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Protocol

Chloroplast DNA Isolation from Higher Plants: An Improved Non-Aqueous Method

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The non-aqueous method of preparing chloroplast DNA (cpDNA) described by Bowman and Dyer (1982), introduced several advantages over the usual aqueous methods for isolating cpDNA. Apart from an improvement in yield, particularly for plants from which cpDNA was difficult to isolate, the method made possible the storage of plant material for long periods, even at ambient temperatures. The chloroplasts are isolated from a homogenate of freeze-dried leaf powder by isopycnic banding in a step gradient of organic solvents.

One drawback of the previously described method is an inverse correlation between yield of cpDNA and contamination with nuclear DNA. Another is a requirement for large quantities of the hazardous solvents, carbon tetrachloride and n-hexane. The present protocol describes modifications to the original method which may help to overcome these problems and employs tools to mechanize the grinding and homogenization of leaves. The method has been applied to a study of the RFLPs of *Oryzeae* spp. (rice), but is applicable to a variety of higher plants.

Abbreviations: cpDNA, chloroplast DNA; RFLP, restriction fragment length polymorphism; NET, 100 mM NaCl, 30 mM EDTA, pH 8.0, 80 mM Tris-HCl; TE, 10 mM Tris, 1 mM EDTA, pH 8; TX, Triton X-100.

Materials, Special Equipment, and Methods

Dangoumill 300 (Prolabo, France) or mortar and pestle.

1,2,4-trichloro-benzene (purum, density 1.45; Fluka)

decahydronaphthalene (*cis* and *trans*, purum, density 0.89; Fluka)

Polytron homogenizer (PMA 20 TN)

NET buffer: 80 mM Tris-HCl, 100 mM NaCl, 30 mM EDTA, pH 8.0.

TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.

Procedure

The following procedure is designed for the preparation of cpDNA from leaves of adult or seedling rice (cultivars or wild species of *Oryza*, *Oryzeae* tribe).

- 10 to 15 g of green leaves[1] are frozen in liquid nitrogen.
- The leaf tissue is pulverized in liquid nitrogen[2] in a Dangoumill at a shaking rate of 700 cpm in a 300-ml steel vessel containing a mixture of small and medium size steel balls (1X diameter, 20 mm; 4X diameter, 12 mm; and 9X diameter, 8 mm). A mortar and pestle can also be used.
- The frozen leaf powder is freeze dried.[3]
- 2 to 4 g of the dry powder is divided into two or three portions and each is blended with 10 ml of cold 4:1 (v/v) mixture of 1,2,4-trichloro-benzene and decahydronaphthalene in a Polytron mixture. Blending is done in a fume hood at room temperature at the lowest setting for 15 to 60 seconds, the interval depending on the state of aggregation of the sample. The diameter of the 50-ml homogenizer cup is 24 mm and that of the blade is 20 mm. The tissue brei is pressed through a nylon mesh of 25-μm pores and the combined residues reblended once or twice more with 5-ml addi-

tions of the solvent mixture. The final volume of the filtrate is about 30 ml and it can be stored for many hours.

- The filtrate is placed in a centrifuge tube, cooled for five minutes in a water bath at 4°C, and carefully overlaid with 10 ml of the 4:1 solvent mix. This step gradient is then overlaid[4] with 2 ml of a 1:3 (v/v) mixture of the same solvents and centrifuged in a swing-out rotor at 2,300 g for 17±2 minutes at 4°C.

- The sharp dark green band at the upper interface is removed from beneath with a pipette with U-shaped tip and transferred into a siliconized 15 ml-Corex tube. At least two volumes of cyclohexane are added, mixed and centrifuged for ten minutes. The supernatant is discarded and the pellet can be stored for many weeks without being dried.

- To the pellet are added, all at 0°C:

  0.5 ml of 20% v/v Triton X-100
  2 ml of NET buffer
  a steel ball, diameter 12 mm

  The tube is vortexed carefully for a few seconds at the lowest possible speed. After complete suspension of the pellet and removal of the ball, the tube is stoppered and placed on ice or on a rotating wheel at 0°C for 1.5 to 3 hrs.

- The lysate is centrifuged at 3,300 g and 0 °C for 10 min or longer and the supernatant poured into a 5-ml tube.

