Theor Appl Genet (1994) 87:821–828

Molecular analysis of plants regenerated from embryogenic cultures of wheat (*Triticum aestivum* L.)

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Received: 28 February 1993 / Accepted: 27 April 1993

Abstract. Total DNAs of plants regenerated from immature embryo-derived 2-month-old embryogenic calli of wheat (cultivars Florida 302, Chris, Pavon, RH770019) were probed with six maize mitochondrial genes (*atpA*, *atp6*, *apt9*, *coxI*, *coxII*, *rrn18-rrn5*), three hypervariable wheat mitochondrial clones (K′, K3, X2), five random pearl millet mitochondrial clones (4A9, 4D1, 4D12, 4E1, 4E11) and the often-used wheat *Nor* locus probe (pTA71), in order to assess the molecular changes induced in vitro. In addition, protoplast-derived plants, and 24-month-old embryogenic and non-embryogenic calli and cell suspension cultures of Florida 302 were also analyzed. No variation was revealed by the wheat or millet mitochondrial clones. Qualitative variation was detected in the non-embryogenic suspension culture by three maize mitochondrial genes (*coxI*, *rrn18-rrn5*, *atp6*). A callus-specific 3.8-kb *Hin*dIII fragment was detected in all four cultivars after hybridization with the *coxI* gene. The organization of the *Nor* locus of the plants regenerated from Florida 302 and Chris was stable when compared to their respective control plants and calli. The *Nor* locus in regenerants of Pavon and RH, on the other hand, was found to be variable. However, *Nor* locus variability was not observed in 14 individual seed-derived control plants from either Pavon or RH sources. In Pavon, a 3.6-kb *Taq*I or a 5.6-kb *Bam*HI + *Eco*RI fragment was lost after regeneration. In one of the RH regenerants, which lost a fragment, an additional fragment was observed.

Communicated by Y. Gleba
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Key words: RFLP – Tissue culture – *Triticum aestivum* – Wheat

Introduction

Wheat is an ancient and yet one of the most important and widely used crops in the world. In addition to the long established conventional breeding methods, wheat can also be improved by cellular and molecular techniques as demonstrated recently by the introduction of a herbicide-resistant gene by particle bombardment (Vasil et al. 1992). The utility of these methods depends on maintaining the genetic fidelity of cells during the long periods of culture and regeneration.

It has been suggested that a vast majority of the variations arising in vitro are neither novel nor significant (Karp et al. 1987; Saleh et al. 1990), and are either epigenetic (D'Amato 1985; Morrish et al. 1990) or perpetuate from the pre-existing polysomatic nature of the donor explant (Breiman et al. 1989; Morrish et al. 1990). Other reports claim that useful morphological, cytogenetical, and molecular variation may be generated in vitro (Larkin et al. 1989). These discrepancies may be due to several factors (Vasil 1987, 1988), such as the nature of the genotypes used (Breiman et al. 1987a, b), the pathways of regeneration (somatic embryogenesis vs organogenesis), and the parameters employed for assessing the effect of in-vitro culture such as gross morphology and cytology (Swedlund and Vasil 1985), field assessment (Rajasekaran et al. 1986), and molecular studies (Breiman et al. 1989; Chowdhury et al. 1990, Saleh et al. 1990; Shimron-Abarbanell and Breiman 1991; Shenoy and Vasil 1992).

DNA restriction fragment length polymorphism (RFLP) has been used frequently to compare wheat regenerants with their parent (Breiman et al. 1987a, 1989; Rode et al. 1987, 1988; Benslimane et al. 1988; Hartmann et al. 1989; Morere-Le Paven et al. 1992a, b). In most of these studies, regenerants were obtained from only a single source, such as calli derived from immature embryos or anthers, and only a few molecular probes were used to assess variation. Recently, Shimron-Abarbanell and Breiman (1991) and Shenoy and Vasil (1992) used a number of probes to examine regenerants of *Hordeum marinum* and *Pennisetum purpureum*, respectively; but in both studies the number of tissue sources used was very small. We have assessed the effect of in-vitro culture by comparing RFLPs of wheat tissues obtained from 17 different sources using 14 organelle DNA probes from maize, wheat and pearl millet and a wheat nuclear DNA probe.

