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Nor loci analysis in progenies of plants regenerated from the scutellar callus of bread-wheat

A molecular approach to evaluate somaclonal variation

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Summary. Progenies of plants regenerated from scutellar callus of bread wheat (*Triticum aestivum* L.) were analysed for the organization of the intergenic spacer of the rRNA genes, located at the sites of the nucleolar organizer region (*Nor* loci). Sexual progenies derived from the regenerated plants of three wheat cultivars were subjected to this analysis. The respective DNAs were digested with the restriction endonuclease *Taq*I, and probed with a specific rDNA fragment by Southern blot-hybridization. The intergenic rDNA spacer could thus be characterized for each of the three cultivars. Thirty-eight progeny plants of the cultivars 'Chinese Spring' and 'Miriam' were found to be stable in their organisation of the *Nor* loci: no changes relative to the *Nor* of control plants from these cultivars were revealed. On the other hand, three progeny plants of 'ND7532' showed reduction in the number of the rDNA spacers. Since no variability in the *Nor* loci could be revealed among control 'ND7532' plants, this seems to indicate that the changes in the progeny of regenerated plants resulted from the in vitro culture of the scutellar callus. Grain glutenin and gliadin profiles of sexual progeny-plants derived from scutellar calli of 'Chinese Spring', of 'Miriam' as well as of 'ND7532' were identical to the respective control plants of these cultivars, indicating low (or no) somaclonal variation in these grain proteins in the analyzed plants.

Key words: Wheat – Ribosomal DNA intergenic spacer – Scutellum – Tissue culture – Somaclonal variation

Introduction

Larkin and Scowcroft (1981) suggested the term 'somaclonal variation' to abbreviate the phenomenon of variability among plants which were regenerated from in vitro cultured cells and tissues. Plant tissue-culture instability has been widely documented on the cytogenetic, morphological and biochemical levels, and comprehensive lists of species in which somaclonal variation has occurred have been reviewed (Karp and Bright 1985; Larkin and Scowcroft 1981; Scowcroft 1985). The occurrence and frequency of somaclonal variation is still a debated issue ranging from reports on uniformity in *Panicum maximum* (Hanna et al. 1984) to 40% variation in rice regenerants and their progeny (Sun et al. 1983). Evidence for somaclonal variation in the organellar and nuclear genomes has been documented in several species. Albino wheat plants regenerated from anther culture had large deletions in the chloroplast genome (Day and Ellis 1984), protoplast-derived potato variants were revealed to be deficient in the number of rRNA genes (Landsmann and Uhrig 1985) and a heritable reduction in the number of rRNA units was observed in regenerated triticale plants (Brettell et al. 1986).

Gosch-Wackerle et al. (1979) and Shimada and Yamada (1979) found independently that in bread wheat (*Triticum aestivum*) plant regeneration was obtained most efficiently from scutellar-calli derived from immature embryos. This method was subsequently adopted as the standard procedure to regenerate wheat from tissue culture. Different degrees of variation in morphological features, yield-components and biochemical traits (e.g. grain-protein composition and amylases) were found among wheat regenerants and their progenies. (Larkin et al. 1984; Ahloowalia and Sherington 1985; Maddock et al. 1985; Cooper et al. 1986).

The genes for 18S, 5.8S and 26S cytoplasmic ribosomal RNAs (*Nor* loci) in eukaryotes are organized in tandem arrays of repeats in the nucleolar organizer regions on one or several chromosomes.

The *Nor* loci of bread wheat were mapped on chromosomes 1B and 6B and studied at the molecular level. The intercultivar variation in spacer length involves the number of the

135 base-pair (bp) repeats which are clustered in the intergenic region. A single *Eco*RI site defines the repeating unit and this entire (approximately 9 kbp) sequence was cloned (Gerlach and Bedbrook 1979) and mapped (Appels and Dvorak 1982).

Since *Nor* loci are variable among wheat cultivars but uniform within each cultivar, we analysed these loci in order to evaluate somaclonal variation in this species. For this evaluation we used a cloned probe (pTA71, see Gerlach and Bedbrook 1979) to assay the spacer-length of these loci in sexual progenies of plants regenerated from the scutellum of immature wheat-embryos. In addition, we analysed the grain-protein profiles of such plants because these proteins show intra-cultivar stability but differ between bread wheat cultivars.

