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Short communication

A Simple and Reliable Method for Preparation of Cross-Contamination-Free Plant Genomic DNA for PCR-Based Detection of Transgenes

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A simplified but reliable method was developed for the polymerase chain reaction (PCR)-based detection of genetically modified (GM) plants. The modified CTAB (mCTAB) method enabled us to prepare a high quality of genomic DNA from several hundred plant leaf samples in one day. Using DNA samples prepared from seven dicots and two monocots, approximately 1.75-kb regions spanning 17 S to 25 S ribosomal RNA genes were successfully amplified in a 2X PCR pre-mix containing BLOTTO. Further fidelity assessment of the mCTAB method by PCR analysis with Roundup Ready soybean (RRS) and non-RRS plants showed that the DNA samples prepared alternately from each of two lines were evidently free of cross-contamination. These results demonstrate that the mCTAB method is highly recommended for the rapid detection of transgenes in large numbers of leaf samples from diverse transgenic plants.

Keywords: Genetically Modified Plant, Genomic DNA, Modified CTAB, PCR, Transgene.

Introduction

Since the polymerase chain reaction (PCR) is a potent technique, which has a conspicuous specificity and sensitivity to detect a given sequence with small amounts of nucleic acid, it can be applied extensively to various fields of molecular biology (Lee *et al.*, 1997; Klimkait, 2000). For rapid PCR-based detection of transgenes in putatively transformed plants, a simple DNA extraction method with high reliability and reproducibility is a prerequisite when it is necessary to process a large number of samples within a limited period. However, the true bottleneck of DNA extraction procedures is the grinding step, which is the most laborious and time-consuming step. In addition, undesirable cross-contamination of DNA often occurs between samples when a limited number of grinding tools, such as a mortar and pestle, homogenizer, and laboratory mixer, were reused in order to pulverize many tissue samples. This contamination may cause a significant problem in the detection of GM plants. This is because PCR amplification is liable to yield a considerable quantity of PCR product, even with a trace amount of DNA contaminated in non-GM samples. To date several DNA extraction methods have been developed (Langridge *et al.*, 1991; Steiner *et al.*, 1995; Aljanabi and Martinez, 1997; Kim *et al.*, 1997; Lin *et al.*, 2000) which could allow the rapid isolation of DNA from plant tissues. However, most of these methods have some drawbacks in terms of sample cross-contamination, low throughput of DNA, or high amounts of residual water-soluble carbohydrate polymers and polyphenolic compounds. In this regard, an alternative method is necessary in order to prepare a high quality genomic DNA that is not only free of cross-contamination, but also suitable for many replicate PCR applications.

Here we present a simple method for DNA extraction by the modification of existing hexadecyltrimethylammonium bromide (CTAB) methods (Gill *et al.*, 1991; Stewart *et al.*, 1993) since CTAB had proven satisfactory to remove endogenous polysaccharides in recalcitrant plant materials. Several buffer adjuvants, such as polyvinylpyrrolidone (PVP-40), sodium bisulfide and b-mercaptoethanol, were incorporated into the extraction buffer to enhance the quality of genomic DNA.

Experimental Procedures

Extraction of genomic DNA (mCTAB method) Fresh leaves of greenhouse-grown plants were used for DNA extraction. Samples were prepared using the lid of an Eppendorf tube to pinch out a disc of leaf material into the tube. The leaf tissues in the tubes can be directly used for DNA extraction, or stored at -80°C until use. Liquid nitrogen was poured into each tube and the