Materials and methods

Plant materials used

Tissue samples were obtained (Table 1) from plants regenerated from immature embryo-derived calli of wheat (*Triticum aestivum*, cultivars Florida 302, Chris, Pavon and RH770019), regenerants from protoplast-derived calli of Florida 302 (FL302), 2-month-old embryogenic calli from all four cultivars, embryogenic, non-embryogenic suspension, non-embryogenic calli, and 24-month-old embryogenic calli from FL302 (for details see Redway et al. 1990a, b; Vasil et al. 1990).

DNA extraction

Tissues were harvested in liquid nitrogen and stored at −80 °C. Frozen tissue from each sample was ground in a cold mortar and pestle and total DNA was extracted using methods described either by Dellaporta et al. (1983) or Saghai-Maroof et al. (1984). DNA was quantified spectrophotometrically by measuring optical densities at 260 and 280 nm (Sambrook et al. 1989).

Preparation of probes

Both nuclear and mitochondrial (mt) probes were used. Maize clones containing the mitochondrial genes for cytochrome c oxidase subunit I (*coxI*, Issac 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 RNAs (*rrn18-rrn5*, Chao et al. 1984) were provided by Dr. C. S. Levings, III (North Carolina State University, Raleigh, N.C.). Pearl millet random mt probes (4A9, 4D1, 4D5, 4D12, and 4E11) were from a mtDNA library generated by Chowdhury and Smith (unpublished). Wheat mtDNA probes designated K′, K3 and X2 (Rode et al. 1987) were provided by Dr. A. Rode (University of Paris, Orsay, France). The wheat *Nor* locus probe (Gerlach and Bedbrook 1979) was provided by Dr. B. S. Gill (Kansas State University, Manhattan, Kan.). Probes (only inserts recovered from the gel using an NA45 membrane, Schleicher and Schuell, Inc.) were labelled using ^{32}P-labelled deoxycytidine 5′-triphosphate (CTP) by the random priming method of Feinberg and Vogelstein (1983).

Restriction endonuclease digestion, blotting and hybridization of DNA

Five micrograms of each DNA preparation was digested with restriction endonucleases using four units of endonuclease per μg of DNA for 4–6 h with the manufacturer's recommended buffer and digestion conditions. The digested DNAs were fractionated by electrophoresis in 0.8% agarose gels using TPE buffer (80 mM Tris-phosphate and 2 mM EDTA, pH 8.0). For fragment-size calculations, *Hin*dIII-digested lambda DNA molecular size markers were included. After electrophoresis, gels were stained for 45 min in 0.5 μg/ml of ethidium bromide, destained for 10 min in deionized water, and photographed under UV light. The migration distance of the size markers was measured. Southern blots were prepared by transferring the DNA to nylon membrane (Hybond-N, Amersham) as described by Sambrook et al. (1989) and covalently binding the DNA to the membrane using UV irradiation. The blots were hybridized overnight at 65 °C, washed twice in 3 × SSC and once in 0.3 × SSC, and autoradiographed using Kodak XAR-5 X-ray film with intensifying screens and variable exposures.

Table 1. Plant materials used for the analysis of wheat cultivars

Control	Florida 302 5(302PL)	Chris 5(CHPL)	Pavon 14(PAVPL)	RH770019 14(RHPL)
Embryogenic callus				
2-month-old (EC)	1	1	1	1
24-month-old (CE)	1	–	–	–
Non-embryogenic callus (CNE)				
24-month-old	1	–	–	–
Embryogenic suspension (SE)				
24-month-old	1	–	–	–
Non-embryogenic suspension (SNE)				
24-month-old	1	–	–	–
Regenerants				
Protoplasts	7	–	–	–
2-month-old (EC)	4	3	6	8