Materials and methods

Establishment, maintenance and regeneration of tissue cultures

Cultures were derived from the scutellum of immature-embryos of three *T. aestivum* cultivars: 'Chinese Spring', 'Miriam' and 'ND7532'. The seeds of the first two cultivars were kindly provided by Professor M. Feldman, of our Department; 'ND7532' was kindly provided by Dr. A. C. Guenzi, Kansas State University, Manhattan, Kansas. The approximately 1 mm long embryos were excised from the caryopses about 12 days after anthesis. The whole spike was surface sterilized by immersion in 70% (v/v) ethanol for 1 min and subsequently in a 30% strength commercial bleaching solution containing 0.05% detergent ('Tween 20') for 15 min. The spikes were rinsed twice with sterile distilled water. The embryos were laid with the scutellum upwards over 8 ml solidified medium in 50 mm Petri-dishes. The medium (VKM) contained macro and microelements of V47 (Binding 1974), the organic constituents of KM (Kao and Michalyuk 1975), 3% sucrose, 0.8% agar and 2 mg/l 2,4-D. Cultures were maintained at 25 °C in the dark for three weeks, the precociously germinated embryos were discarded and the scutellar calli were cultured for an additional three weeks at the same conditions. The calli were then transferred to VKM containing 1 mg/l IAA and 1 mg/l zeatin, and kept in 16 h light provided by 'white' fluorescent tubes (approximately 50 $\mu E\ m^{-2}\ s^{-1}$). Under these conditions about 85 percent of the calli regenerated plants. The latter were transferred to 'Jiffy' turf-pots, kept for one week under plastic cover in the culture room, and subsequently planted into 5-l pots in the greenhouse.

Material analysed

Self-fertilization of the regenerated plants (SC_1) lead to seeds from which SC_2 plants were obtained. A subsequent sexual cycle lead to SC_3 plants. SC_2, SC_3, as well as control plants from regular seeds of the respective cultivars were used for the *Nor* loci analyses.

DNA extraction

Total DNA was prepared according to Appels and Dvorak (1982) with minor modifications. Seedlings were germinated aseptically for about 10 days. Ten to 20 seedlings having a total fresh weight of about 1 g were crushed to fine powder with a pestle and mortar in liquid nitrogen in the presence of a small quantity of acid-washed sand. The powder was mixed with 5 ml of DNA isolation buffer (0.1 M NaCl; 0.1 M Tris-HCl, pH 8.5; 0.05 M EDTA; 0.2% SDS; 0.1 mg/ml proteinase K). The homogenate was extracted twice with phenol-chloroform (1:1, v/v) and once with chloroform-isoamyl alcohol (24:1) and the aqueous phase was ethanol precipitated in the presence of 0.3 M sodium acetate. The DNA was redissolved in 0.5 ml of TE buffer (0.01 M Tris-HCl pH 8.0; 0.001 M EDTA) and incubated for 2 h at 37 °C with 100 µg/ml RNAse A and 60 units/ml RNAse T1. The aqueous phase was reextracted with phenol-chloroform (1 : 1, v/v) and chloroform as previously mentioned. The DNA was ethanol precipitated in the presence of 0.3 M sodium acetate and the precipitate was vacuum dried and resuspended in 100 µl TE buffer.

Restriction-endonuclease digestion, blotting and hybridization

One microgram of each DNA preparation was digested with 10 units of endonuclease for 16 h according to the manufacturers instructions. The digested DNA's were fractionated by electrophoresis in 1% agarose slab-gels and the gels were transferred to a nitrocellulose filter by the procedure of Southern (1975). Each filter was baked at 80 °C for 2 h and incubated in the prehybridization solution (6×Denhart, 4×SSC, 0.1% SDS and 100 µg/ml sonicated salmon sperm DNA; 1×Denhart is 2% ficoll, 0.2% polyvinylpyrollidone and 0.2% Bovine serum albumine; 1×SSC is: 0.15 M NaCl, 0.015 M sodium citrate). The prehybridization solution was replaced with the hybridization solution containing the same components and a ^{32}P labelled pTA71 plasmid. The pTA71 contains a 9 kbp *Eco*RI fragment which includes the 18S, the 25S and the spacer sequences of wheat rRNA, inserted into the pAC184 vector (Gerlach and Bedbrook 1979). The plasmid was labelled by nick translation (Rigby et al. 1977). The filters were hybridized at 65 °C for 16 h then washed once in 3×SSC and twice in 0.3×SSC at 65 °C and finally dried and autoradiographed overnight.