- 170 µl of 20% w/v SDS and 30 µl of proteinase K (10 mg/ml) are added and incubated at 60 to 65°C overnight (minimum two hours).

- Two extractions with chloroform/phenol (1:1 v/v) and one with chloroform are carried out (Maniatis et al., 1982). After the first extraction, 190 µl of 3 M Na acetate buffer, pH 6.2, are added.

- The DNA is ethanol-precipitated in four 1.5-ml reaction tubes, pelleted, ethanol-washed and dried as usual. Each pellet is allowed to dissolve in 25 µl of TE buffer at 4°C for at least one hour or preferably overnight.

Notes

1. It is not necessary to destarch rice; the leaves may be collected directly in the morning. It is also possible to dry the leaves overnight at 60°C, but with a reduced yield of cpDNA. See *Results and Discussion: alternative procedures.*
2. One may also grind previously lyophylized or heat-dried tissue at ambient temperature.
3. The dry powder can be stored *in vacuo* over P_2O_5 at -20°C for many months.
4. The first of the two overlays, serving to filter the ascending chloroplast material, is not strictly necessary. The second one protects the chloroplast band against condensed water.
5. In this solvent mixture the mitochondria appear to be a little denser than the chloroplasts. CpDNA prepared according to the above protocol shows little contamination with mitochondrial DNA as judged from Southern-blot hybridization with a probe of cytochrome oxidase II from wheat mtDNA. A still higher density mixture of the same solvents can be used to get a greater proportion of mtDNA and still avoid excessive nuclear DNA contamination (Zhang et al., 1988).

Results and Discussion

Original method

Chloroplast DNA was isolated from rice (*Oryza sativa*) leaves by the non-aqueous method originally described by Bowman and Dyer (1982), the DNA digested with *BstI*, and the fragments separated by agarose gel electrophoresis. The restriction pattern is shown in lane *OM* of Fig. 1. Approximately 25 fragments are resolved against a smear of nuclear DNA covering especially large cpDNA fragments. When a detergent extraction step was introduced in this method, there was a noticeable decrease in the intensity of the background, and the disappearance of two medium size fragments corresponding to nuclear ribosomal DNA as shown by hybridization with a rDNA probe (unpublished results). We interpret this to mean that the organellar membranes are lysed by Triton X-100, whereas the nucleosomes are less attacked. So, within a certain time, most of the cpDNA is solubilized, whereas the chromatin can be pelleted by centrifugation, resulting in a purification of cytoplasmic DNA with an overwhelming proportion of cpDNA compared to mitochondrial DNA.

Revised procedure

Due to the introduction of the subsequent purification step into the revised procedure, a higher density of the solvent mix can be used, making the separation by centrifugation less restrictive and so resulting in a greater recovery of chloroplast material, without a notable increase in final nuclear DNA contamination. (See note 5 above).

The densities of the solvent mixes used in the "selective layer" (i.e., the one directly below the uppermost thin protective layer) of the step gradients were accurately 1.31 for the original method (Fig. 1, lanes *OM* and *OM-TX*) and about 1.35 for the revised method (lanes *RM-TX* to *HD*). Under these conditions, the differential solubilization by Triton X-100 can be seen by comparing lanes *RM-TX* and *RM+TX0°*; the smear disappears while the cpDNA yield remains high.

A similar use of Triton X-100 for resuspending the chloroplast pellet was made by Lehväslaiho (1987) in an aqueous procedure: he added 4% Triton X-100 in NET buffer to the pellet for 30 to 60 min at room temperature. He did not, however, need differential solubilization. The essential importance of maintaining low temperature for this effect is shown by lanes *RM+TX37°* and *RM+TX15°* in Fig. 1: both samples are prepared with the new method, with the only modification that, in lane *RM+TX37°*, incubation of the pellet with Triton and NET was carried out at 37°C but in lane *RM+TX15°* at 15°C.

In the new procedure, 1,2,4-trichlorobenzene and decahydronaphthalene are used instead of carbon tetrachloride and n-hexane because they are less volatile and rather viscous, characteristics which are more suitable for grinding in the solvents. Furthermore, these solvents allow mechanical blending of the freeze-dried powder with a Polytron homogenizer, which liberates more usable material than by hand grinding, especially in the case of hard, fibrous leaf tissues.

