Plant Molecular Biology Reporter **22:** 435a–435h, December 2004

Publish by Abstract

An Improved Semiautomated Rapid Method of Extracting Genomic DNA for Molecular Marker Analysis in Cocoa, *Theobroma cacao* L.

RANJANA BHATTACHARJEE*, MARIA KOLESNIKOVA-ALLEN, PETER AIKPOKPODION, SUNDAY TAIWO and IVAN INGELBRECHT
Central Biotechnology Laboratory, International Institute of Tropical Agriculture, Ibadan, PMB 5034, Nigeria

Abstract. DNA extraction is a time-consuming and expensive component of molecular marker analysis, constituting about 30-60% of the total time required for sample processing. Furthermore, the procedure for extracting high-quality DNA from tree species such as cocoa differs from extraction protocols suitable for other crop plants. This is accompanied by problems in collecting leaf tissues from field-grown cocoa trees, where storage facilities are not available and where transporting samples to laboratory for immediate refrigeration is usually impossible. We preserved cocoa leaf tissues in the field in an NaCl-CTAB-azide solution (as described in Rogstad, 1992), which did not require immediate refrigeration. This method also allowed preservation of leaf tissues for a few days during transportation and protected leaf tissues from bacterial and fungal attacks. Once transported to the laboratory, the samples were stored at 4°C for almost 1 y. To isolate good-quality DNA from stored leaf tissues, a rapid semiautomated and relatively high-throughput protocol was established. The procedure followed a modified CTAB/β-mercaptoethanol method of DNA extraction in a 96-well plate, and an automated system (i.e., GenoGrinder 2000) was used to grind the leaf tissues. The quality of DNA was not affected by long storage, and the quantity obtained per sample was adequate for about 1000 PCR reactions. Thus, this method allowed isolation of about 200 samples per day at a cost of $0.60 per sample and is a relatively high-throughput, low-cost extraction compared with conventional methods that use manual grinding and/or expensive kits.

Full text†: This article, in detail, is available only in the electronic version of the *Plant Molecular Biology Reporter*

Key words: ball bearing, cocoa, DNA extraction, high-throughput, SSR

*Author for correspondence. e-mail: r.bhattacharjee@cgiar.org; fax: +234 2 2412221; ph: +234 2 2412626.
†Editor's note: Although the scientific content of this article has been reviewed, the full-text Web publication has not been edited in detail.

Abbreviations: CTAB, hexadecyltrimethylammonium bromide; CIA, chloroform-isoamylalcohol; Na-azide, sodium azide; NaCl, sodium chloride; PCI, phenol-chloroform-isoamylalcohol; PCR, polymerase chain reaction; SSR, simple sequence repeat; TE, Tris-EDTA.

Introduction

Isolation of pure and high-quality DNA suitable for restriction analysis, cloning, and polymerase chain reaction (PCR) is now routinely done for most plant species with fresh, cryogenically preserved, or freeze-dried tissue of appropriate developmental stages. However, DNA extraction still remains a cumbersome step in molecular studies, constituting about 30-60% of the total time required for sample processing. This becomes more difficult when working with perennials or tree plant species. Several factors limit isolation of sufficiently pure DNA from tree species that are rich in impurities, such as terpenes, polyphenols, and polysaccharides. These contaminants are in abundance in foliage of perennials, and they coextract with the DNA. As a consequence, many tree species require more complex extraction methods than do annual plants, requiring special grinding procedures (Scott and Playford, 1996; Shepherd et al., 2002). Furthermore, because most of the tree species are grown in locales where storage and refrigeration facilities are not immediately available, collection of leaf samples becomes almost impossible. In such cases, the protocol described by Rogstad (1992) for preserving leaf tissues in an NaCl-CTAB-azide solution is the best solution. This method does not require immediate transportation for refrigeration of samples and can preserve leaf tissues without damage from bacteria or fungi for few days.

Cocoa (*Theobroma cacao* L.) is a perennial tree species grown at locations far away from laboratory facilities, and it is impossible to transport samples immediately. Therefore, the collected leaf samples were preserved in the NaCl-CTAB-azide solution following the procedure described in Rogstad (1992) before they were transported to the laboratory and refrigerated at 4°C.