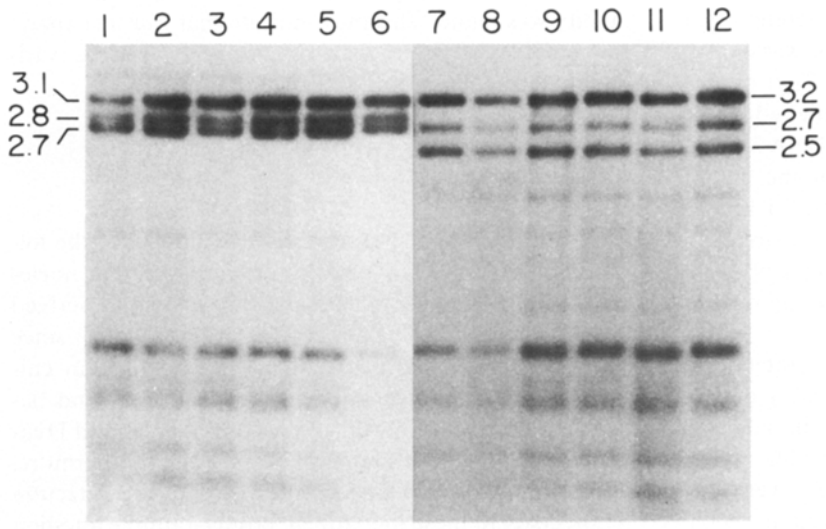

Fig. 1. Southern hybridization analysis of total genomic DNA from control plants and progenies of regenerated (SC_2) plants of *T. aestivum* cv. 'Chinese Spring' and 'Miriam', digested with *Taq*I and hybridized to pTA71. Size indicators are in kbp. *Lanes 1–6:* 'Chinese Spring', lane *1* = control; lanes *7–12:* 'Miriam', lane *7* = control

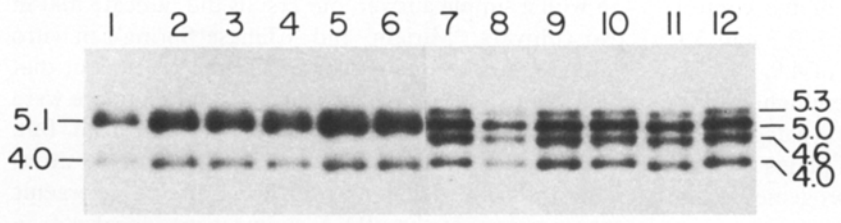

Fig. 2. Southern hybridization analysis of total genomic DNA from control plant and progenies of regenerated (SC_2) plants of 'Chinese Spring' and 'Miriam', digested with *Eco*RI-*Bam*HI and hybridized to pTA71. Size indicators are in kbp. *Lanes 1–6:* 'Chinese Spring', lane *1* = control; lanes *7–12:* 'Miriam', lane *7* = control

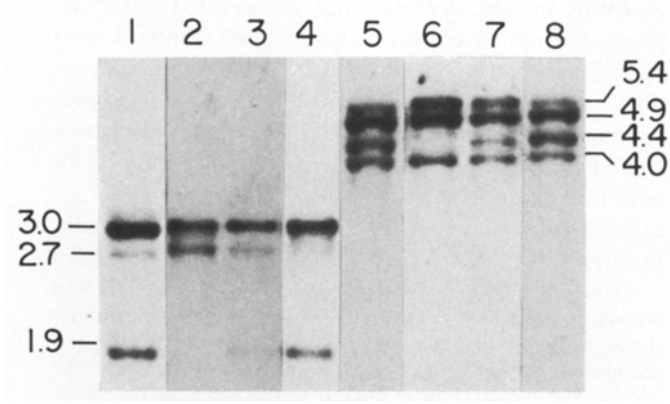

Fig. 3. Southern hybridization analysis of total genomic DNA from control plant and progenies of regenerated (SC_2) plants of 'ND7532' digested with *Taq*I and *Eco*RI-*Bam*HI and hybridized to pTA71. Size indicators are in kbp. *Lanes 1–4:* TaqI digests, lane *1* = control; lanes *5–8:* EcoRI-BamHI digests, lane *5* = control

Analysis of grain proteins

This analysis was performed according to Galili and Feldman (1983). Briefly, total endosperm proteins of single grains, after removal of the embryos, were extracted in aluminum-lactate buffer and subjected to one dimensional discontinuous sodium-dodecyl-sulphate polyacrylamide gel-electrophoresis (SDS-PAGE).

