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Biochemical and molecular analysis of plants derived from embryogenic tissue cultures of napier grass (*Pennisetum purpureum* K. Schum)

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Summary. We have investigated the extent of biochemical and molecular variation in 63 plants of napier grass (*Pennisetum purpureum* K. Schum.) regenerated from 3- to 24-week-old embryogenic callus cultures. The calli were derived from cultured basal segments of young leaves and immature inflorescences obtained from a single field-grown donor plant. The entire population was analyzed for the activity of 14 isozyme systems, but no qualitative variation was found at any of the loci examined. Similarly, no restriction fragment length polymorphisms (RFLPs) were detected in the mitochondrial, plastid and nuclear genomes in a representative sample of regenerated plants. Our results confirm earlier reports of the genetic uniformity of plants derived from somatic embryos and highlight their value both for clonal propagation and for genetic transformation.

Key words: Gramineae – napier grass- Somatic embryogenesis – Variation – *Pennisetum purpureum*

Introduction

Organized meristems and "germ line" cells are generally immune to epigenetic and permanent genetic (mutation) changes that occur during cell division and differentiation in vivo and in vitro. On the other hand, most cell cultures and differentiated plant tissues are polysomatic and genetically heterogeneous (D'Amato 1985; Vasil 1987). Tissue cultures have been said to be useful for recovering pre-existing as well as tissue-culture-generated variability (somaclonal variation), which could be used in plant breeding (Larkin and Scowcroft 1981). Unfortunately, much of the variation recovered and described from tissue cultures thus far has proven to be neither novel nor agronomically useful. As a result, to date there is not a single instance where somaclonal variation has resulted in the introduction of a genetically stable and superior plant variety of any major crop that is grown commercially on a large scale and can not be obtained by conventional breeding (Vasil 1990).

Tissue culture methods are now widely used for the clonal propagation of plants. However, the presence of considerable genetic variation in the cultures as well as in the plants derived from them is a matter of serious concern in clonal propagation and genetic transformation. Therefore, any system which significantly reduces or eliminates tissue culture generated variation can be of much practical utility. In most species of the *Gramineae*, plant regeneration in vitro takes place through the formation of somatic embryos (Vasil and Vasil 1986; Vasil 1988). The presence of variant cells in the embryogenic tissue and cell cultures of gramineous species is not uncommon. It is significant, however, that no phenotypic or cytological variation is seen in plants regenerated from such heterogeneous cultures (Swedlund and Vasil 1985). It has thus been proposed that there is stringent selection against cytologically aberrant cells during the formation of, and the regeneration of plants from, somatic embryos (Swedlund and Vasil 1985; Vasil 1987). Recent studies have provided further evidence to support this theory (Cavallini et al. 1987; Cavallini and Natali 1989; Gmitter et al. 1991).

The recovery of plants which are phenotypically and/or cytologically similar to the donor plants has been described from embryogenic tissue cultures of *Pennisetum glaucum* (= *P. americanum*, Vasil and Vasil 1981; Swedlund and Vasil 1985) and *P. purpureum* (Haydu and Vasil 1981; Wang and Vasil 1982; Chandler and Vasil

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1984; Rajasekaran et al. 1986). In the few instances where variation was recovered, it was shown to be a manifestation of the pre-existing variation within the explants used for culture (Morrish et al. 1990).

The present study was undertaken to determine if plants derived from embryogenic cultures of *Pennisetum purpureum* K. Schum. (napier grass) contain, or are free of, any single gene mutations that could be screened by biochemical and molecular analyses and probes. We carried out a biochemical and molecular characterization of 63 regenerated plants by isozyme analyses and by screening for restriction fragment length polymorphisms (RFLPs) in the mitochondrial, plastid and nuclear genomes.

