction has become a bottleneck for large-scale applications of DNA markers and transgenic detection [9]. Based on previous reports [10-11], we further simplified the process of DNA extraction, and a protocol for rice DNA isolation is reported here.

MATERIALS AND METHODS

Materials

Seeds of the following conventional and hybrid rice varieties were used in this study: indica hybrid rice Xieyou 92 (Xieqingzao/Hui 92) F_1 and Xieyou 46 (Xieqingzao A/Milyang 46) F_1, the cytoplasmic male sterile line Xieqingzao A, the restorer line Milyang 46, and the transgenic japonica rice KMD1 (with a *cry1Ab* gene) and its control variety Xiushui 11.

DNA extraction

DNA was extracted using a modified CTAB method [12] and a simplified protocol, which was developed in this study. Seeds were immersed at 28-30˚C. After germination for 6 days in dark, the etiolated seedlings of about 2-3 cm in height were collected for DNA isolation. Our simplified protocol included the following steps: (1) 10-20 mg etiolated seedlings were cut and put into a 1.5-mL eppendorf tube and homogenized using a glass pestle in 200 μL of 0.5 mol/L NaOH; (2) 200 μL of 1 mol/L Tris-HCl (pH=8.0) was added into the tube and mixed well; (3) after centrifugation for 10 min at 12 000 r/min, the supernatant was used directly for PCR analysis.

PCR and products analysis

PCR amplification of DNA fragments of the *cry1Ab* gene in transgenic rice and of the microsatellite within the *Wx* gene in hybrid rice were conducted using the DNA templates isolated by modified CTAB method and our own simplified protocol. The sequences of forward primer and reverse primer for the *cry1Ab* gene were 5'-TTCCTTGGACGAAATCCCACC-3' and 5'-GCCAGAATTGAACACATGAGCGC-3', targeting a fragment of 559 bp. The forward primer of SSR of *Wx* gene was 5'-CTTTGTCTATCTCAAGACAC-3' and the reverse primer 5'-TTGCAGATGTTCTTCCTGATG-3', which were designed according to Blight et al [14]; the forward primer was labeled with fluorescent Carboxytetramethylrhodamine when the PCR product was analyzed in capillary electrophoresis. The final reaction volume was 20 μL, containing 1 μL template DNA, 2.0 μL 10×PCR buffer, 0.3 μL 10 mmol/L dNTPs, 1.6 μL 25 mmol/L $MgCl_2$, 1.0 μL 4.0 μmol/L primer (0.4 μmol/L for labeled primer), 0.3 U *Taq* DNA polymerase and ddH_2O. The PCR was carried out using a PCR Express PX2 (Hybaid). For amplification of the *cry1Ab* gene, DNA was pre-denatured for 4 min at 94˚C, followed by 30 cycles of reaction: 94˚C for 30 s,

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54˚C for 60 s and 72˚C for 90 s with a final extension for 7 min at 72˚C. For the amplification of SSR of *Wx* gene, it was done as follows: a pre-denature at 94˚C for 4 min, followed by 35 cycles of 94˚C for 30 s, 55˚C for 60 s and 72˚C for 45 s; and finally at 72˚C for 7 min. The reaction without adding template DNA was set up as a negative control for PCR analysis every time.

PCR products were separated in agarose gel electrophoresis or capillary electrophoresis. In agarose gel electrophoresis, the product amplified from *cry1Ab* gene and the SSR from *Wx* gene were separated by electrophoresis on 1.5% agarose gel for 1.5 h at 70 voltage and on 3% agrose gel for 2 h at 90 voltage in TAE buffer, respectively. A 100 bp DNA Ladder Plus (MBI) was used as the standard marker. The gel was stained in EB solution and visualized using Gel Doc-1000 image system (BIO-RAD).

