about 4–5 cm and be a smeary band near the dye front.

An alternative to the phenol extraction for DNA purification, is centrifugation on a CsCl gradient. The digested lysate may be diluted to the desired volume (determined by the size of the ultracentrifuge tube) and made 1.7 g/ml with CsCl. The solution is centrifuged as 50,000 rpm in the VTi80, and then fractionated by dripping from the bottom. The nucleic acid-containing fractions are analyzed by electrophoresis, and the desired fractions are dialyzed and nucleic acids collected by ethanol precipitation.

Solutions:

1) HB (homogenization buffer). 0.4 M mannitol, 1 mM EDTA, 0.05% cysteine, and 10 mM TES-Na^+, pH 7.2. Autoclave and store at 4°C. Supplement with 0.1% BSA and 0.03% beta-mercaptoethanol before use. Add BSA to the surface and allow to dissolve, do not stir.
2) Sucrose solutions are percent (w/v) prepared in 10 mM TES-Na^+ pH 7.2, 10 mM EDTA and autoclaved.
3) 50 mM Tris-HCl, 10 mM EDTA (autoclave).

59. Non-aqueous method for purification of cytoplasmic DNA from individual rice plants for RFLP analysis

Shang-Hong ZHANG, Andreas M. DALLY and Gérard SECOND
Centre Emberger, CNRS, B. P. 5051, 34033 Montpellier Cedex, France

A non-aqueous method for the purification of chloroplast DNA (ctDNA) was developed and successfully applied to wheat (Bowman and Dyer 1982). However, in our experience, it can hardly be applied directly to rice. Also, there is no report on the isolation of mitochondrial DNA (mtDNA) from dried plants, which has some advantages: easier sampling, possibility of mailing and long term conservation, etc.

Several important improvements have been made in the method of purification of ctDNA of rice in non-aqueous media (Dally 1988). Among them are 1) the less dangerous 1, 2, 4-trichlorobenzene (CB) and decahydronaphthalene (HN) are used instead of carbon tetrachloride and n-hexane in the isolation of chloroplasts, allowing mechanical blending in addition, and 2) to prevent nuclear contamination, 4% Triton X-100 is used for the first lysis. At a low temprerature, this detergent destroys preferentially the organellar membranes, so that most of cytoplasmic DNA may be recovered in the supernatant while (the contaminant) nuclear DNA can be precipitated.

A 4:1 (v:v) mixture of CB and HN is used, with a protective layer of 1:3 mixture overlaid. After centrifugation, a band containing chloroplasts is collected at the 4:1/1:3 interface. Eventually, there are mitochondria in this collection, but they are in negligible quantities compared with chloroplasts. In our trials,