Results

Assessment of tissue culture-induced variability

Total DNA was isolated from individual plants, callus or suspension cultures. One gram of immature plant tissue yielded approximately 0.3–0.5 mg of DNA and that of callus or suspensions approximately 0.5–1.0 mg of DNA. A summary of the hybridization patterns obtained with the 15 probes (six maize mt genes, five random pearl millet mt clones, three hypervariable wheat mt clones, and the wheat *Nor* locus) hybridized to the blots containing total DNA digested with either *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, *Taq*I, or double digested with *Bam*HI + *Eco*RI, is presented in Table 2.

Analysis of mitochondrial gene polymorphism

Of the six maize mt gene probes (*atpA*, *atp6*, *atp9*, *coxI*, *coxII*, *rrn18-rrn5*) hybridized to the blots containing 31 samples of wheat total DNA (listed in Table 1) three (*atp6*, *coxI*, *rrn18-rrn5*) revealed variation (Fig. 1). Most of these variations were found in DNA isolated from the non-embryogenic suspension culture. Figure 1A (*coxI* probe hybridized to *Hin*dIII blot) shows three bands (10.7, 9.4, 5.7 kb) missing in non-embryogenic suspension culture (302SNE). A 3.8-kb fragment is present in the suspensions and calli of all the four cultivars but is absent in all the other lines. When the *rrn18-rrn5* probe was hybridized to the *Hin*dIII-digested blot, a 10.1-kb fragment was missing in 302SNE and also in one of the protoplast-derived plants (302RP1) but was present in all the other lines at variable intensities (Fig. 1B). A 3.1-kb fragment present in all the lines except 302SNE is shown in Fig. 1C (*coxI* probe hybridized to *Bam*HI + *Eco*RI blot) and a 6.9-kb fragment present in 302SNE is absent in all the other lines (Fig. 1D, *atp6* probe hybridized to *Hin*dIII blot).

Assessment of polymorphism using random mt DNA probes

Five random mt DNA probes from pearl millet (4A9, 4D1, 4D12, 4E1, 4E11) hybridized to either the *Hin*dIII- or *Eco*RI-digested total DNAs of wheat did not reveal any variation (data not shown).

Autordiograms obtained from the hypervariable wheat mt DNA probes (K′ hybridized to the *Hin*dIII- and *Sal*I-digested blots, X2 to the *Sal*I-digested blot,

Table 2. Results obtained from 15 probes hybridized to DNA of wheat cultivars and their regenerants digested with restriction endonucleases (N = no, Y = yes and * refers to the presence of variation)

Probe	Res. enz.	No. of bands	Band sizes in kb	Variation
atpA	*Eco*RI (E)	1	4.2	N
	*Pst*I	1	7.4	N
atp6	*Hin*dIII	5	9.4, 6.9*, 3.4, 1.5, 0.8	Y (qual)
	*Bam*HI (B) + E	4	7.9, 6.7, 3.6, 2.9	N
atp9	*Eco*RI	2	14.2, 4.2	N
	*Hin*dIII	4	13.5, 7.6, 3.2, 1.6	N
coxI	*Eco*RI	3	9.9, 3.6, 2.8	N
	*Hin*dIII	5	8.2*, 7.2*, 5.7*, 3.8*, 2.2	Y (qual)
	B + E	2	3.1*, 2.6	Y (qual)
coxII	*Pst*I	1	8.2	N
	*Eco*RI	2	4.1, 2.8	N
	*Hin*dIII	2	3.5, 2.3	N
18*S*	*Hin*dIII	4	10.1*, 5.5, 4.9, 3.6	Y (qual)
4A9	*Hin*dIII	2	9.4, 2.0	N
4D1	*Hin*dIII	2	5.4, 3.0	N
4D12	*Eco*RI	2	6.0, 5.3	N
4E1	*Hin*dIII	3	8.9, 3.7, 1.8	N
4E11	*Eco*RI	3	2.9, 1.7, 1.2	N
K′	*Eco*RI	2	9.6, 7.8	N
	*Hin*dIII	5	16.5, 4.1, 3.5, 1.7, 0.8	N
	*Sal*I	2	14.4, 10.9	N
	B + E	5	5.9, 5.2, 4.5, 3.9, 3.4	N
K3	*Eco*RI	4	7.2, 5.5, 4.7, 2.6	N
	*Hin*dIII	4	6.7, 3.6, 2.7, 1.8	N
	*Sal*I	2	19.0, 11.5	N
	B + E	6	7.4, 4.9, 4.3, 2.8, 2.2, 1.8	N
X2	*Sal*I	1	3.6	N
	*Eco*RI	4	20.9, 13.3, 5.3, 1.8	N
Nor	*Taq*I	7	3.6*, 3.1*, 2.9*, 2.7*, 1.7,	Y (qual)
	B + E	6	5.6*, 5.1*, 4.9, 4.5, 39, 1.1	Y (qual)