The substituted solvents are less hazardous for routine handling and laboratory work, are less expensive and can be used more economically.

For the pelleting of the recovered chloroplast material, n-hexane is replaced by the less toxic but similarly inert and volatile solvent cyclohexane.

On average, approximately 10 µg of cpDNA were recovered from 12 g (fresh weight) of adult leaves of various species and varieties of rice with a large variation according to the genotype, the developmental stage and the physiological state of the leaves (Dally, 1988). A higher yield can be obtained by using young leaves from seedlings.

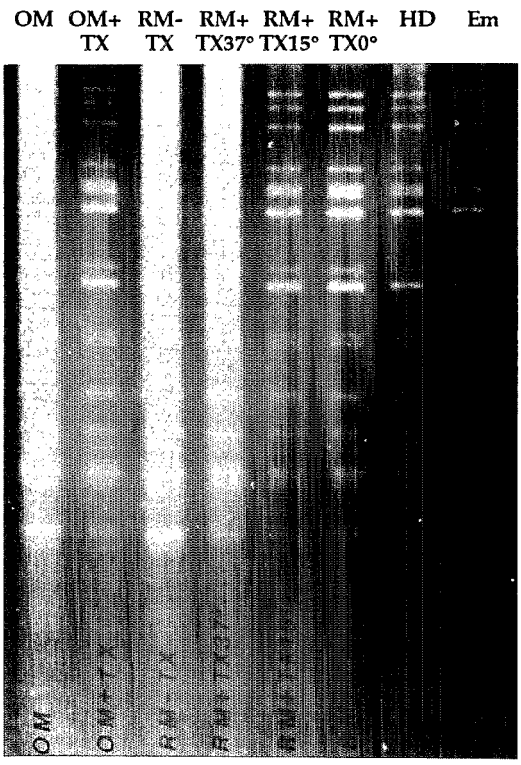

Fig. 1. ***Bst I*** **digests of cpDNA from the** ***Oryza sativa japonica*** **strain 521,** obtained by different procedures (from the left to the right): OM: original method without TX; OM + TX: original method with TX at 0°C; RM-TX: revised method without TX; RM + TX 37°: revised method with TX at 37°C; RM + TX 15°: revised method with TX at 15°C; RM + TX 0°: revised method (with TX at 0°C); HD: extracted from heat dried leaves; Em: direct extraction by emulsion. Further explanations are in the text. The DNA load of each lane (about 1 µg) corresponds to the yield obtained from 1 g of fresh leaves, except lane 7, which contains all the DNA obtained from 5 g of fresh leaves.

A comparison with the aqueous method as used in rice

A physical map of rice cpDNA was previously determined with DNA isolated from leaves blended in liquid nitrogen followed by extraction with aqueous solutions (Hirai et al., 1985). Recently the total sequence of rice cpDNA was reported (Shimada et al., 1988). Our restriction patterns with *BamHI* and *PstI* are similar to those of Hirai et al. (1985).

It should be noted that a large amount of two-week-old seedlings (typically 50 g fresh weight) are used to extract cpDNA with the aqueous method, whereas our protocol allows us to extract similar amounts from leaves of a single plant. This is of prime importance when comparing many different genotypes or hybrids and sterile plants as in studies on population biology or plant breeding.

Southern blots of restricted total DNA also allow the use of DNA extracted from a single plant. In this case, however, the resolution of the

bands is poorer than with their direct observation under ethidium bromide fluorescence. Good resolution is of great importance when differences among restriction patterns may be related to small additions or deletions as found in rice (Dally, 1988).

Alternative procedure

It is possible to extract cpDNA with the described procedure after heat drying the leaves (overnight at 60°C). This should open the way to using leaves collected in the field under conditions where neither liquid nitrogen nor freeze drying are available. In a limited number of trial, we obtained irregular results with a generally low yield of cpDNA and the DNA sometimes degraded, especially the larger restriction fragments. A good result is shown in lane *HD* of Fig. 1.