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frozen tissue was pulverized with a 1000-µl plastic pipette tip sealed with flame. Each ground sample was mixed well with 300 µl of a modified CTAB (mCTAB) buffer containing 2% (w/v) CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 1% (w/v) PVP-40 (Sigma), 0.5% (w/v) sodium bisulfide, and 1% (v/v) β-mercaptoethanol (add just prior to use). The sample was then incubated at 65°C for 20 min with occasional mixing followed by the addition of 300 µl of chloroform to each sample. The tube was then vortexed for 10 s at maximum speed and spun down for 5 min at 12000 g at room temperature. The upper (DNA containing phase was transferred to a sterile tube) was precipitated for 5 min with an equal volume of isopropanol at room temperature, and centrifuged for 5 min at 12000 g. Afterwards, the pellet was dried on the lid of a waterbath (65°C) and resuspended in a 50 µl sterile 0.1X TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) containing RNase A (50 µg/ml). An A_{260}/A_{280} reading monitored the quality of DNA with a UV spectrophotometer (BioSpec-1601, Shimadzu, Japan). In order to measure the quantitative recovery, the genomic DNA, as well as serially diluted lambda DNA (10 ng to 1 µg) used as a mass references, were separated in 0.8% (w/v) agarose and the quantity was calculated using a CCD camera combined with TotalLab 1D image analysis software (Phoretix, UK).

PCR Analysis We prepared a ready-to-use 2X PCR mix (1 ml) containing 200 µl of 10X PCR buffer (without $MgCl_2$), 200 µl of 25 mM $MgCl_2$, 200 µl of 50% (v/v) sterile glycerol, 160 µl of dNTPs (2.5 mM each), 10 µl of dimethyl sulfoxide (DMSO), 186 µl of sterile distilled water, 20 µl of BLOTTO [10% (w/v) skim milk powder, 0.2% (w/v) NaN_3], 4 µl of 1% (w/v) xylene cyanol FF, and finally 20 µl of *Taq* DNA polymerase (5 units/µl). When it is necessary to use different *Taq* DNA polymerase, its compatible 10X PCR buffer that is provided by the manufacturer can be employed in place of the 10X PCR buffer. BLOTTO was added to the pre-mix to attenuate the PCR inhibition by polyphenols (De Boer *et al.*, 1995) that were potentially coextracted with the genomic DNA. *Taq* polymerase in this pre-mix was stable for at least 3 months when stored at -20°C. Each PCR amplification (20 µl) employed 10 pmol of each primer and 1 µl of DNA template and performed on the GeneAmp® PCR System 9700 (PE Applied Biosystems, Norwalk, USA).

Sequence Analysis All PCR products produced in this study were subcloned into the pGEM-T easy vector (Promega, Madison, USA). DNA sequencing was performed using a ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Norwalk, USA) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, USA).

Spray Analysis After collecting the leaf tissues for DNA extraction, the plants were sprayed with 0.3% (w/v) glyphosate, and grown for another 7 days to monitor the herbicide tolerance.

Results and Discussion

We applied the mCTAB method to extract genomic DNA from young leaves of seven dicot plants, soybean (*Glycine max* Merr.), tomato (*Lycopersicon esculentum* Mill.), perilla (*Perilla frutescens* L.), pepper (*Capsicum annuum* L.), potato (*Solanum tuberosum* L.), cucumber (*Cucumis sativus* L.), Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis*), and two monocot plants, maize (*Zea mays* L.), and rice (*Oryza sativa* L.). Each disc of the leaf tissue samples ranged in size from 6.6 to 19.1 mg, depending on the plant species (Fig. 1). The quality of genomic DNA extracted from a disc of leaf tissue from each plant species was verified by agarose gel electrophoresis, as well as by absorbance spectra. In all cases, single high-molecular weight bands of undegraded genomic DNA were visible (Fig. 1) and the spectrophotometric values of A_{260}/A_{280} were higher than 1.8. This indicates that the resulting DNAs are of high enough quality to be employed for PCR applications or other DNA manipulations. When quantification of genomic DNA was done using serially diluted lambda DNA as mass references, total DNA yields were in a range of 0.715 to 2.410 µg depending on the plant species and fresh weights of the starting materials. Thus,

