Short Technical Reports

Chloroplast DNA Extraction from Herbaceous and Woody Plants for Direct Restriction Fragment Length Polymorphism Analysis

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ABSTRACT

The technique described here is a fast and simple method of extracting chloroplast DNA (cpDNA). It overcomes the need for differential centrifugation using density gradients. The leaves do not have to be kept in the dark and lyophilized before extraction, but lyophilization is still possible. The chloroplasts are specifically lysed in a cell extract of leaves, using a non-ionic detergent. After isolation by centrifugation, the cpDNA is purified by the combined action of proteolytic enzymes and detergents, followed by the elimination of proteins using a mixture of chloroform and isoamyl alcohol. This method provided good quality restriction profiles for all species analyzed.

INTRODUCTION

Chloroplast DNA (cpDNA) is used extensively in molecular systematic and phylogenetic studies with plants (8,10,13). This small molecule varies from 120–217 kb depending on the species (7) and is generally inherited through the maternal lineage. It can be inherited through the paternal (11) or biparental (4) lineages, however.

The utility in obtaining an enriched extract of cpDNA is that, if it is sufficiently pure, the small size of this DNA makes it possible to directly observe the restriction fragment length polymorphism (RFLP) of entire molecules (2). Most of the techniques described for extracting cpDNA are based on either a preliminary isolation of intact chloroplasts, which are then lysed to extract the DNA or on the purification of the cpDNA taken for a total cellular DNA extract. The isolation of the chloroplasts is generally carried out on density gradients in an aqueous or organic solution (1,3,5), which makes it possible to separate the chloroplasts from the other genomic compartments. The purification of the cpDNA from other genomes (based on the difference in the nucleotide base content of DNA) requires the use of a CsCl density gradient (6,12).

The use of a density gradient step, which is common to these extraction methods, is often delicate, time- and reagent-consuming and has also to be modified for each different species being studied. Moreover, it requires equipment such as an ultracentrifuge, which is not available in every laboratory. We describe a very simple and fast method of extracting cpDNA in an aqueous phase, without centrifugation through a gradient. The cpDNA is extracted from a fraction containing both nuclei and chloroplasts by a differential chloroplast lysis using a non-ionic detergent. The extract of cpDNA obtained is sufficiently purified such that high-quality RFLP can be obtained. This method has given good results on all the herbaceous and woody species analyzed and with all of the restriction enzymes used.

MATERIALS AND METHODS

Plant Material

We experimented with leaves of different ages to obtain the best yield with cpDNA of good quality. Leaves were collected from healthy plants. Extraction from too young leaves (<5 days) reduced the yield from the cpDNA quantity of chloroplasts (both because of fewer plastids per cell and fewer cpDNA copies per plastid). On the other hand, extraction from old leaves (>15 days for several species of *Pennisetum*) affected the quality and the digestibility of the extract because of the presence of large quantities of pigments and polysaccharides. The best results were obtained with young green leaves of 5–10 days for all the species studied. The method was tested on phylogenetically distant species: 10 herbaceous species [*Cenchrus ciliaris* L., *Poa alpina* L., *Cucurbita pepo* L., *Pe. glaucum* (L.) R.Br., *Pe. purpureum* Schum., *Pe. polystachion* (L.) Schult., *Pe. subangustum* (Schum.) Stapf & Hubbard, *Pe. pedicellatum* Trin., *Pe. hordeoides* (Lam.) Steud. and *Pe. setosum* (Swartz) L. Rich.] and 2 woody species [*Delonix regia* (Boj.) Raf. and *Gmelina arborea* Roxb.]. Moreover, for all species of the genus *Pe. Brevivalvula* section, several individuals were analyzed.

Extraction Procedure

Preparing a suspension of cell extract. The cells are ground mechanically and then suspended in a buffer, which ensures that the integrity of the nuclei and chloroplasts is maintained.