Materials and methods

Induction of callus and plant regeneration

Young leaves and inflorescences from a field-grown clone of *Pennisetum purpureum* (accession number PP10) were used to induce embryogenic callus on semi-solid basal MS medium (Murashige and Skoog 1962) supplemented with 1 mg/l NAA (α-naphthaleneacetic acid), 0.5 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.5 mg/l BAP (6-benzylaminopurine), 5% v/v coconut milk and 0.2% w/v Gelrite (Scott Laboratories). The induction of embryogenic callus and its selection were as described by Haydu and Vasil (1981). The white and compact embryogenic callus was transferred to regeneration medium (semi-solid MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP) 3, 6, 12, 18 and 24 weeks after the initiation of cultures and maintained in an illuminated growth chamber at 27 °C and under a 16-h light cycle for 4 weeks. The plantlets were transferred to tubes [150 mm (l) × 25 mm(dia)] containing the same medium and maintained for 3 weeks to allow for root and shoot elongation before transfer to soil [4 parts Metromix 300 (Grace Horticultural Products) to 1 part Perlite (Chemrock Industries)] in the greenhouse. Eventually, the plants were planted out in the field alongside the parental clone. Plants regenerated from leaf-derived callus were identified by their callus pedigrees, and individual regenerants in each pedigree were assigned ascending numbers (i.e. C1R1, C2R1, C2R2, ... etc.). A total of 57 plants were obtained from 11 leaf-derived callus pedigrees, and 6 plants were obtained from inflorescence-derived callus pedigree Inf3.

Isozyme analysis

Fresh leaf tissue was collected from the field on dry ice and stored at −80 °C for later use. The procedures described below were optimized by Mr. L. F. Pedrosa (unpublished results). Samples were ground in the cold in a home-made multi-well acrylic grinding unit in 0.5 v/w grinding buffer (0.2 *M* TRIS-HCl pH 7.8, 60% glycerol and 0.2% β-mercaptoethanol). The crude extract was soaked up on 1.5 mm × 12 mm strips of GB003 blotting paper (Schleicher and Schuell). Excess wicks from individual samples were stored at −80 °C for future use. Starch gels were prepared using 13% w/v starch (Sigma Chemicals, catalog # S-4501) in a home-made mould with gel dimensions 184 mm × 158 mm × 6 mm and buffer systems B (histidine-citrate pH 5.7), C (boric acid-lithium hydroxide pH 8.3) and D (histidine-citrate pH 6.5) as described by Stuber et al. (1988). Sample wicks were loaded on the gel and run for 15 min, after which the wicks were discarded. The activity of each enzyme system, along with its power requirements, were optimized using the different gel buffer systems mentioned above. The gels were run at 35 mA constant current for 4 h at 4 °C. To keep the gel cool during the run, a glass plate with an icepack was placed on the gel. Gels were sliced into 3-mm-thick sections and stained for enzyme activity. Recipes for activity staining of different enzymes were obtained from Vallejos (1983) and Stuber et al. (1988). The enzymes visualized for activity are listed in Table 1. Extracts from all the regenerants were run on two gels, with each containing a representative lane of the parental clone.

Table 1. Isozymes stained for positive activity

Acid phosphatase α- (α-ACP)
Acid phosphatase β- (β-ACP)
Alcohol dehydrogenase (ADH)
Aspartate aminotransferase (AAT)[a]
Esterase α- (α-EST)
Esterase β- (β-EST)
Endopeptidase (ENP)
Glutamate dehydrogenase (GDH)
Hexokinase (HEX)[a]
Malate dehydrogenase (MDH)
Malic enzyme (ME)
6-Phosphogluconate dehydrogenase (PGD)
Phosphohexose isomerase (PHI)
Shikimic acid dehydrogenase (SAD)

[a] Refers to enzymes stained according to procedures described by Vallejos (1983), other stains according to Stuber et al. (1988)

Mitochondrial DNA analysis

Mitochondrial DNA (mtDNA) was extracted according to the procedures of Chowdhury and Smith (1988) from the soft basal regions (including young leaves and stem) of field-grown tillers. Tissues were ground in saline buffer using a Waring blender, and intact mitochondria were fractionated from the mixture by differential centrifugation. Extramitochondrial DNA was eliminated with a DNase treatment. The mtDNA was then isolated after treatment with potassium acetate to eliminate carbohydrates and phenol-chloroform and chloroform to remove proteins. DNA was precipitated using isopropanol. MtDNA was isolated from a total of 24 samples, including the parental clone PP10. Each of the callus pedigree groups were represented, and plants were selected at random from pedigrees of more than 2 individuals. MtDNA extracted from the callus as well as the inflorescence pedigrees was subjected to restriction analyses using four different endonucleases (*Bam*HI, *Hin*dIII, *Pst*I and *Sal*I). Restriction of mtDNA was carried out using 10–20 units of endonuclease at 37 °C for 3–4 h and buffers specified by the manufacturer. The restricted samples were run on 0.8% w/v agarose gels made with TPE buffer (80 m*M* TRIS-phosphate and 2 m*M* EDTA, pH 8.0) in a large gel unit (25 cm × 20 cm) at 2 V/cm for 16 h. *Hin*dIII-digested DNA fragments from bacteriophage lambda were run in a lane alongside as size markers. Following the run the gels were stained with 0.5 μg/ml ethidium bromide for 45 min and destained for 10 min in deionized water. Stained gels were photographed on a Fotodyne UV transilluminator (model 3-3500). These gels were then depurinated in 0.25 *M* HCl for 10 min, denatured for 30 min in a 0.6 *M* NaOH and 1.5 *M* NaCl solution and neutralized for 30 min in a 1 *M* TRIS and 1.5 *M* NaCl solution. The DNA was bidirectionally blotted onto Hybond-N nylon membrane (Amersham Corp)