For capillary electrophoresis, the PCR products were purified and denatured before loading. The amplifications in tubes were participated at 4℃ for 30 min by adding 1/10 volume of 7.5 mol/L NH_4OAc and 2.5 times volume of ethanol. After centrifugation at 12 000 r/min for 30 min, the pellets were washed two times with 70% ethanol, dried in air and dissolved in 3 μL of ddH_2O. Then 0.5 μL of purified DNA solution was mixed well with 4.5 μL loading buffer, denatured at 95℃ for 2 min and then cooled immediately on ice. Capillary gel preparation, pre-electrophoresis, sample loading and electrophoresis were carried out according to the operation protocol for Mega BACE1000 Sequencer (Amersham Bio Sciences) and the data were automatically collected by the equipment.

RESULTS

Detection of the transgene

A DNA fragment of the transgene *cry1Ab*, with the expected size, was amplified through PCR using the DNA extracted by both the modified CTAB method and our simplified method (Fig. 1). The results indicated that the DNA isolated using our protocol could be used as DNA template for PCR analysis of transgenes in transgenic plants.

SSR amplification

The DNA isolated using the two methods were further used for the amplification of the SSR of the *Wx* gene. Results showed that the SSR fragment of *Wx* gene was amplified using DNA extracted by our simplified method and the modified CTAB method. A single fragment was amplified from each parent, while mixed bands from the hybrids (Fig. 2). Capillary electrophoresis also showed that it was useful for documenting the amplified fragments from hybrid rice Xieyou 46, where two

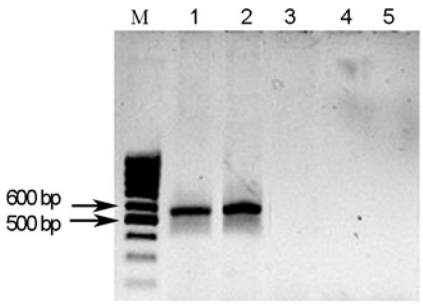

Fig. 1. Electropherogram of PCR products of *cry1Ab* fragment.

Lane M, Marker for molecular weight; Lanes 1 and 3, KMD1 and Xiushui 11 amplified from DNA extracted by the simplified method; Lanes 2 and 4, KMD1 and Xiushui 11 amplified from DNA extracted by CTAB method; Lane 5, Blank control.

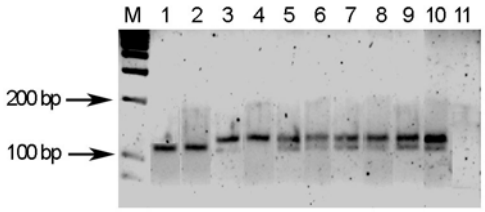

Fig. 2. SSR marker electropherogram of *Wx* gene in rice.

Lane M, Marker for molecular weight; Lanes 1, 3, 5 and 7, Xieqingzao A, Milyang 46, Xieyou 46 (F_1) and Xieyou 92 (F_1) DNA extracted by the simplified method, respectively; Lanes 2, 4, 6 and 8, Xieqingzao A, Milyang 46, Xieyou 46 (F_1) and Xieyou 92 (F_1) DNA extracted by CTAB method, respectively; Lanes 9 and 10, Xieyou 46, DNA stored at -20℃ for 60 days after being extracted by the simplified and CTAB methods, respectively; Lane 11, Blank control.

peaks appeared in capillary electropherogram (Fig. 3), which implied that the DNA extracted using our simplified protocol could also be used for PCR and subsequent analysis using capillary electrophoresis.