Collect young green leaves (5–10 days) from healthy plants. They may need to be washed in water to remove the coarser impurities. There are two ways of making the extract, as follows: *(i)* grind in liquid nitrogen either fresh leaves or leaves frozen in liquid nitrogen and stored at - 50°C, 10–15 g until a fine powder is obtained. Transfer this powder into a tube containing 25 mL of sorbitol buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA, pH 7.8) with 0.5% sodium bisulfite at 4°C; or *(ii)* freeze whole or coarsely cut leaves in liquid nitrogen and then freeze-dry on hot plates. Take 0.8–1.5 g of lyophilized leaves, grind 2 or 3× for 10 s in a coffee grinder, then suspend in 25 mL sorbitol buffer containing 0.5% sodium bisulfite at 4°C.

Filtering the suspension. To eliminate large size cell fragments, conduct filtration at 0°C–4°C (on crushed ice). Transfer the suspension of homogenized leaves to one layer of Scrynel Polyester filter of 33 µm mesh size that has been placed in a mortar. Twist the cloth and force out the maximum amount of filtrate. Add sorbitol buffer and repeat this process 5 or 6× to rinse the residue, combining the filtrates after each filtration (in a flask at 4°C) until a final volume of 100 mL is obtained.

Lysing the chloroplasts and extracting the cpDNA. To eliminate the mitochondria, centrifuge at low speed. The pellet, which contains both nuclei and chloroplasts, is resuspended and then submitted to a chloroplast-specific lysis to liberate the cpDNA. The liberated cpDNA is then isolated from the nuclei and non-lysed chloroplasts by centrifugation. The supernatant is then submitted to the combined action of proteolytic enzymes and detergents to

dissociate the cpDNA from proteins.

Centrifuge the extract at 4000× *g* for 25 min at 4°C. Discard the supernatant and carefully resuspend the pellet in 100 mL sorbitol buffer without sodium bisulfite, using a glass stirring rod. Centrifuge the resulting suspension at 1000× *g* for 20 min. Keep all elements used (tubes, suspension and solutions) during resuspension at between 0°C and 4°C. Discard the supernatant and resuspend the pellet in 4 mL of NET buffer (100 mM NaCl, 80 mM Tris-HCl, 30 mM EDTA, adjusted to pH 8.0 with 1 M NaOH) at 4°C in a 15 mL Corex tube. Add 1 mL of 20% Triton® X-100 (Sigma, St. Louis, MO, USA) solution and 200 µL of 2-β-mercaptoethanol: at this stage, the volume of the solution is approximately 6 mL. Conduct lysis at 0°C for 90 min with a three-dimensional gyratory rocker at slow speed. Centrifuge the solution at 4800× *g* for 10 min at 0°C. Transfer the supernatant into 15 mL Corex tubes containing 150 µL of pronase (10 mg/mL; Sigma), 7 µL of proteinase K (20 mg/mL; Appligène Oncor, Illkirch, France) and 160 µL of 20% SDS. Enzyme lysis is conducted at 37°C for 3 h. Centrifuge the solution at 4800× *g* for 10 min at 25°C. Transfer the supernatant into 15 mL glass centrifuge tubes containing 2.2 mL of CTAB buffer (20 mM EDTA, 2.8 M NaCl, 4% Cetyltrimethylammonium bromide, 100 mM Tris-HCl, pH 8.0), 100 µL of 2-β-mercaptoethanol and 0.05 g of insoluble PVP (Sigma) and then blend slowly on the rocker for 10–15 h at 55°C (PVP decreases the effect of polyphenols, quinones and tannins).

Purifying the cpDNA. Removal of protein is carried out by extracting the aqueous solution of cpDNA using chloroform-isoamyl alcohol (24:1; Sigma).

Bring the volume of the solution to 15 mL with chloroform-isoamyl alcohol. Mix by turning the tubes upside-down manually 10×, then centrifuge at 4000× *g* for 5 min at 15°C. Carefully pipet the aqueous phase, taking care not to remove anything from the interface. Add 1/10 the volume of 3 M sodium acetate (pH 5.2), so the concentration is now at 0.3 M. Bring the volume to 15 mL with chloroform-isoamyl alcohol. Mix, then centrifuge a second time at 4000× *g* for 10 min at 15°C. Once again, carefully pipet the aqueous phase and bring the volume to 15 mL with chloroform-isoamyl alcohol. Mix, then centrifuge a third time at 4000× *g* for 10 min at 15°C. Again, carefully pipet the aqueous phase.