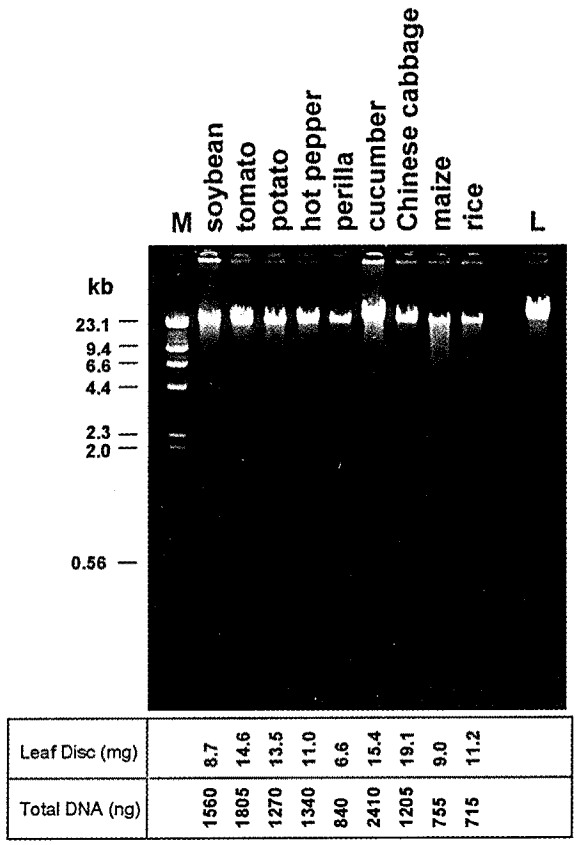

Fig. 1. Agarose gel electrophoresis of DNA extracted from young leaves of diverse plants. Ten microliter aliquot of each preparation (50 µl) was subjected to 0.8% (w/v) agarose gel electrophoresis containing 0.5 µg/ml of ethidium bromide. The weight of a leaf disc and DNA yield was indicated below each lane. Lane M and L indicate a *Hin*dIII-digested and undigested lambda DNA (500 ng), respectively. The amounts of leaf samples and DNA were measured from at least three independent trials.

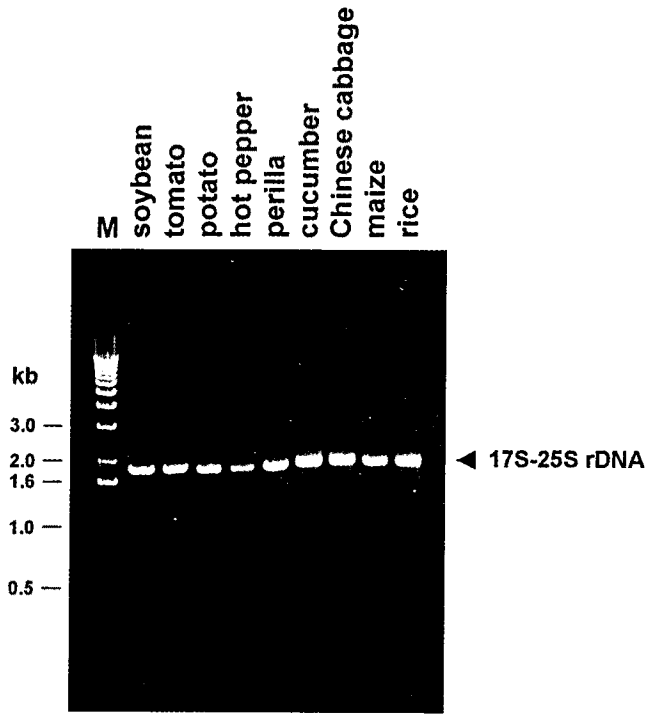

Fig. 2. PCR analysis of ribosomal DNA sequences. PCR products (10 µl each) were analyzed on a 1.0% (w/v) agarose gel containing 0.5 µg/ml of ethidium bromide. The 17S-25S rDNA indicates the PCR products that were amplified from the region spanning 17 S to 25 S ribosomal gene. M indicates 1-kb DNA ladder (Gibco BRL).

quantity in each preparation was sufficient to perform more than 35 typical PCR analyses when 20 ng of DNA is used as a template.