Several DNA extraction protocols already available for cocoa have proven unsuitable because of the presence of high levels of polysaccharides and polyphenolic compounds within the tissues. Furthermore, the published protocols developed specifically for cocoa are complex, requiring extensive configuration steps, often with the use of cesium chloride gradients (Couch and Fritz, 1990; Figueira et al., 1992; Laurent et al., 1993; Lanaud et al., 1995; Ronning et al., 1995). Recently, researchers have started using kits to extract DNA (Perry et al., 1998); these kits are costly, and use of them restricts the number of samples that can be extracted per day. Currently, the most common procedure for isolating DNA from cocoa involves dry leaf tissues that require manual grinding, which is not only time-consuming but also cumbersome. Therefore, we describe a relatively high-throughput protocol to extract genomic DNA from cocoa by use of a semiautomated system (GenoGrinder 2000) for grinding leaf tissues. This allowed grinding of 192 leaf samples in a format of 96 × 2 plates at a time and resulted in rapid extraction of DNA from leaf tissues per person per day.

The entire procedure followed a modified CTAB/β-mercaptoethanol–based DNA extraction protocol, as described by Mace et al. (2003).

Materials and Methods

Plant material

About 8-10 leaf disks 6 mm in diameter per field-grown cocoa tree were excised from young, green, fleshy leaves that showed no or minimal damage from microorganisms or insects. Disks were placed inside 2.0-mL Eppendorf tubes containing 1.8 mL of saturated NaCl-CTAB-azide solution (70 g of NaCl, 3 g of CTAB, 0.04 g of Na azide, and 200 mL of distilled water). Samples were kept in Eppendorf tubes without any specific storage conditions during collection in the field and transportation to the laboratory, which lasted as long as 3-4 d in a tropical climate. At the laboratory, samples were refrigerated at 4°C until DNA extraction was performed.

DNA extraction reagents

DNA extraction buffer (CTAB buffer), consisting of 7% CTAB, 1.4 M NaCl, 20 mM EDTA-Na_2, 100 mM Tris-HCl (pH 8.0), with the addition of 0.2% β-mercaptoethanol just before use

- TE buffer, consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)
- CIA, consisting of 24 parts chloroform: 1 part octan-1-ol (isoamylalcohol)
- Isopropanol, stored at –20°C
- Sodium acetate, 3 M, stored at room temperature
- Ethanol, 70% and 100%, stored at –20°C
- RNase A (10 mg/mL)
- Low-salt TE (10 mM Tris, 0.1 mM EDTA [pH 8])

Initial sample preparation

Leaf disks were stored at 4°C, and in most cases, the storage was for at least 1 y in NaCl-CTAB-azide buffer as described in Rogstad (1992). Four leaf disks were then removed from Eppendorf tubes with the help of forceps; washed vigorously; and placed inside 1.2-mL polypropylene strip tubes with strip caps, also called coster 1.2-mL tubes (Marsh Biomarket, available as 12- × 8-well strips), containing 2 prechilled 4-mm chrome-plated grinding ball bearings (Spex CertiPrep, as described by Karakousis and Langridge, 2003; Mace et al., 2003; Rehman et al., 2001). Balls were dispensed by an automatic ball dispenser 1 d before DNA extraction and kept at 20°C. Two more balls were then put on top of the leaf disks to ensure proper grinding of the leaf tissues.

Basic extraction protocol

1. Turn on the 65°C water bath 1 h before starting the extraction procedure.
2. Aliquot enough CTAB buffer for 200 samples (450 µL per sample plus 10% extra) into a disposable 50-mL Falcon tube. Incubate buffer in a 65°C water bath.
3. Add 450 µL of warm CTAB buffer to each sample and cap tightly with polyethylene (chloroform-resistant) strip caps.
4. When loading the samples onto the grinding device, GenoGrinder 2000 (Spex CertiPrep), balance the tubes across 2 racks (each with 96 samples).

