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Short Communication

Improved RAPD amplification of recalcitrant plant DNA by the use of activated charcoal during DNA extraction

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With 2 figures

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Abstract

An efficient procedure has been developed for DNA extraction from cotton by modifying the original CTAB method. The major improvement concerns the use of activated charcoal to bind resinous and coloured compounds which copurify with the DNA. The efficiency of amplification by RAPD was used as a criterion to evaluate the action of activated charcoal. Twenty-five random decamers from Operon Technologies were used to compare DNA samples extracted in the presence or absence of activated charcoal. The results in terms of amplification suggest that the use of activated charcoal in DNA extraction enhances RAPD amplification. This technique was initially developed for cotton but it has been applied successfully to other recalcitrant plants such as coffee, rubber tree, cassava and banana.

Key words: *Coffea arabica* — *Gossypium hirsutum* — *Hevea brasiliensis* — *Manihot esculenta* — *Musa accuminata* — activated charcoal — CTAB — DNA — RAPD

Progress in the molecular genetics of cotton is conditioned by the availability of a simple and inexpensive method for DNA extraction. The few protocols that have been described for this purpose may yield high quality DNA but none of them simultaneously minimize time, cost, amounts of chemicals and plant material. Using a slightly modified procedure of Murray and Thompson (1980), the DNA solution that we obtained was brown and sticky with A_{260}/A_{280} ratios between 1.2 and 1.4, due to the presence of contaminants such as polysaccharides, polyphenolics and other secondary compounds (Kanazawa and Tsutsumi 1992, Lodhi et al. 1994). These compounds, which are especially abundant in cotton (Paterson et al. 1993), must be removed from DNA because they can irreversibly interact with proteins and nucleic acids (Dabo et al. 1993). Polysaccharides have been reported to inhibit DNA polymerase (Shioda et al. 1987) and their presence leads to unreproducible RAPD results (Mejjad et al. 1994). We decided, therefore, to adapt the method mentioned above to cotton DNA extraction. We increased the concentrations of PVP-40, β-mercaptoethanol and NaCl from 1 to 2%, 0.2 to 5% and 1.4 to 2 M, respectively, but the essential modification was the use of activated charcoal. This reagent can absorb resinous matter and coloured impurities in aqueous phase at high temperatures (Vogel 1956). Oakeley et al. (1994) used activated charcoal to remove polyphenolics from tobacco callus extracts before assaying GUS activity. Our study sought to find an easy and inexpensive cotton genomic DNA extraction method before beginning a marker-assisted selection programme with various cotton species and hybrids.

Plant material: Genomic DNA was extracted from *G. hirsutum*, *G. thurberi*, G. *raimondii*, *G. sturtianum*, two trispecific hybrids and 20 of their first backcrosses to *G. hirsutum*. All the plants used in the experiments (cotton but also coffee, rubber tree, cassava and banana) were grown in a greenhouse. For all plants, young leaves were used when available, but mature leaves were also used.

The DNA extraction buffer: was composed of 2% (w/v) CTAB (hexadecyltrimetylammoniumbromide), 2% (w/v) PVP-40 (polyvinylpyrrolidone, Sigma, St. Louis, MO, USA), 2.0 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 5% β-mercaptoethanol (added in a fume hood just before use). The other reagents were activated charcoal powder (Sigma), washing buffer (80% ethanol, 10 mM ammonium acetate), chloroform : isoamyl alcohol 24 : 1 (v/v), RNase A 10 μg/μl in water and TE buffer.

Protocol: (1) Preheat the extraction buffer at 60°C in a water bath to dissolve CTAB. (2) Grind the tissue sample in liquid nitrogen using a pre-cooled mortar and pestle. (3) Transfer the powdered tissue to a 50 ml tube, wait until all the liquid nitrogen has evaporated and add 7.5 ml pre-heated extraction buffer per g of tissue. (4) Add 25 mg of charcoal per g of initial tissue sample; put efficiently in suspension by gentle vortexing. (5) Incubate the mixture at 60°C for 30–60 min in a water bath with periodic shaking. (6) Add 1 volume of chloroform : isoamyl alcohol (24 : 1) at room temperature and mix by inverting the tubes periodically for 10 min. (7) Centrifuge for 10 min at 16 300 *g* at room temperature to separate the phases; avoiding the interface, recover the aqueous phase using a sterile, wide-bore plastic pipette. (8) Add 2/3 volume of isopropanol kept at room temperature; invert the tubes 10 to 15 times and allow to precipitate for 10–60 min at room temperature. (9) Centrifuge for 5 min at 650 *g* at room temperature and remove the supernatant. (10) Add 10 ml of washing buffer per g of tissue sample and put on a vertical shaker for 30–60 min. (11) Centrifuge for 5 min at 650 *g* at room temperature and remove the supernatant. (12) Vacuum dry the pellet for 2–5 min or air dry for 30 min and resuspend the DNA in 0.3 to 1 ml TE. (13) Treat the DNA solution with 1 μl RNase A/100 μl DNA and incubate for 30 min at 37°C to eliminate RNA; store at 4°C (short term) or at −20°C (long term).