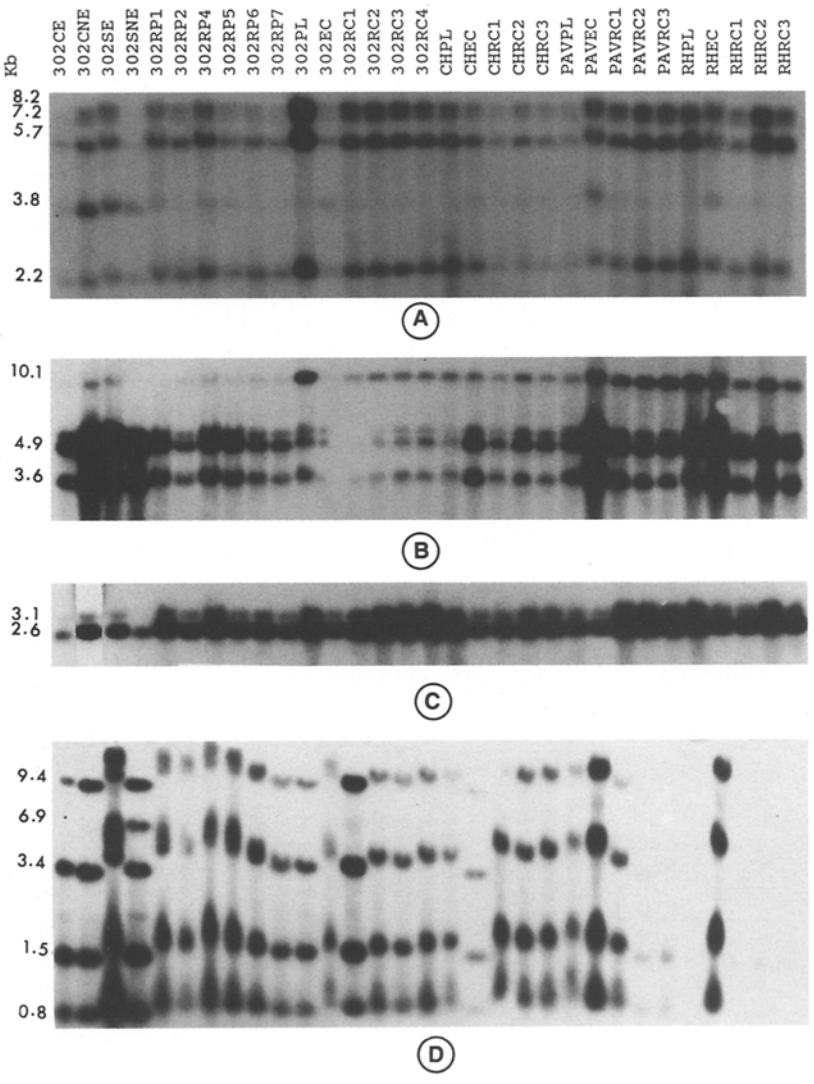

Fig. 1A–D. Hybridization patterns of maize mitochondrial genes to the total DNAs of wheat materials, listed in Table 1, digested with the restriction endonuclease *Hin*dIII hybridized by *coxI* (**A**), 18*s* (**B**), *atp6* (**D**), and with *Bam*HI + *Eco*RI hybridized by *coxI* (**C**)