and covalently linked to the membrane by UV irradiation for 3–5 min. The nylon blots were prehybridized overnight at 65 °C in a 0.5 *M* Na_2HPO_4 buffer (pH 7.2) containing 7% SDS (sodium dodecyl sulfate) and 1% BSA (bovine serum albumin). Radioactively labelled probes were prepared using ^{32}P-labelled dCTP and the random priming method of Feinberg and Vogelstein (1983). Hybridization was also carried out overnight at 65 °C after adding the radioactively labelled, denatured probe to the pouch containing the membrane. The membranes were subjected to two 15-min washes in 3 × SSC (1 × SSC-0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0) at 65 °C. Washed blots were exposed to Kodak XAR-5 X-ray film with Cronex Hi-Plus (E. I. Dupont de Nemours and Co) intensifying screens. The duration of exposure to the X-ray film was dependant on the signal strength, which was typically from 1 day to 1 week, or sometimes longer.

Plasmids containing maize mitochondrial clones were digested with the appropriate endonuclease, and the fragment(s) of choice was removed from the gel using a NA45 membrane (Schleicher and Schuell), as specified by the manufacturer. Maize clones with mitochondrial genes cytochrome c oxidase subunit I (*coxI*, Isaac et al. 1985), subunit II (*coxII*, Fox and Leaver 1981), F1-F0 ATPase subunit 6 (atp6, Dewey et al. 1985a), subunit 9 (atp9, Dewey et al. 1985b), subunit alpha (atpA, Braun and Levings 1985) and 18S-5S ribosomal RNA genes (18S, Chao et al. 1984) were provided by Dr. C. S. Levings, III (North Carolina State University, Raleigh, N.C., USA). Maize mitochondrial cosmid probes 2A8, 6B9 and 4D12 were provided by Dr. D. R. Pring (University of Florida). Wheat mtDNA probes K', K3 and X2 were provided by Dr. A. Rode (University of Paris, Orsay, France).

Analysis of total DNA

Total DNA was extracted from leaf tissue previously ground in liquid nitrogen and stored at −80 °C. The methods used for the isolation of total DNA were those of Dellaporta et al. (1983) and a modified Appels and Dvorak (1982) procedure as described by Brears et al. (1989). Total DNA from both the callus and inflorescence pedigree types was digested using restriction endonucleases *Eco*RI, *Hin*dIII and *Pst*I. The DNA was analyzed by blotting onto nylon membranes and hybridizing with probes from the maize chloroplast genome, the wheat nuclear genome and the napier grass nuclear genome. The restriction of total DNA was carried out using approximately 10 µg DNA with 40–60 units of endonuclease, this was digested at 37 °C for 4–6 h. Restricted DNA was run on 0.8% w/v agarose gels in TPE buffer at 1 V/cm for 16 h. Staining and photographing of the gels was done as described above. DNA from the gels was blotted onto Hybond-N nylon membranes using the Southern blotting technique described by Maniatis et al. (1982). Hybridization techniques were the same as described for mtDNA.

Random maize cpDNA cosmid probes CtA5 and CtB9 were provided by Dr. D. R. Pring (University of Florida) The wheat *Nor* locus gene was provided by Dr. B. S. Gill (Kansas State University, Manhattan, Kan.). Random nuclear probes 1141, 1181 and 1185 from the *P. purpureum* genome were provided by Dr. M. K. U. Chowdhury (University of Florida).

Results and discussion

To determine the genetic fidelity of a population derived from in vitro culture, it is important to examine biochemical and molecular aspects in addition to cytological and morphological characters. Since enzymes are coded for by genes, any disruption in the coding sequence even at the single base level could force variations in the expression of the enzyme, leading to an altered individual. Any aberrations in the DNA structure that lead to an altered expression in enzymes belonging to major biochemical pathways would most likely be lethal and hence not expressed. This study, therefore, involved the use of a large number of enzymes and random DNA probes from nuclear as well as extranuclear sources to scrutinize the regenerants.