5. Process samples in a GenoGrinder 2000 following the manufacturer's instructions, at 500 strokes per minute for 10 min.
6. Incubate the samples for 30 min in a 65°C water bath with occasional mixing.
7. Remove tubes from the water bath and add 400 µL of CIA to each sample. Tightly cap samples and invert 2-3 times to mix well.
8. Centrifuge plates at 2250*g* (Eppendorf centrifuge model 5810 with Swing-bucket rotor model A-2-DWP) for 20 min.
9. Transfer about 300 µL of the supernatant into fresh strip tubes without disturbing the interface. Remove the balls and discard the used strip tubes.
10. Add 0.7 vol (210 µL) of ice-cold isopropanol to the supernatant and mix by inverting the tubes to precipitate the DNA.
11. Centrifuge plates at 2250*g* for 30 min.
12. Carefully decant the supernatant without disturbing the pellet and air-dry for 20 min.
13. Add 200 µL of low-salt TE and 3 µL of RNase A per sample and place at 37°C for 1 h (or overnight at room temperature).
14. Add 200 µL of phenol-chloroform-isoamylalcohol (PCI) (25:24:1) to each sample and mix well by inverting the tubes. Centrifuge at 2250*g* for 15 min.
15. Transfer the supernatant to freshly labeled strip tubes by use of multichannel pipettes and add 200 µL of CIA. Mix well by inverting the tubes 2-3 times.
16. Centrifuge at 2250*g* for 15 min and transfer the supernatant in freshly labeled strip tubes. Add 315 µL of ethanol-acetate solution (30 mL of cold ethanol and 1.5 mL of 3 M sodium acetate [pH 5.2]) to each sample. Place the samples at –20°C for 10 min.
17. Centrifuge samples at 2250 *g* for 20 min and carefully decant supernatant without disturbing the pellet.
18. Wash pellets with 200 µL of 70% ethanol and quickly spin the plate at 2250*g* for 10 min. Carefully pour off supernatant and air-dry the pellet for 1 h.
19. Resuspend dried pellet in 100 µL low-salt TE and store at 4°C.

Notes

- Preferably, the extraction buffer should be freshly prepared.
- The 10-min grinding procedure was repeated to ensure sufficient disruption and homogenization of leaf tissue.
- Strip tubes should be tightly closed before mixing.
- Racks must be precisely balanced before centrifuging.
- Chrome-plated balls, after being used, should be cleaned in soapy water for 1 h and then with 0.2 M HCl for 10 min. The balls should then be rinsed in distilled water and dried to reuse.

DNA quantification

Aliquots of 3 µL of freshly extracted genomic DNA were electrophoresed on 0.8% agarose gels, stained with ethidium bromide, and visualized under an ultraviolet transilluminator for quality and yield assessment. DNA quantity was estimated from the regression of band intensity compared with the aliquots of

standard amounts of λ genomic DNA (Boehringer Mannheim, catalog no. 84836720) used as marker ladder.

PCR amplification using cocoa single sequence repeat (SSR) primers

Nine samples of isolated DNA were randomly selected and subjected to PCR amplification with 10 cocoa SSR primers (Lanaud et al., 1999). The total volume of the PCR reaction was 10 µL, which contained 1 µL of freshly extracted DNA (~2.5 ng), 1 µL of 10× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 1 µL of 25 mM $MgCl_2$, 0.5 µL of each forward and reverse primer, 0.2 µL of 10 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 0.1 µL of 5 U of *Taq* polymerase (Bioline). Amplifications were carried out in a gradient cycler PTC 200 (MJ Research). The PCR cycle consisted of initial denaturing at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 51°C annealing for 1 min, and 72°C for 1 min. This was followed by further primer extension at 72°C for 7 min. PCR products were then stored at 4°C.

Amplification products were viewed by electrophoresis on conventional 1.2% agarose gels with the standard ladder marker (Hyperladder V, Bioline) and automated capillary electrophoresis (ABI 3100, Applied Biosystems).

Results and Discussion

A semiautomated DNA extraction procedure has been standardized to isolate DNA from preserved cocoa leaf samples. The protocol was then routinely used to extract DNA from 2000 cocoa germ plasm accessions under study. The GenoGrinder 2000 grinding device was used for automated grinding of leaf tissues. This device reduced the hassle of manual grinding, allowed homogenization of 192 samples per day per person, and thus made the entire process rapid.