and K3 to the *Eco*RI-digested blot) are presented in Figs. 2A, B, C and D, respectively. Except for K3, none of the probes detected DNA sequence differences in any of the lines tested. In addition, no variation was revealed by the K3 probe hybridized to blots of *Hin*dIII-, *Sal*I- or *Bam*HI + *Eco*RI-digested DNAs or by the K′ hybridized to the blots containing DNAs digested with either *Eco*RI, *Hin*dIII, *Sal*I or *Bam*HI + *Eco*RI (Table 2). The K3 probe detected a 4.7-kb *Eco*RI band missing in the protoplast-derived regenerants but all the other regenerants had the same pattern as their respective control plant. Except for *Sal*I, all the other restriction endonucleases used in this investigation digested the DNA completely. Even after obtaining the *Sal*I enzyme from two sources (BRL and Promega) and digesting for 8 h using the supplied buffer at the recommended temperature, the quality of *Sal*I-digested DNAs was not as good as the other enzyme-digested DNAs.

Assessment of Nor locus DNA polymorphism

When the *Nor* locus DNA was used to probe blots of total DNAs digested with *Taq*I, and double digested with *Bam*HI + *Eco*RI (Figs. 3A, B), no differences in hybridization patterns were revealed between the embryogenic and non-embryogenic cultures (calli or suspension), between the long term (24-month-old) and short term (2-month-old) calli, or between the regenerants derived from protoplasts, embryogenic calli, and the control plants of FL302. Similarly, the hybridization patterns of the Chris control plant, its 2-month-old embryogenic calli, and calli-derived regenerants, are the same for both the enzymes. Of the four cultivars