Isozyme analysis

Isozyme studies have been especially useful in tissue culture analysis. Enzymes like ADH, AAT and EST, amongst others, have been used for the analysis of tissue-culture-derived progeny. The present investigation has scrutinized a larger number of enzymes than most other studies, including those that detected variation (Brettell et al. 1986; Davies et al. 1986; Allichio et al. 1987; Dahleen and Eizenga 1990). The uniformity observed in the banding patterns of all the isozymes analyzed in this report shows the absence of biochemical aberrations in the regenerants at the tested loci.

No qualitative differences were found in the banding patterns of any of the samples tested, demonstrating the absence of variation at the loci examined. Occasional discrepancies in the intensity of bands between individual lanes were attributed to the inherent nature of the starch gel technique, where the proteins loaded on each wick can not be quantified, and hence quantitative differences were disregarded. Figures 1 and 2 represent the gels stained for ADH and HEX activity, respectively.

Mitochondrial DNA analysis

Each of the mtDNA restriction profiles yielded between 30 and 50 bands on the gel. However, none of the gels showed any differences between the restriction profiles of individual lanes. Figure 3 shows the restriction profile of the mtDNA digested with *Pst*I. Hybridization of the mitochondrial probes atpA, atp6, atp9, *cox*I, *cox*II, 18S (Figs. 4, 5) and the random maize mitochondrial probes 2A8, 6B9, and 4D12 to mtDNA blots did not yield any variant patterns, confirming the absence of any qualitative differences in the banding patterns of representatives from both pedigree types.

Nuclear and cytoplasmic genomes have been previously used in the analysis of variability arising in vitro. In the Texas type male-sterile cytoplasm of maize, a reversion to fertility is exclusively associated with the deletion of the T-*urf*13 mitochondrial gene or a disruption in its activity (Brettell et al. 1980; Pring and Lonsdale 1989). Such a rearrangement, induced in vitro, is characteristic

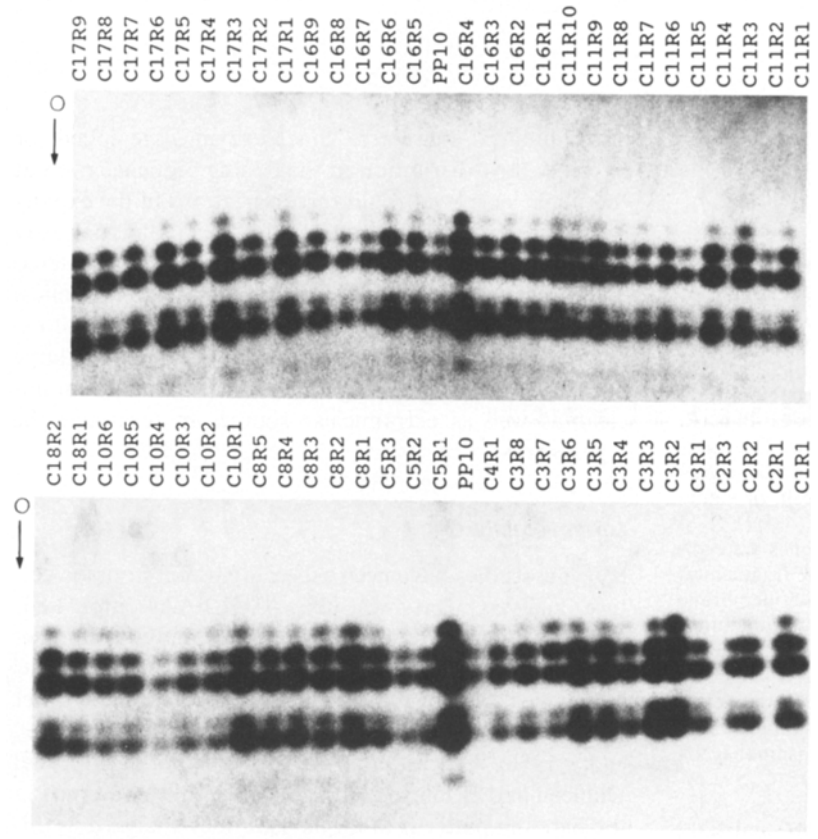

Fig. 1. Starch gel representing all the individuals of the leaf-derived callus pedigree, stained for ADH activity. *PP10* is the parental clone used as the control