DISCUSSION

DNA markers are of great potential for marker assisted selection, germplasm characterization and gene mapping because of its advantages over other genetic markers [1, 13, 16] During the past decade, significant progress has been made in DNA marker development, and consequently, more and more abundant and accurate DNA markers are now available for various applications. However, the application of DNA markers in applied agricultural research, especially in plant breeding, is still limited because of cost and effectiveness [17-18]. With regard to the whole process of marker development, a simple DNA extraction method has become the critical step that needs special attention, because the detection and analysis steps are

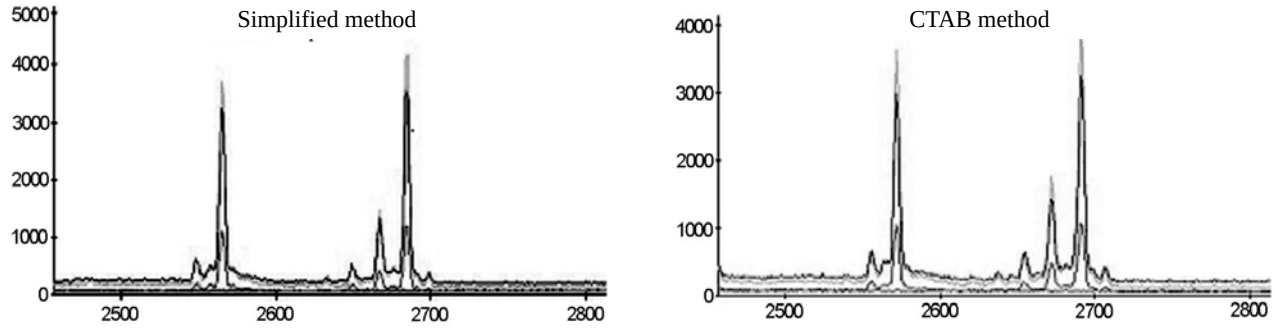

Fig. 3. Capillary electropherogram of SSR *Wx* in hybrid rice Xieyou 46 from DNA extracted by the simplified method and CTAB method.

now relatively efficient and cost-effective.

Although various conventional DNA extraction protocols are now available, the process is still tedious, time-consuming and needs toxic regents [7-8], e.g. CTAB and chloroform are needed for CTAB method. Simplification of DNA extraction has been a major subject and as a result various methods are now available including commercial kits. In rice, the protocol reported by Zhang et al [11] and Wang et al [19] were regarded as the most simplified and rapid ones. Compared with the traditional methods, the simplified protocols or kits are convenient or efficient to some degrees, but further improvement and simplification are still needed. On the principle of alkaline hydrolysis, we developed a very simple protocol, which only involves one tube and two steps, in which the DNA was extracted only by grinding and centrifuging in one tube and could be used as the template in PCR, requiring simple facilities and convenient exercise. By this method, the extraction of DNA becomes very rapid, and DNA extraction could be done for several hundreds of samples per day per person, and thus significantly reduces the cost and time for DNA isolation (Table 1).

Transgenic technology and DNA molecular marker techniques have become the core of molecular breeding, an important research field of plant breeding. Among DNA molecular markers, the SSR marker is considered one of the excellent genetic markers, because of its co-dominant inheritance, relative abundance, high polymorphism, reproducibility, and easy analysis [20-21]. Thus the detection of transgenes and analysis of SSR markers are rapidly becoming important tools in plant variety improvement. We proved that the DNA isolated by our simplified protocol could be used as DNA template for PCR, and subsequent analysis of agrose gel electrophoresis or capillary sequencing electrophoresis. Therefore, the protocol should have wide application in DNA extraction of other populations for other genes, and thus could be useful for high throughput screening of transgenic rice lines, marker assisted selection, rapid variety authenticity test and germplasm characterization.

Table 1. Comparison on cost and efficiency between the simplified method and CTAB.

Item	Simplified method	CTAB method
Time per 20 samples (h)	0.3	5.8-6.0
Total steps of extraction	2	10-11
Waste liquid per 20 samples (mL)	6	33-35
Cost (Yuan/sample)	0.15	0.55

Although the extraction process in our method is very simple, attentions still should be paid to avoid DNA degradation, e.g. rice seedlings should be ground powerfully and quickly, and equal volume Tris-HCl buffer (pH=8) be added immediately after the grinding process in NaOH being completed. Meanwhile, once the supernatant is transferred to a fresh tube and stored at -20˚C, the DNA becomes much stable and can be in good quality for several months.

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