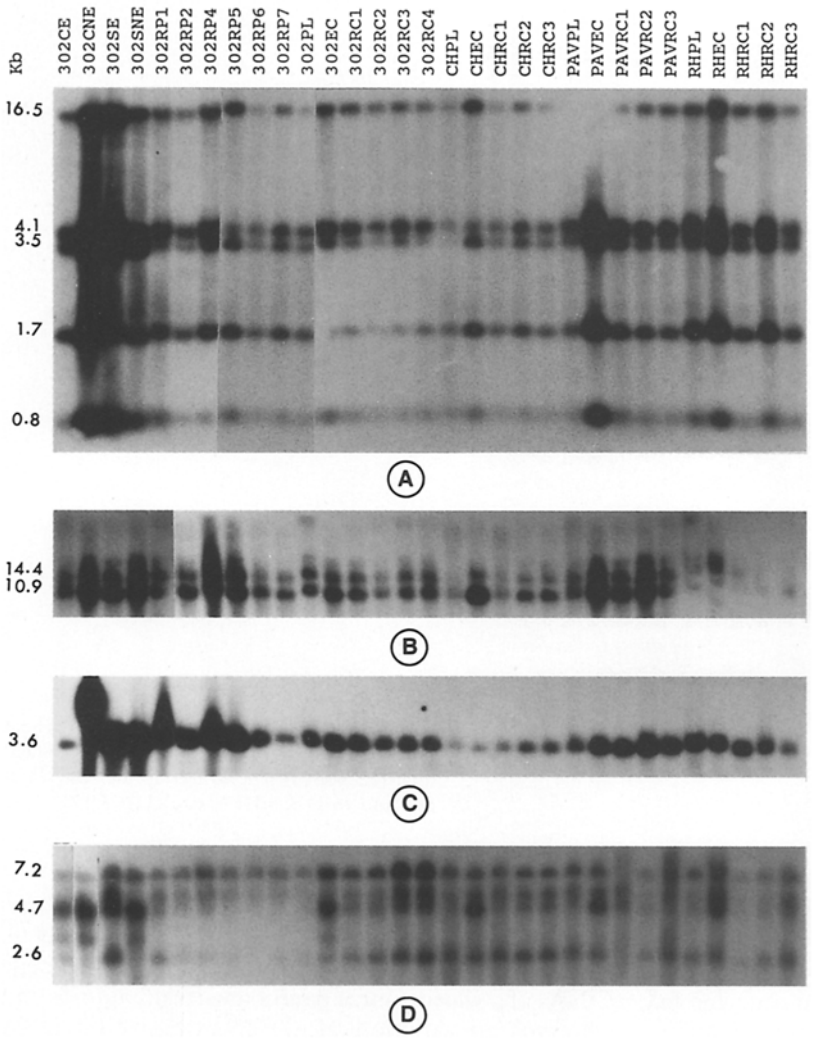

Fig. 2A–D. Hybridization patterns of hypervariable wheat mitochondrial DNA probes to the total DNAs of wheat digested with restriction endonuclease *Hin*dIII hybridized by K′ (**A**), *Sal*I by K′ (**B**) and X2 (**C**), and *Eco*RI by K3 (**D**)

(FL302, Chris, Pavon, RH) tested, two distinct hybridization patterns (one for FL302 and Chris, and the other for Pavon and RH) are revealed by both the enzymes. A 3.6-kb *Taq*I fragment present in Pavon and RH is absent in FL302 and Chris (Fig. 3A). Similarly, Fig. 3B shows that a 5.6-kb *Bam*HI + *Eco*RI (B + E) fragment present in Pavon and RH is absent in FL302 and Chris. No variation between the source parent and its calli was observed in any of the cultivars. Although no variation between the source parent and its regenerants was observed in FL302 or Chris, both Pavon and RH regenerants showed variation.

A 3.6-kb *Taq*I fragment present in the Pavon source plant and its calli was missing in PAVRC1 and PAVRC2, and only weakly present in PAVRC3 (Fig. 3A). In the RH cultivar, on the other hand, RC2 and RC3 have the same hybridization pattern as the source plant and its calli, but three *Taq*I fragments (3.6, 3.1, 2.9 kb) were missing in RC1 which also showed an additional 2.7-kb fragment. A 5.6-kb B + E fragment present in the Pavon source parent and its calli was absent in all the three regenerants (Fig. 3B). The same fragment was also present in the RH source parent, its calli and its regenerants RC2 and RC3. In RC1, the 5.6-kb fragment is missing but an additional 5.1-kb fragment was observed.

To verify whether the variation revealed in the regenerants of both the Pavon and RH cultivars was pre-existing in the source parents in a heterozygous form, DNA from a total of 14 individual plants from each source was double digested with B + E and hybridized with the *Nor* locus DNA probe. Hybridization patterns for all the individual plants for both the cultivars were the same, indicating no residual heterozygosity for the *Nor* locus in the source parents (Fig. 3C). Three more regenerants from Pavon and five more regenerants from RH analyzed for *Nor* locus variability showed patterns similar to their parents.