Preservation of leaf samples in CTAB-NaCl-azide solution permitted high-quality DNA to be obtained (most samples, Figure 1), although the leaves showed some discoloration with time. In fact, the preservation procedure confirmed that cocoa leaf tissues can be stored almost the same as fresh tissue in CTAB buffer for a long time and do not require immediate isolation of DNA. The quality of genomic DNA is evident from high-molecular-weight bands and the absence of RNA along with other polysaccharides that usually affect migration during electrophoresis. Although some shearing of DNA was apparent, which might be due to grinding of tissues with steel balls, the quantity of DNA obtained was high, sufficient for about 1000 PCR reactions. Because of bactericidal and detergent properties of CTAB, field preservation in CTAB-NaCl-azide solution not only avoided bacterial and fungal proliferation during relatively prolonged dehydration in air but also facilitated careful cleaning of leaves during washing in the laboratory before DNA extraction (Rogstad, 1992). The storage in CTAB buffer further helped in softening the leaf tissue so that grinding with ball bearings was easier, and it may also have dissolved some of the polyphenols and polysaccharides present in the tissue. In general, this procedure enabled isolation of DNA from fresh leaves rather than from dry leaves, which is the most common tissue material used for DNA extraction from cocoa.

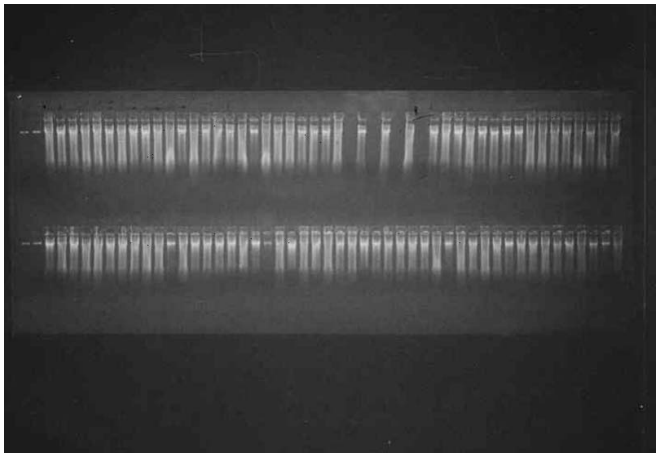

Figure 1. Agarose gel (0.8%) analysis of genomic DNA extracted from leaf tissues of 96 cocoa genotypes.

DNA from 9 randomly selected samples was subjected to PCR amplification. Amplification products (Figure 2) were obtained for all the DNA samples with different cocoa SSR primers, except for the primer mTcCIR18. Interestingly, none of the DNA samples showed amplification products for this particular primer. Further, as seen in Figure 1, DNA could not be isolated from few samples, perhaps because of smaller amounts of tissue material used in DNA extraction or excess DNA used in PCR. Therefore, standardizing the amount of tissue material used for DNA extraction and the quantity to be used in PCR is critical. A smaller amount of tissue material can result in isolation of no or very low quantities of DNA, and excess DNA in the PCR reaction can sometimes inhibit the process (Dilworth and Frey, 2000). Hence, use of 4-5 leaf disks is sufficient to obtain a sufficient quantity of DNA in this extraction procedure.

We also tested in parallel DNA extraction from dry leaf tissue, and we obtained variable and inconsistent results. The DNA quality was poor and/or the yield was low, which affected the possibility of generating PCR products for all samples under investigation.

By using the established protocol, we routinely obtained good quality and yield of DNA from 2000 samples. Sample-to-sample variation was negligible compared with other extraction procedures. PCR amplification has been completed for 1000 samples, which resulted in good clear PCR products in 75% of the samples. Therefore, a second round of phenol extraction was done that took care of the remaining polyphenolic compounds present in the samples.

An additional and relevant feature of this extraction protocol is the cost per extraction and the number of samples that could be processed per day. The time required to extract DNA from such a large number of samples has been reduced by at least 80% compared with previous extraction procedures, and the material costs (including labor cost) for the extraction procedure is reduced by 5- to 6-fold, making the cost per sample $0.60. This simple, rapid, cost-effective, semi-automated, high-throughput protocol can therefore be used for large-scale DNA