PCR was performed using genomic DNA prepared as above to demonstrate reliability of our extraction method. The primers were designed on the basis of tomato 17 S and 25 S ribosomal RNA genes: 17S-F, 5'-TTCTATGGGTGGTGGTG CAT-3' (at positions 1256 to1275, Genbank X51576) and 25S-R, 5'-CAGACTCCTTGGTCCGTGTT-3' (at positions 655 to 674, Genbank X13557). One microliter of each DNA sample was subjected to PCR analysis (20 µl of total volume) using the 2X PCR pre-mix containing commercially available *Taq* DNA polymerase and 10X PCR buffer (Takara, Japan) under the following conditions: denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min with a final extension step of 72°C for 5 min. We obtained approximately 1.75-kb PCR products for all nine plants used in this study (Fig. 2). This indicates that the mCTAB method and the PCR pre-mix are suitable for PCR applications.

Further experiments were performed to evaluate two crucial parameters of the extraction method: prevention of sample cross-contamination throughout DNA extraction and the constant yields of PCR amplicons among samples. For these purposes we used two parallel lines of soybean varieties: glyphosate-tolerant RRS (AG2702, Monsanto) and glyphosate-sensitive soybean (A3244, Monsanto, referred to as non-RRS). Monsanto Korea Co. kindly provided these. The RRS contains a transgene expressing a modified 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) under the control of a cauliflower mosaic virus (CaMV) 35S promoter, hence the plant should show tolerance to the herbicide glyphosate (Rogers, 1998). To prevent cross-contamination of DNA among samples during the grinding step, we used disposable plastic pipette tips individually to