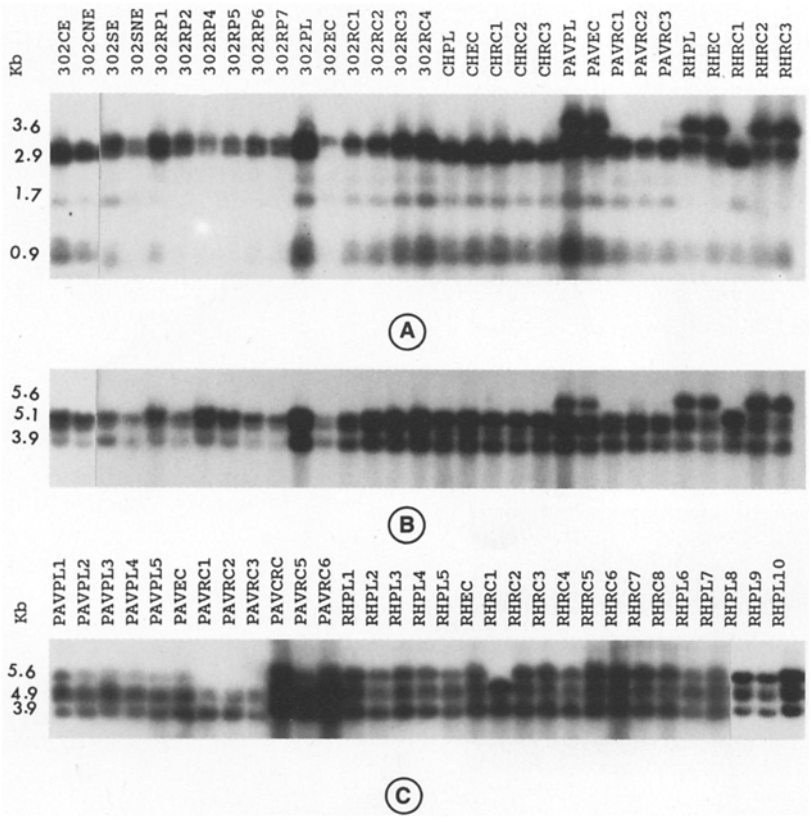

Fig. 3A–C. Hybridization patterns of wheat Nor locus probe to the total DNAs of wheat digested with restriction endonuclease *Taq*I (**A**), and with *Bam*HI + *Eco*RI (**B, C**)

Discussion

Our results show that no major variation occurred in wheat mt DNA during short-term culture. Similar results have been reported in rice (Saleh et al. 1990), wheat (Rode et al. 1985), barley (Breiman et al. 1987b; Shimron-Abarbanell and Breiman 1991), Napier grass (Shenoy and Vasil 1992) and sugarcane (Chowdhury and Vasil 1993). Chowdhury and Vasil (1993) used six maize mt genes and three wheat hypervariable mt clones to examine regenerants of three cultivars of sugarcane and reported only minor variation in cell suspension cultures and second cycle regenerants. Similarly, Shenoy and Vasil (1992) detected no RFLPs in plants regenerated from young leaf-derived calli of *P. purpureum*. Shimron-Abarbanell and Breiman (1991) assessed tissue-culture-derived genetic variability among normal (green) regenerants of *H. marinum* using three random barley mt clones containing only non-coding sequences and the coding sequences of five of the mt genes used in the present study. They reported relative stability when assayed with coding sequences and one mutant was detected by one of the non-coding sequence clones. Saleh et al. (1990) found no variation in the *Bam*HI mt DNA restriction profile of leaves, a 2-month-old regenerable and a 19-month-old non-regenerable suspension culture of rice. Rode et al. (1985) compared the restriction patterns of organelle DNAs of a wheat source parent and its dihaploids and observed no variation. However, extensive mt DNA variations were detected by the three hypervariable mt clones in somatic tissue cultures initiated from immature embryos of two wheat cultivars (Hartmann et al. 1987; Rode et al. 1987, 1988).

In the present study qualitative variation was revealed in long-term non-embryogenic suspension cultures when *coxI* was hybridized to *Hin*dIII or *Bam*HI + *Eco*RI digests, when *rrn18-rrn5* was hybridized to a *Hin*dIII digest, and when *atp6* was hybridized to a *Hin*dIII-digested blot. Loss of fragment(s) was detected by the *coxI* and *rrn18-rrn5* probes, and a gain was detected by the *atp6* probe. Similar differences between control plant or embryogenic and non-embryogenic cultures were also reported in Chinese Spring and Aquila cultivars of wheat (Rode et al. 1988) and in rice (Chowdhury et al. 1988). In Chinese Spring, an 8-kb mt DNA region was missing in the non-embryogenic cultures in comparison to embryogenic cultures and a 17-kb region was missing in comparison to the control plant. In rice, both restriction and hybridization pattern variations were observed between a non-regenerable suspension culture and its control