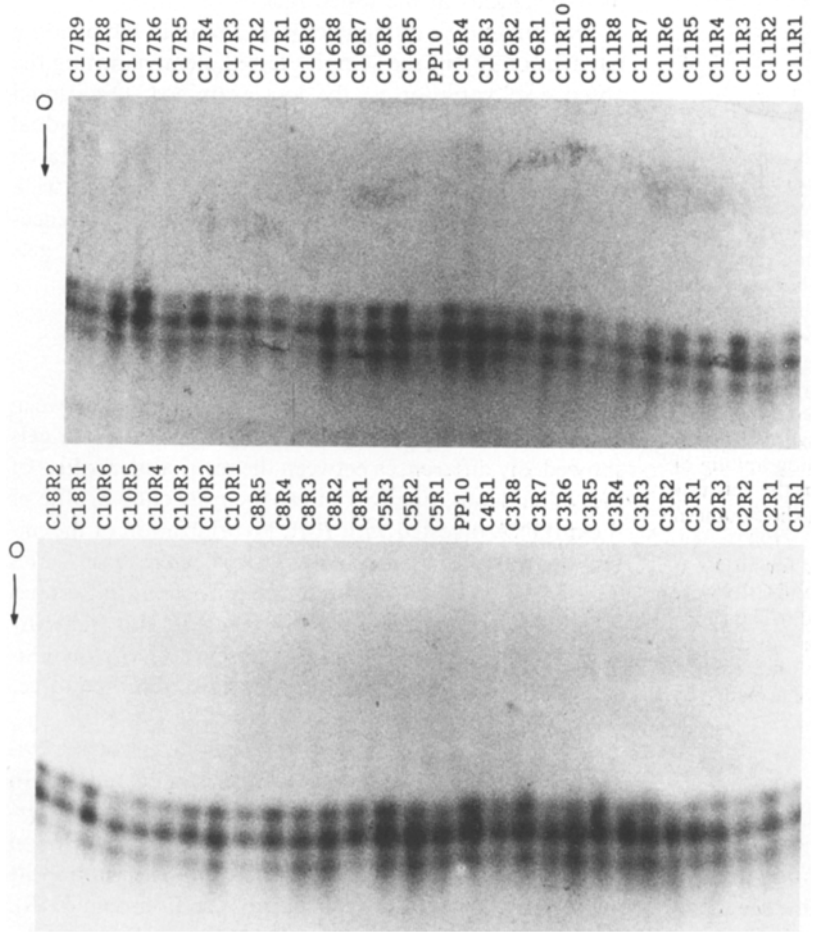

Fig. 2. HEX activity of the population from leaf callus, observed on a starch gel. *PP10* is the parental clone used as the control