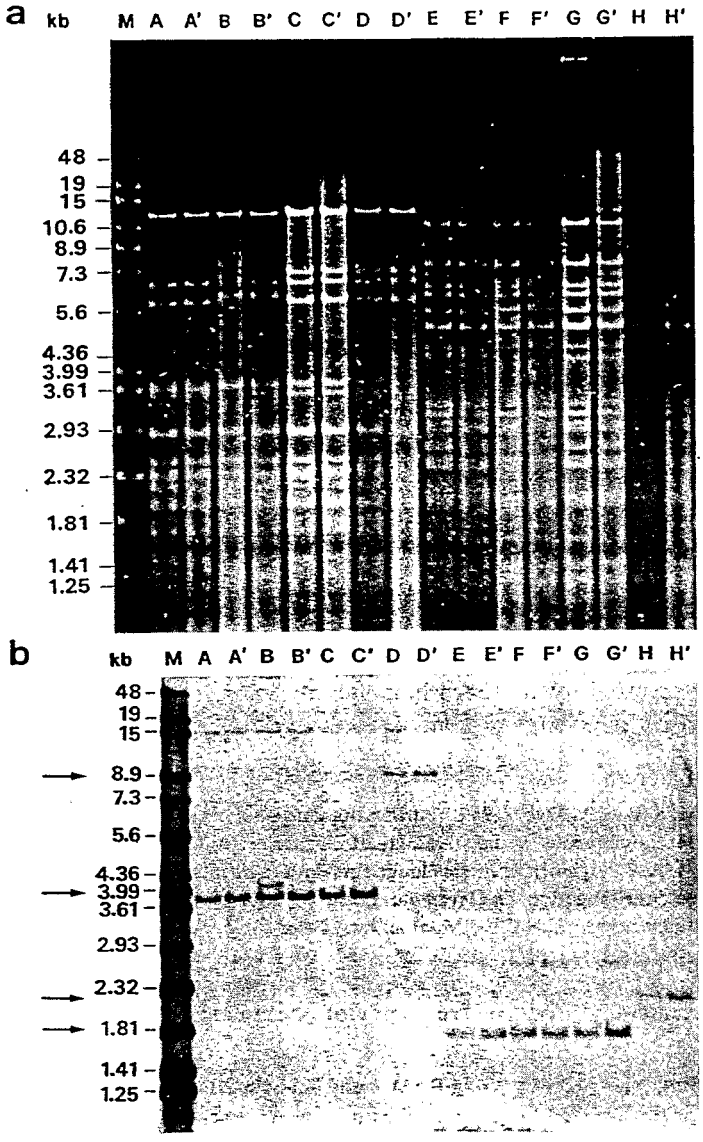

Fig. 1. a) Rice cytoplasmic DNA digested by EcoRI (A–D) and AvaI (E–H).
b) The same pattern revealed by Southern blot hybridization with a cytochrome oxidase II (COX II) probe from wheat.

In both a) and b), M is a size marker (Raoul from Appligene). The samples used are: *O. rufipogon* (OR42) in A and E, *O. officinalis* (W1198)in B and F, *O. sativa* (V20A, male sterile) in C and G, and *O. rufipogon* (W1187) inD and H. In each sample, lanes A, B, etc. are from the upper band (lighter band), and lanes A′, B′, etc. are from the inferior band (denser band) in the preparation. In b), arrows on the left indicate the major hybridization fragments which should correspond to mtDNA. Note that the inferior band gives a better yield, especially when the DNA-quantities are small (D versus D′; H versus H′). The origin of other fragments revealed by the probe are uncertain, perhaps they are nuclear DNA, but are not ctDNA. In B. thet wo additional fragments which do not exist in other lanes are perhaps due to an incomplete digestion.

the results of Southern blot hybridization with a wheat mtDNA probe were irregular with this preparation.

Contrary to the situation in aqueous solution, we found that the mitochondria are a little denser than the chloroplasts in the mixture of organic solvents used. Thus, for the enrichment of mitochondria, various combinations of solvent mixtures such as 1:0 (pure CB) with 4:1, 9:1 with 4: 1, 8:1 with 6:1 were tried, but the following procedure gave the best results, using a 8:1 mixture of the same solvents (d=1.38) for blending and intercalating a layer of 4:1 mixture (d=1.34) in between the 8:1 and 1:3 layers.

Two bands appear after centrifugation at the interface between 4:1 and 1:3 (upper band), and at the interface between 8:1 and 4:1. Though the contamination of chloroplasts is inevitable (Fig. 1a), the inferior band is enriched with mitochondria as can be judged from Southern blot hybridization with a nonradioactively labeled wheat mtDNA cytochrome oxidase II probe (Fig. 1b). In addition, from the DNA obtained from the inferior band, traces of restriction fragment pattern of mtDNA can in some cases be seen directly along the ctDNA pattern in the ethidium bromide stained electrophoresis gel (Fig. 1a).

So the upper band recovered by the centrifugation may be used for the preparation of cytoplasmic DNA, in which mtDNA concentration is sufficiently high to be easily revealed by Southern blot hybridization.

References

Bowman, C. M. and T. A. Dyer, 1982. Purification and analysis of DNA from wheat chloroplasts isolated in nonaqueous media. Analytical Biochemisstry **122**: 108–118.

Dally, A. M., 1988. Polymorphisme des longueurs des fragments de restriction de l'ADN chloroplastique dans la section *Eu-Oryza* du genre *Oryza* (riz) et implications phylogénétiques. Thèse de Doctorat. U.S.T.L., Montpellier. 160p.

60. Enhancement of division frequency of protoplasts and plant regeneration in rice

Bao-Lin SUN, Xiang-Hui LI, Yong-Ru SUN and Shi-Min ZHAO

Institute of Genetics, Academia Sinica, Beijing 100012, China

Calli were initiated from young inflorescences of *Oryza sativa* L. on MS medium supplemented with 2 mg/l 2,4-D, 3% (w/v) sucrose and 1% (w/v) agar adjusted to pH 5.6–5.8. The calli of 1–1.5 g were introduced into a flask containing 30 ml of AA liquid medium. The suspension cell line was established after being cultured for 7–8 weeks on a gyratory shaker at 100 rpm.

Protoplasts were isolated from established cell line at 3–4 days after subculture. Protoplasts were cultured at densities between $2-5\times10^5$/ml in KM8p media solidified with 0.8% (w/v) agarose. KM8p liquid medium was added when the agarose drops were solidified. The percentage of the protoplasts having divided