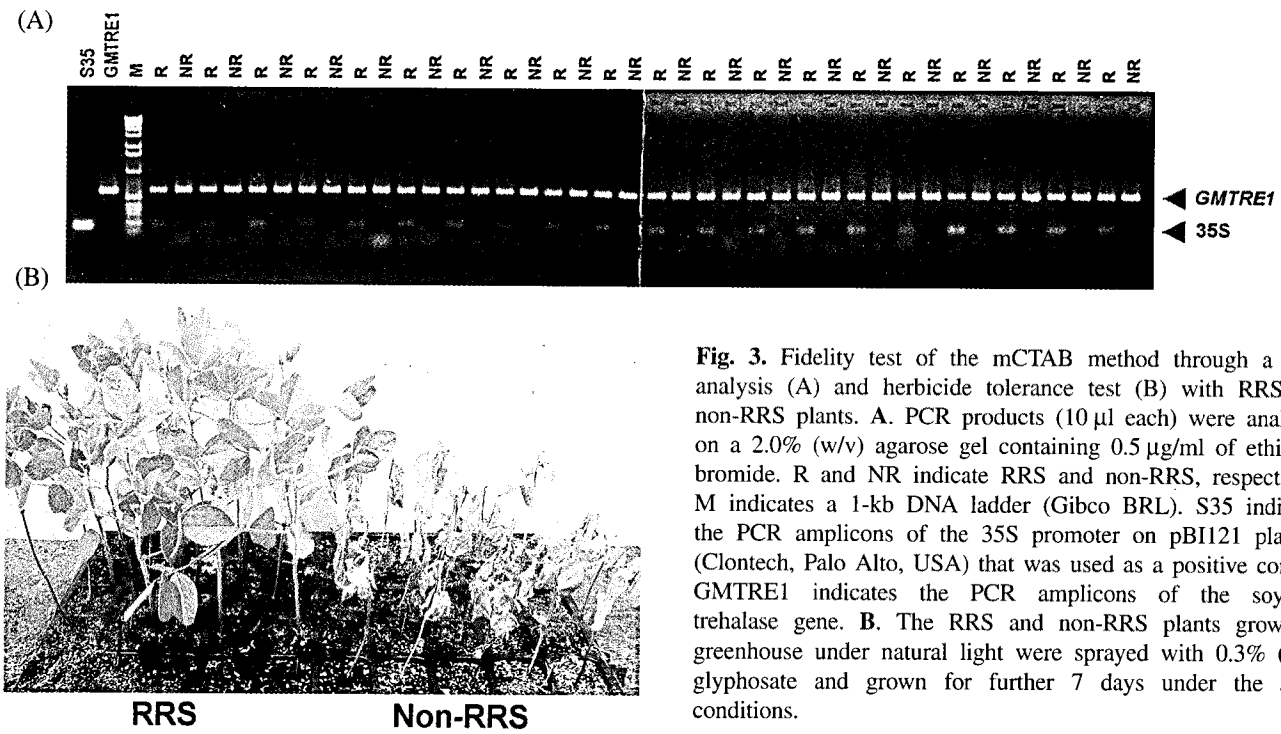

Fig. 3. Fidelity test of the mCTAB method through a PCR analysis (A) and herbicide tolerance test (B) with RRS and non-RRS plants. **A.** PCR products (10 µl each) were analyzed on a 2.0% (w/v) agarose gel containing 0.5 µg/ml of ethidium bromide. R and NR indicate RRS and non-RRS, respectively. M indicates a 1-kb DNA ladder (Gibco BRL). S35 indicates the PCR amplicons of the 35S promoter on pBI121 plasmid (Clontech, Palo Alto, USA) that was used as a positive control. GMTRE1 indicates the PCR amplicons of the soybean trehalase gene. **B.** The RRS and non-RRS plants grown in greenhouse under natural light were sprayed with 0.3% (w/v) glyphosate and grown for further 7 days under the same conditions.

pulverize each leaf disc. Genomic DNA was prepared in an alternating order from each leaf disc of twenty RRS and twenty non-RRS plants. PCR was performed using the 2X PCR pre-mix containing AmpliTaq® Gold polymerase and 10X Gold PCR buffer (PE Biosystems) under the following condition: denaturation/activation at 95°C for 10 min followed by 45 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s with a final extension step of 72°C for 7 min. A pair of primers (Lipp *et al.*, 1999) specific for the CaMV 35S promoter was used: 35S-1, 5'-GCTCCTACAAATGCCATCA-3' and 35S-2, 5'-GATAGTGGGATTGTGCGTCAT-3'. For an internal PCR, an additional pair of primers specific for a single copy gene *GMTRE1* encoding soybean trehalase (Aeschbacher *et al.*, 1999) was employed simultaneously for the same reaction: GMTRE1-F, 5'-GCCGGCTTCGAAATCGCTGTC-3' and GMTRE1-R, 5'-GCGTATGTAAGTCTGGCTGTG-3'. As shown in Fig. 3A, we obtained a single band (0.6-kb of *GMTRE1*) for non-RRS plants. While the expected length of PCR amplicon of the *GMTRE1* gene was 449-bp on the basis of its cDNA sequence (Genbank AF124148), we obtained a 593-bp product that revealed a 144-bp intron by sequence analysis (data not shown). PCR amplification for RRS plants with two pairs of primers also yielded considerable amounts of PCR products (0.6-kb of *GMTRE1* and 0.2-kb of 35S promoter) in all cases. The results of PCR amplification were reproducible for all samples examined and low variations in the amounts of PCR products were attained. In addition, no cross-contamination was detected in any case. This indicates that our method is highly recommended for PCR applications when handling large numbers of samples simultaneously.

In order to validate the fidelity of the PCR results (Fig. 3A), we performed the herbicidal tolerance experiment. The RRS and non-RRS plants used above for PCR analyses were sprayed with 0.3% (w/v) glyphosate. Herbicide tolerance was observed for all RRS plants, whereas all of the non-RRS plants showed susceptibility to the herbicide (Fig. 3B). This indicates that the results of herbicidal tolerance experiments exactly corresponds to the PCR analyses.

In conclusion, the modified CTAB method in this study was developed to avoid the potential cross-contamination of samples by means of disposable plastic pipette tips. In addition, this simplified and quick procedure enabled us to prepare genomic DNA from hundreds of leaf samples in one day with high reproducibility. Furthermore, several adjuvants were employed into the extraction buffer to enhance the quality of genomic DNA by preventing oxidation by polyphenolics and co-precipitation with polysaccharides. The quantity and the quality of the DNA extracted by this method were enough to perform scores of PCR-based reactions. When the sample sizes were increased, our scaleable extraction method yielded enough DNA to be processed for other downstream purposes, including restriction endonuclease digestion, Southern blotting and construction of a genomic DNA library (data not shown). We currently use these procedures to identify transgenes introduced into various plant species; including soybean, maize, perilla, hot pepper, tomato, and potato. We routinely obtained reliable results in every case.

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