Precipitating the cpDNA. Add an equal volume of isopropanol to the aqueous phase. After mixing, let stand at -50°C for 2 h, then centrifuge at 9000× *g* for 10 min at 25°C. Discard the supernatant and dissolve the pellet of cpDNA in 500 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), then reprecipitate in a microfuge with 1/10 (vol/vol) of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol. Centrifuge at 9000× *g* for 10 min at 25°C. Discard the supernatant and wash the pellet of cpDNA in 1.5 mL of 70% ethanol to remove salts. Centrifuge at 9000× *g* for 10 min at 25°C. Dry the resulting pellet of cpDNA under a vacuum in a desiccator, then redissolve in a volume of TE buffer sufficient to produce a concentration of 0.1 µg/µL (20–100 µL, depending on the size of pellet). Conduct the redissolving at room temperature for approximately 12 h. Store at -20°C.

Digestion and analysis of restriction fragments. The cpDNA extracts are digested by restriction enzymes in accordance with the suppliers' instructions. The quantity of cpDNA needed for digestion by a restriction endonuclease having a hexanucleotide recognition sequence is of the order of 0.8 µg. Each digestion is conducted in the presence of spermidine (4 mM) and RNAse (300 U; 12 U/µL), then stopped after 6–7 h by adding 1/10 volume of bromophenol blue-containing, gel-loading buffer.

The electrophoretic separation of the restriction fragments is conducted on 0.85% agarose gel (9).