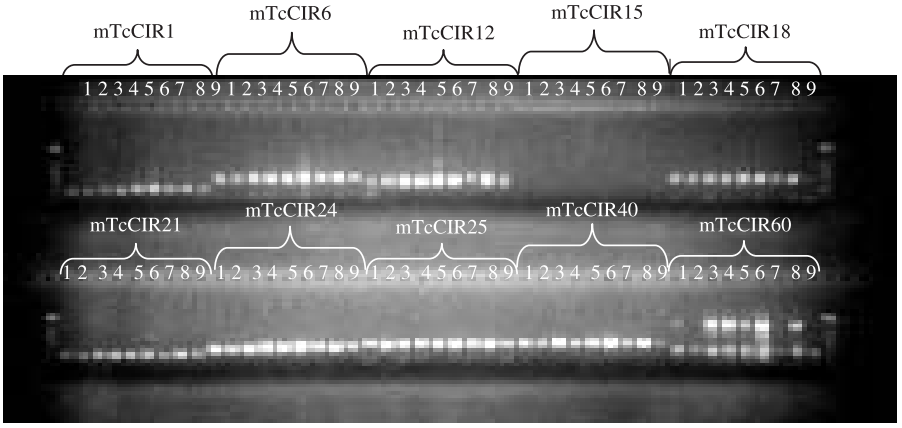

Figure 2. PCR amplification of DNA isolated from nine cocoa genotypes with various oligonucleotide primers (indicated in the figure). The first and last lanes represent DNA standards. mTcCIRx is the nomenclature for cocoa primers developed at CIRAD, France. The letter x stands for the number (1-9) of the following genotypes: *1*: Genotype 1 (AKP1); *2*: Genotype 2 (AMAZ10); *3*: Genotype 3 (CS2H1); *4*: Genotype 4 (C-Clone); *5*: Genotype 5 (Scavina6); *6*: Genotype 6 (T-Clone); *7*: Genotype7 (Amelonado); *8*: Genotype 8 (CTIS); *9*: Genotype 9 (Parinari-Clones).

extraction in diversity and marker-assisted selection studies of cocoa, which require large sample sizes.

Acknowledgments

This protocol standardization at the International Institute of Tropical Agriculture was supported by grants from the USDA/USAID-Masterfoods in the framework of the cocoa genetic diversity project for the West African region.

References

Couch JA and Fritz PJ (1990) Isolation of DNA from plants high in phenolics. Plant Mol Biol Rep 8: 8-12.

Dilworth E and Frey JE (2000) A rapid method for high throughput DNA extraction from plant material for PCR amplification. Plant Mol Biol Rep 18: 61-64.

Figueira A, Janick J, and Goldsbrough P (1992) Genome size and DNA polymorphism in *Theobroma cacao.* J Am Soc Hort Sci 117: 673-677.

Karakousis A and Langridge P (2003) A high-throughput plant DNA extraction method for marker analysis. Plant Mol Biol Rep 21: 95a-95f.

Lanaud C, Risterucci AM, N'Goran AKJ, Clement D, Flament MH, Laurent V, and Falque M (1995) A genetic linkage map of *Theobroma cacao* L. Theor Appl Genet 91: 987-993.

Lanaud C, Risterucci AM, Pieretti I, Faique M, Bouet K, and Lagoda PJL (1999) Isolation and characterization of microsatellites in *Threobroma cacao* L. Mol Ecol 8: 2141-2152.

Laurent V, Risterucci AM, and Lanaud C (1993) Variability for molecular ribosomal genes within *Theobroma cacao*. Heredity 71: 96-103.

Mace ES, Buhariwalla HK, and Crouch JH (2003) A high-throughput DNA extraction protocol for tropical molecular breeding programs. Plant Mol Biol Rep 21: 459a-459h.

Perry MD, Davey R, Brian Power J, Lowe KC, Frances H, Bligh J, Roach PS, and Jones C (1998) DNA isolation and AFLP genetic fingerprinting of *Theobroma cacao* (L.). Plant Mol Biol Rep 16: 49-59.

Rehman A, Raman R, Read B, and Raman H (2001) High throughput DNA isolation method for routine marker-assisted selection in barley. In: Proc 10th Aust Barley Tech Symp, Melbourne, 12-16 September, pp. 1-4. Available at: http://www.regional.org.au/au/abts/2001/t4/rehman.htm. Accessed December 3, 2004.

Rogstad SH (1992) Saturated NaCl-CTAB solution as a means of field preservation of leaves for DNA analysis. Taxon 41: 701-708.

Ronning CM, Schnell RJ, and Kuhn DN (1995) Inheritance of random amplified polymorphic DNA (RAPD) markers in *Theobroma cacao*. J Am Soc Hort Sci 120: 681-686.

Shepherd M, Cross M, Stokoe RL, Scott LJ, and Jones ME (2002) High-throughput DNA extraction from forest trees. Plant Mol Biol Rep 20: 425a-425j.

Scott KD and Playford J (1996) DNA extraction technique for PCR in rainforest plant species. Biotechniques 20: 974-978.