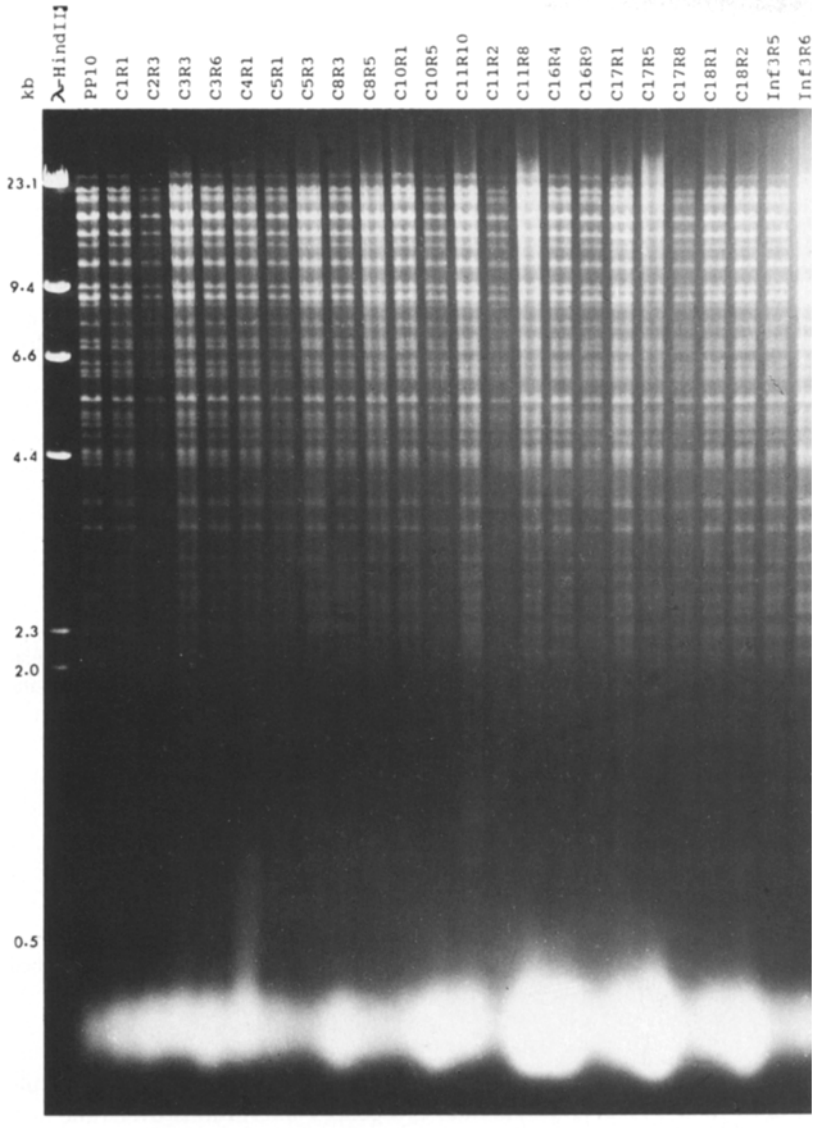

Fig. 3. Restriction profile of the mitochondrial genome of *P. purpureum* (accession *# PP10*) after digestion with endonuclease PstI

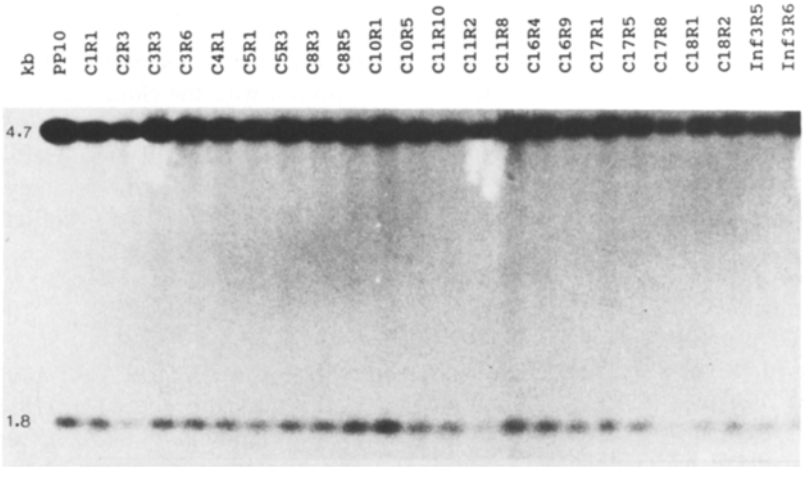

Fig. 4. HindIII-digested mtDNA blot probed with the maize mitochondrial gene *coxII*