RESULTS AND DISCUSSION

To confirm that the DNA being analyzed originated mainly from chloro-

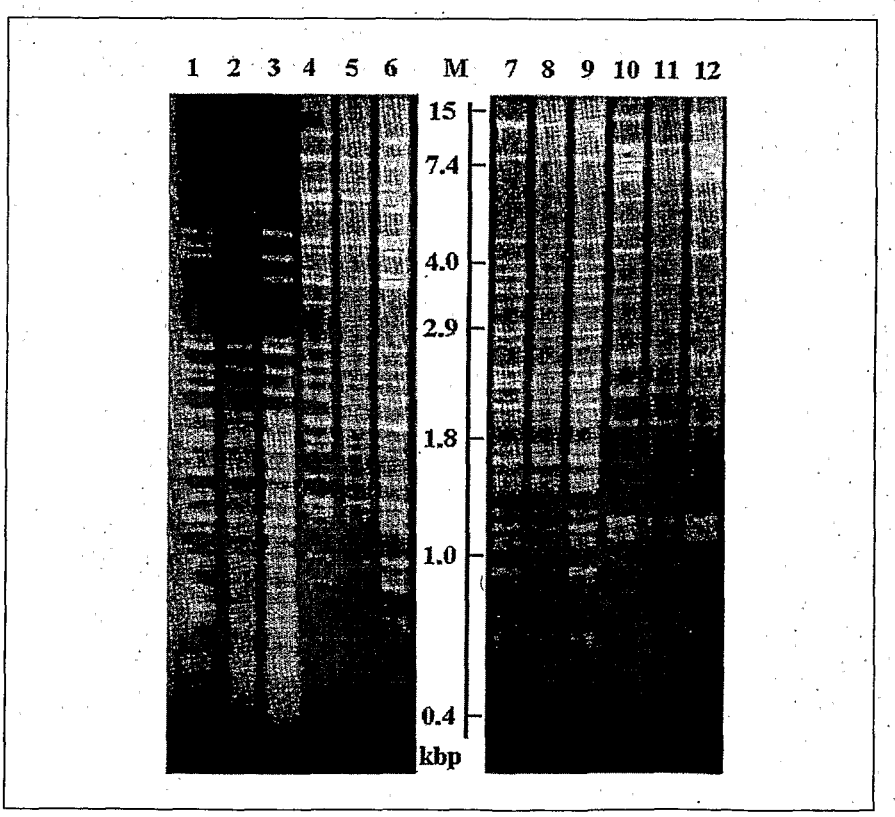

Figure 1. Example of restriction fragment patterns obtained by digestion of cpDNA. *Hae*III (lanes 1, 2 and 3); *Dra*I (lanes 4, 5 and 6); *Eco*RI (lanes 7, 8 and 9) and *Hind*III (lanes 10, 11 and 12) in *Pe. subangustum* (lanes 1, 4, 7 and 10 for individual A and 3, 6, 9 and 12 for individual B) and *Pe. polystachion* (lanes 2, 5, 8 and 11). Intraspecific polymorphism is observed between individual A and B of *Pe. subangustum* with *Hae*III and *Dra*I.

plasts, several individuals of *Poa alpina* and *Pe. glaucum* were extracted using both the aqueous extraction method described here and the non-aqueous technique described by Michaud et al. (5). RFLP profiles were always identical in both techniques, confirming that it was cpDNA in both cases (data not shown).

The method presented here yields cpDNA of good quality for RFLP analyses in all the taxa tested. Quantification of the cpDNA was carried out by agarose gel electrophoresis, which also verified the absence of degradation. The quantity of DNA obtained by this method (between 2 and 10 µg per extraction) varied from species to species and depended on the physiological state of the plant. Lyophilization made sample storage easier, and it also improved the quality of the grinding and thus increased the yield of cpDNA extract.

All 12 of the species studied were analyzed using the endonucleases *Bgl*II, *Cfo*I, *Dra*I and *Hind*III. The grasses were also analyzed using the enzymes *Bsp*XI, *Eco*RI, *Hae*III and *Xho*I. In all cases, high-quality restriction profiles were obtained with this method (Figures 1 and 2). No technical adaptation to the different species tested was necessary, unlike other techniques previously described, which required the use of gradients that had to be readjusted for each species studied (2,5,6,10,12).

Futhermore, the specificity of chloroplast lysis using a non-ionic detergent such as Triton X-100 decreases as temperature and lysis time increase. Therefore, the level of these factors condition the purity of the cpDNA extract (1).

The method presented here is remarkably reliable, efficient on a large number of species, simple to implement (little specialized equipment needed) and use (extraction in an aqueous phase without density gradients and unnecessary destarching step). The extraction of the cpDNA is conducted directly without any purification step from the extract of total DNA, decreasing both the cost and the extraction time. This property makes it easily transferrable for any laboratory wishing to analyze cpDNA polymorphism. It is currently being used routinely in the plant genetics laboratory of the Institut de Recherche pour le Développement, Niamey, Niger on diverse species.

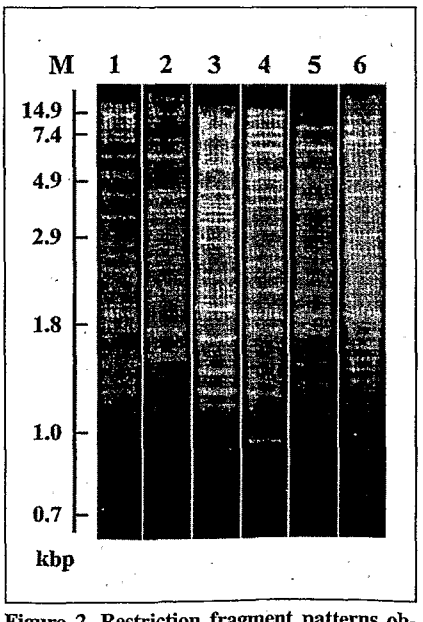

Figure 2. Restriction fragment patterns obtained by digestion of cpDNA with *Dra*I. *Delonix regia* (lane 1), *Cucurbita pepo* (lane 2), *Gmelina arborea* (lane 3), *Pennisetum purpureum* (lane 4), *Pe. pedicellatum* (lane 5) and *Poa alpina* (lane 6).