plant. A callus-specific mt DNA fragment was also revealed in this investigation when *coxI* was hybridized to *Hin*dIII-digested DNA. A similar fragment was seen in callus cultures of Chinese Spring (Hartmann et al. 1987) but it disappeared after the first subculture (2 month) whereas in FL302 it persisted even in the 24-month-old cultures (present study). In the cultivar Aquila, however, a loss of at least one mt DNA fragment was reported in callus cultures (Rode et al. 1987). The gain or loss of fragment(s) in callus cultures could be due to mutations in the repeated sequences of the genome. Such mutations might trigger further genomic changes such as amplification, translocation, chromosome breakage, and transposition (Walbot and Cullis 1985).

The *Nor* locus probe contains both the non-transcribed spacer (NTS) and transcribed regions. The NTS region has been found to be variable between cultivars and species of wheat and barley but is relatively uniform within a cultivar (May and Appels 1987). It is thus an ideal marker to identify cultivars and to investigate in-vitro culture effects (Saghai-Maroof et al. 1984; Brettell et al. 1986; Benslimane et al. 1988). The *Nor* locus probe used in our investigation detected variation between the cultivars and also between regenerants of the Pavon and RH cultivars and their respective control plants. Variation between regenerants and control plants revealed by the *Nor* locus probe was also reported in wheat (Breiman et al. 1987a, 1989; Benslimane et al. 1988), and in triticale (Brettell et al. 1986).

None of the 15 probes used in the present investigation detected any variation in the regenerants of FL302 and Chris (immature embryo-derived). Only the *Nor* locus probe detected variation in three of the six regenerants tested in the Pavon, and in one of the eight regenerants tested in the RH, cultivars. These results indicate a genotypic influence on the revelation of variation rather than the in-vitro culture itself. Similar genotypic difference in tissue-culture-induced variation was reported earlier in wheat (Karp and Bright 1985; Breiman et al. 1987a; Benslimane et al. 1988; Mohmand and Nabors 1990) and in triticale (Brettell et al. 1986). Although Rode et al. (1987) reported the variation observed in the doubled haploids of spring wheat cv 'Cesar' as a tissue-culture effect, Benslimane et al. (1988) conducted a similar study in another genotype of wheat and concluded that the occurrence of *Nor* locus variation in response to in-vitro androgenesis could not be considered a general rule, but depended more upon the nature of the genotype used.

It is clear that the presence or absence, and the extent, of variation in tissue-culture regenerants is dependent on the genotype used (Breiman et al. 1987a, b; Mohmand and Nabors 1990) and the genetic heterogeneity of the explant (Benslimane et al. 1988; Breiman et al. 1989; Morrish et al. 1990). A number of recent molecular studies (Shimron-Abarbanell and Breiman 1991; Shenoy and Vasil 1992; Chowdhury and Vasil 1993; Valles et al. 1993) provide additional support for the view that there is a strong selection in favor of plant regeneration from normal cells in embryogenic calli (Vasil 1987, 1988). In the present investigation, variation was detected in two cultivars only by the *Nor* locus probe. We tested the presence of *Nor* locus heterogeneity in the control plants of these two cultivars by analyzing individual plants. Since no variation was observed in the individual control plants, the most likely explanation of the *Nor* locus variation present in the regenerants could be an in-vitro culture-induced variation. However, a similar conclusion drawn by Breiman et al. (1987a) from a comparable experiment based on the absence of polymorphism in a population of control plants was later questioned by them (Breiman et al. 1989) following experiments which permitted the detection of *Nor* locus heterozygosity in the individual control plants of the same cultivar (ND7532). Breiman et al. (1989) concluded that the question of *Nor* region variability in tissue-culture regenerants should be treated with caution.

Acknowledgements. We thank Dr. Phil Bruckner for growing our plant material in the field at Tifton, Georgia. Supported by the Monsanto Co. (St. Louis, Mo.). Florida Agriculture Experiment Station Journal Series No. R-03502.

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