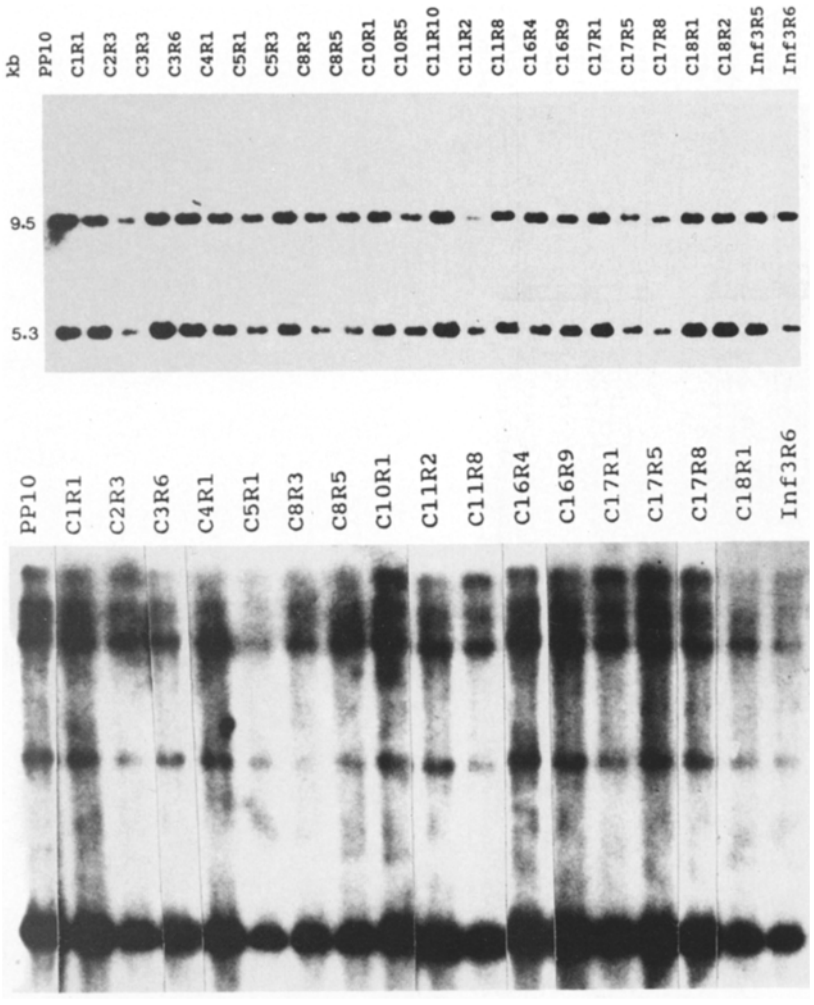

Fig. 5. Blot containing BamHI-digested mtDNA probed with the maize mitochondrial gene 18-S

Fig. 6. SalI-digested mtDNA blot probed with wheat mitochondrial probe K′

of only the T-type male-sterile cytoplasm in maize and has not been shown to occur elsewhere. In the mitochondrial genome of *Brassica campestris*, the restriction profile from in vitro cultures showed the presence of rearrangements. However, the native plant tissue also showed similar rearrangements at a much lower level, and it was concluded that there was a selection towards this pattern and an amplification in tissue culture (Shirzadegan et al. 1989). Long-term cultures of wheat (Hartmann et al. 1987, 1989) and rice (Chowdhury et al. 1988; Saleh et al. 1990) have been reported to possess an altered mtDNA profile when compared to mtDNA from short-term cultures. These cultures were also reported to lose their ability to form embryogenic callus. In the present study such differences were not observed in plants recovered from 3- to 24-week-old embryogenic callus cultures. This supports the view that there is a direct selection in vitro towards genetically normal cells in the formation of somatic embryos, and the plants obtained from somatic embryos are stable. It also seems to corroborate, at a molecular level, the evidence presented by Murashige and Nakano (1966, 1967), that in vitro cultures accumulate cytogenetic aberrations over time and consequently lose their regenerative capacity.

A region of the wheat mitochondrial genome that has been reported to undergo extensive rearrangements when the plants are introduced into culture has been termed as a "hypervariable" region (Hartmann et al. 1987, 1989; Rode et al. 1987). Blots from the *Sal*I-digested DNA were subjected to hybridization with the clones K′, K3 and X2 characterized from the hypervariable region of the wheat mitochondrial genome (Rode et al. 1987; Hartmann et al. 1987). These probes also did not detect any aberrations in the mtDNA hybridization profiles (Fig. 6).

Analysis of total DNA

The random maize chloroplast cosmid probes CtA5 and CtB9 and random napier grass nuclear probes 1141, 1181 and 1185 did not detect any variation in all the samples analyzed (Fig. 7). The nucleolar organizer region (*Nor*)

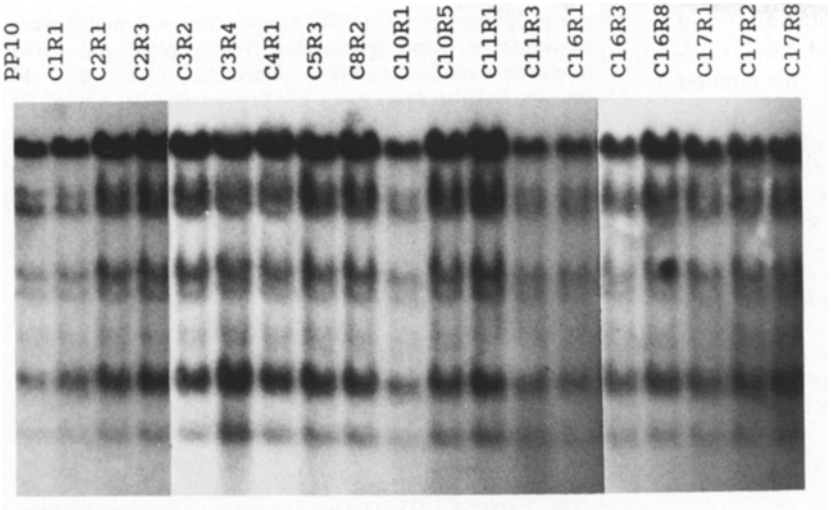

Fig. 7. Blot of total DNA digested with *Eco*RI and probed using a random cosmid probe from the maize chloroplast genome