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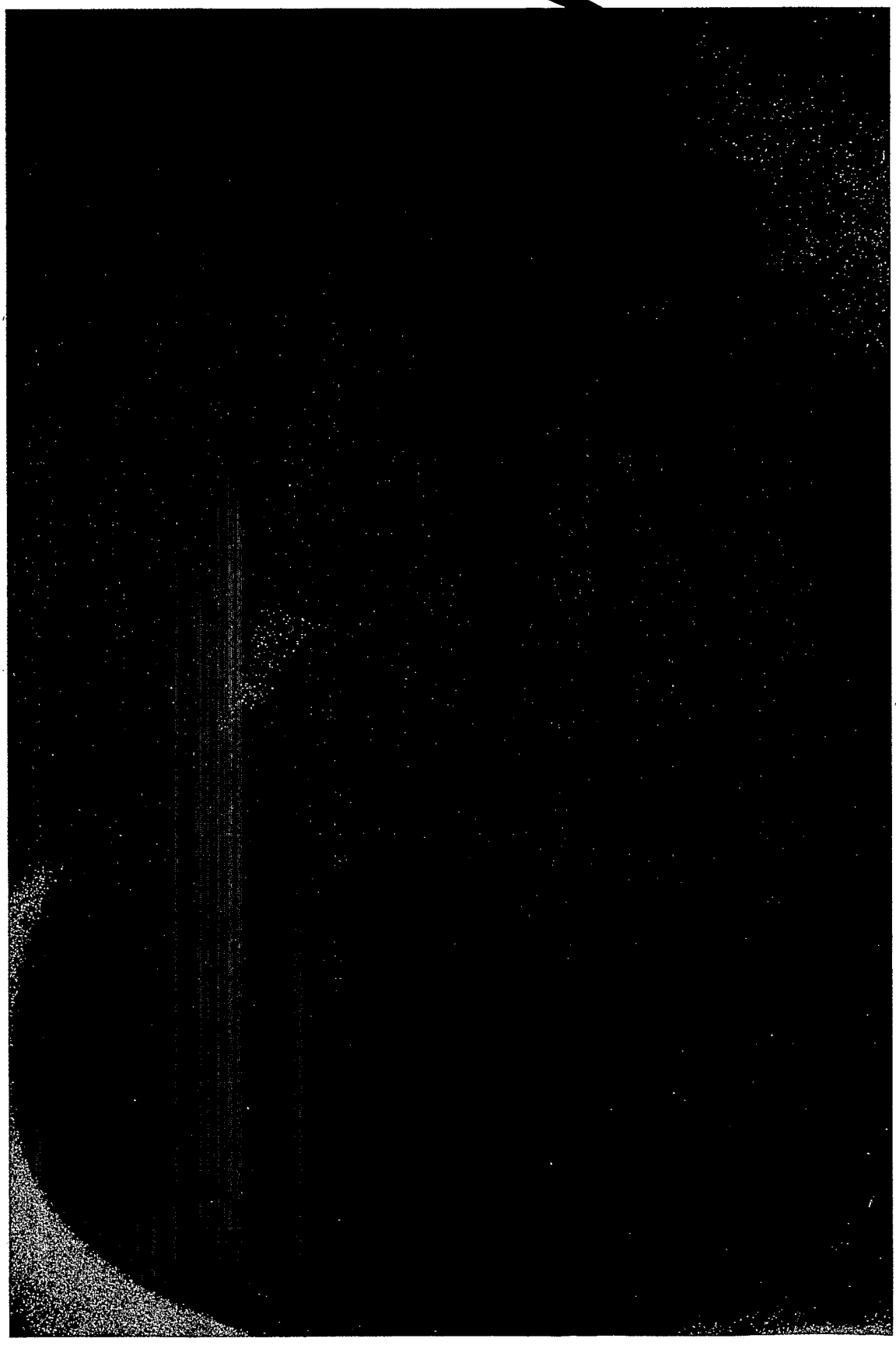

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