The modified non-aqueous preparation method for cpDNA proposed here represents only one of several possibilities concerning the use of alternative solvents. One can, for example, avoid the problem of dispersing tough, e.g., vacuum-dried, chloroplast pellets into aqueous solutions as follows: The pellet is dispersed in a small volume of 1,1,1-trichloroethane, which is easily done in half a minute of careful shaking. Then the organic phase is extracted on a rotating wheel with one or two volumes of the NET buffer containing 3 or 4% Triton X-100 for several hours at 0°C. The presence of this relatively mild solvent liberates organellar DNA and, when an emulsion forms, the DNA is transferred into the aqueous phase, from which the extraction can be continued in the usual way. This procedure requires, however, careful timing, because extraction for too long results in nuclear contamination of the preparation and one that is too short results in a considerable decrease in yield. Fig. 1, lane *Em* shows cpDNA sample obtained in this way.

As [^{35}S]-end labeling of restriction fragments using the Klenow enzyme fragment allows the reduction in amount of cpDNA used to as little as 0.04 µg per restriction digest (cf. Lehväslaiho, 1987, verified in our experience) and, as the DNA can be extracted from air-dried leaf material, it could eventually be possible to include well-preserved herbarium material in comparative genetic screenings.

Reliability of the method

For rice, the method described in this paper has turned out to be quick, safe and reliable. It made possible a large-scale investigation on the Section Eu-*Oryza* of the genus *Oryza*, analyzing the cpDNA of more than 300 plants representing 247 varieties of 15 wild and cultivated species

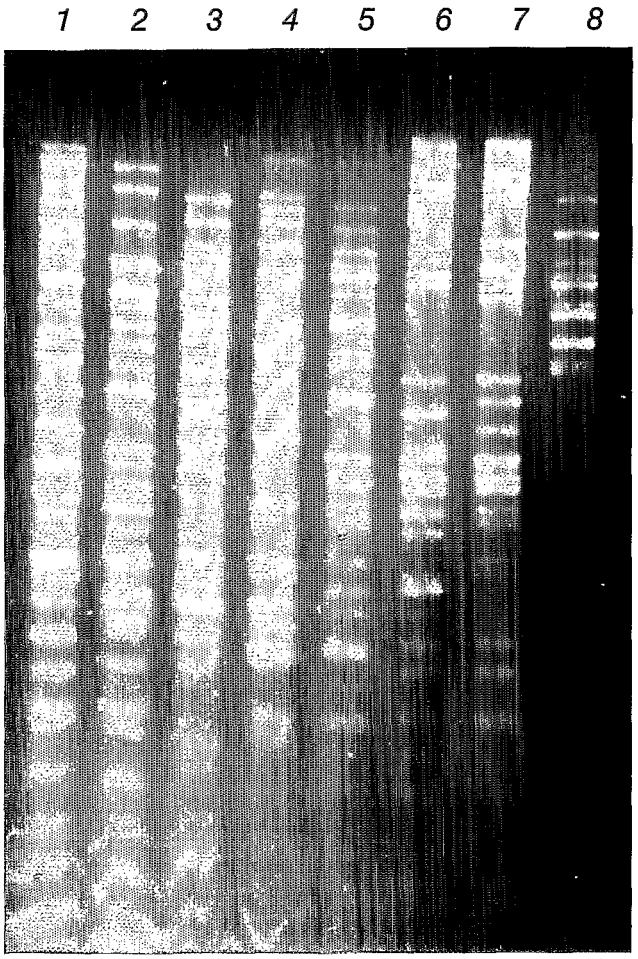

Fig. 2. ***Bst I*** **digests of cpDNA** (from the left to the right): 1) *Arum maculatum*, 2) *Asphodelus fistulosus*, 3) *Avena sterilis*, 4) *Chikusichloa brachyathera*, 5) *Eichornia crassipes*, 6) *Gossypium australe*, 7) *Hibiscus* sp., 8) *Scilla Lilio-Hyazinthus*. The DNA load on each lane corresponds to the yield from 2 g of fresh leaves.

and distinguishing between 32 different chloroplast genomes (Dally, 1988, Dally & Second, in preparation).

The described improvements are not uniquely adapted to rice. In a brief test of the unmodified cpDNA extraction procedure on 12 plants of different genera, nine gave positive results (*Plantago lanceolata* plus samples shown in Fig. 2). *Thymus vulgaris*, *Quercus ilex*, and *Iris* sp. gave negative results.

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