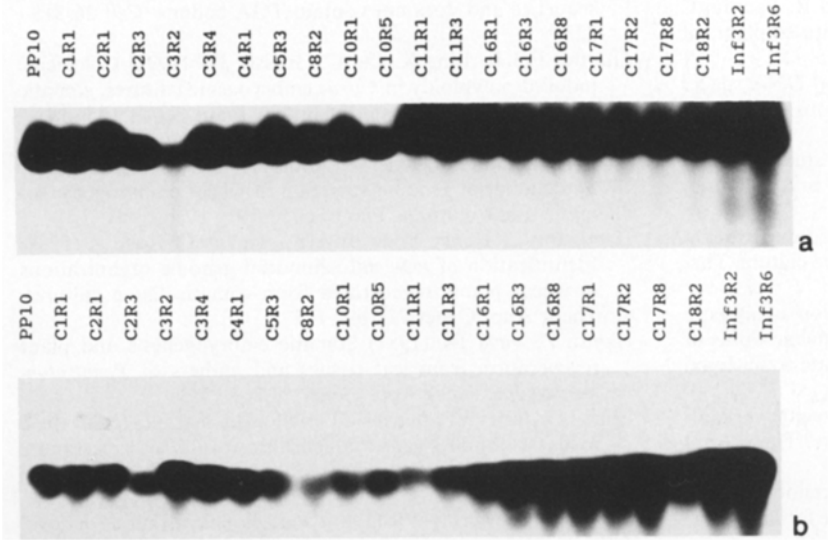

Fig. 8. **a** Blot of *Eco*RI-restricted total DNA probed with the *Nor* locus gene from wheat. **b** *Hin*dIII-digested total DNA blot probed with the *Nor* locus gene from wheat

locus gene from the wheat nuclear genome was of particular interest in the identification of aberrations. However, the variability of the *Nor* locus in tissue-culture-derived wheat plants (Breiman et al. 1987) has been questioned by Breiman et al. (1989) since they reported the occurrence of such abnormalities at low frequencies in the parental population. The lack of variation at the *Nor* locus was reflected in the blots digested with the three restriction enzymes (*Eco*RI, *Hin*dIII and *Pst*I) and probed using the wheat *Nor* locus gene (Fig. 8). The absence of variability at the *Nor* locus in our study further emphasizes the uniformity of the regenerants.

Different populations of *P. purpureum* have been shown to exhibit distinct RFLPs between them (Smith et al. 1989) when probed using random nuclear DNA probes from the napier grass genome. DNA blots of total DNA restricted with all the three restriction enzymes (*Eco*RI, *Hin*dIII and *Pst*I) were probed with the probes (1141, 1181, 1185) used by Smith et al. (1989) but did not expose any RFLPs.

In conclusion, plants derived from somatic embryos are stable at the biochemical as well as molecular levels. This observation is based on the activity stains of 14 isozyme systems, four mtDNA restriction profiles and more than 30 mitochondrial, chloroplast and nuclear DNA hybridization profiles. Our results show that regenerants from somatic embryos of *P. purpureum* do not contain mutations at any of the loci examined and directly support the view that plants derived from somatic embryos are genetically stable and true to type. Such a predictability of the outcome is imperative for genetic manipulations and can be achieved by carefully selecting somatic embryos from biotechnological manipulations. Combined with earlier evidence of the lack of any phenotypic and cytogenetic variation in plants derived from embryogenic cultures (Haydu and Vasil 1981; Wang and

Vasil 1982; Chandler and Vasil 1984; Swedlund and Vasil 1985; Rajasekaran et al. 1986; Cavallini et al. 1987; Cavallini and Natali 1989; Gmitter et al. 1991), this method can be a powerful means for maintaining the genetic fidelity of plants regenerated from tissue culture.

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