The protocol was applied by adding activated charcoal (a), to the hot mixture just after the incubation at 60°C for 5–10 min (b), before the incubation at 60°C to test the elimination of contaminants at high temperature and (c), without activated charcoal. The three samples of DNA (a, b, c) were submitted to RAPD (random amplified polymorphic DNA) using 25 random 10-mer from Operon Technologies Inc. (Ala-

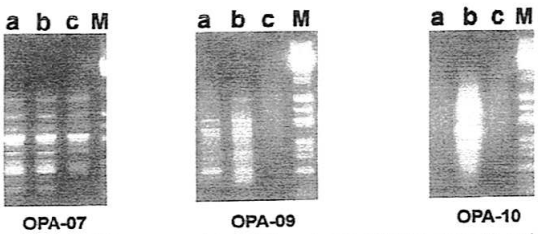

Fig. 1: RAPD patterns obtained by primers OPA-07, OPA-09 and OPA-10 from the three DNA samples: a. DNA extracted with activated charcoal added after the incubation at 60°C; b. DNA extracted with activated charcoal added before the incubation; c. DNA extracted without activated charcoal; M, molecular size marker (λDNA/*Hin*dIII + $\phi \times$ 174RFDNA/*Hae*III)

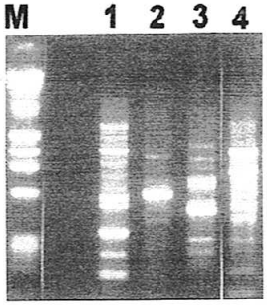

Fig. 2: RAPD patterns of *Coffea arabica* (1), *Musa accuminata* (2), *Hevea brasiliensis* (3) and *Manihot esculenta* (4). RAPD markers were amplified with primer OPA-01; M, molecular size marker (λDNA/*Hin*dIII + $\phi \times$ 174RFDNA/*Hae*III); each lane contains 7 µl amplification product

meda, CA, USA). Amplification reaction volumes were 25 µl, each containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 200 µM each dNTP (Pharmacia, Uppsala, Sweden), PHC-3 0.8 µM random primer, 1 U *Taq* Polymerase (Pharmacia) and 50 ng of *G. hirsutum* DNA, overlayed with mineral oil and exposed to the following conditions using a Techne DNA thermal cycler: 94°C for 5 min, followed by 45 cycles of 1 min at 94°C, 1 min at 34°C, 2 min at 72°C, finished by 7 min 30 s at 72°C and then kept at 15°C till electrophoresis. The RAPD products were resolved on 1.8% agarose gel and visualized by ethidium bromide staining.

Taking all the samples into consideration, a total of 132 different RAPD fragments were amplified. Of these RAPD products, 131 fragments were obtained when activated charcoal was added before incubation, 85 fragments were amplified when activated charcoal was added after incubation and 80 fragments were amplified when activated charcoal was not used. Figure 1 shows an example of RAPD results that were used to score the fragments. The amplification varies between primers; this difference in amplification strength could be due to the fact that some primers may be more sensitive to the presence and amount of impurities than others (Fritsch et al. 1993). In all the RAPD reactions, the DNA extracted with activated charcoal before incubation was equally or more amplifiable than the two other DNA samples. Since the three DNA samples are from the same species (*G. hirsutum*) and great care was taken to avoid manipulation artefacts by repeating each reaction at least twice, the most likely source of variation between the samples under study is the presence of contaminants. The results suggest that activated charcoal is effective in removing coloured and resinous matter which inhibit DNA polymerase during amplification.

Digestible DNA was extracted from other recalcitrant plants such as coffee, banana, cassava and rubber tree. For all species tested, the A_{260}/A_{280} ratio ranged from 1.60 to 2.0 and the highest yield expressed in µg/g of fresh leaf was 150 for *G. hirsutum*, 100 for *Coffea arabica*, 300 for *Manihot esculenta*, 20 for *Musa acuminata* and 300 for *Hevea brasiliensis*.

The amplification conditions described were successfully used in RAPD reactions with the DNA extracted from coffee, rubber tree, cassava and banana; an example is shown in Fig. 2. The advantages of this CTAB modified method are its simplicity and rapidity; it does not require special conditioning of the tissues and DNA can be isolated from 500 mg of leaf without large quantities of reagents. The amount of activated charcoal to be added before incubation should be adapted to the amount of contaminants present in the tissues. On average, the best results in cotton are obtained by the addition of 10 to 40 mg of activated charcoal per gram of fresh leaf.

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