Results

Total DNA was extracted from seedlings originating from seeds of three cultivars: 'Chinese Spring', 'Miriam' and 'ND7352'. The DNA was digested with *Taq*I or double digested with *Eco*RI-*Bam*HI, fractionated by electrophoresis in agarose gels, transferred to nitrocellulose and hybridized with the ^{32}P labelled pTA71 plasmid in order to assess the rDNA spacer-length polymorphism within and among these cultivars. The *Taq*I endonuclease was chosen since it cleaves the wheat rDNA to small fragments with the exception of the variable intergenic spacer region where *Taq*I sites are rare, therefore producing long *Taq*I fragments characteristic of the spacer length (Appels and Dvorak 1982). The *Eco*RI-*Bam*HI endonucleases cleave the intergenic spacer and the ribosomal coding genes into fragments of variable and fixed-length fragments, respectively (Appels and Dvorak 1982). Hybridization patterns revealed distinct combinations in spacer length of the rRNA genes for each cultivar (Figs. 1–3), but no within-variation could be detected in the three varieties (data not shown). *Taq*I fragments of 2.7, 2.8 and 3.1 kbp were revealed for 'Chinese Spring', fragments of 2.5, 2.7 and 3.2 kbp for 'Miriam' and fragments of 1.9, 2.7 and 3.0 kbp for 'ND7532'. To reveal *Nor* diversity in scutellar-calli derived 'Chinese Spring' plants, we extracted the DNA from siblings

(SC_2) which resulted from selfing of 25 different regenerated plants (SC_1). Each of these SC_1 plants was derived from a different callus. Southern blots of *Taq*I or *Eco*RI-*Bam*HI digests hybridized with radiolabelled pTA71 indicated that the spacer-lengths in all 25 DNA samples were identical to spacer-lengths found in the control plants (2.7, 2.8, 3.1 kbp for *Taq*I or 4.0 and 5.1 kbp for *Eco*RI-*Bam*HI). A sample of DNA from SC_2 siblings is shown in Figs. 1 and 2 (lanes 1–6). Progenies of one of these plants (SC_3 generation) did not show any new variation in spacer length (data not shown).

Nor diversity in 'Miriam' plants regenerated from scutellar-callus was assayed in siblings (SC_2) derived from selfings of 9 different SC_1 plants; in addition, siblings (SC_3) from selfings of 3 different SC_2 plants were analyzed. When the respective DNAs were processed as indicated above they all showed the same hybridization patterns (Figs. 1 and 3, lanes 7–12). These patterns were identical to the patterns of the control plants from this cultivar (fragments of 2.5, 2.7 and 3.1 kbp after *Taq*I digestion and fragments of 4.0, 4.6, 5.0 and 5.3 after *Eco*RI-*Bam*HI digestion were lighted-up). Contrary to the uniformity in *Nor* loci revealed in the progenies from the scutellar-calli derived plants of 'Chinese Spring' and 'Miriam' – such progenies from 'ND7532' did show *Nor* diversity. The spacer-region analysis of SC_2 plants from the latter cultivar is shown in Fig. 3. The diversity was expressed in qualitative and/or quantitative changes in *Taq*I fragmented spacer length. In one plant (Fig. 3 lane 2) the 1.9 kbp fragment was missing and was not detectable even after long exposure. The ratio between the 2.7 and 3 kbp *Taq*I fragments of this plant was obviously different from the respective ratio in the control. In another plant (lane 3), the hybridization pattern indicated reduced amounts of the 1.9 kbp *Taq*I fragment. A third plant (lane 4) lost the 2.7 kbp *Taq*I fragment. The variation in length of the rDNA repeats is probably due to a reduction in the number of 135 bp repeats because the latter repeats occupy most of the DNA sequences between these sites (Appels and Dvorak 1982). The assay discriminating between the progenies of regenerated 'ND7532' plants by the *Eco*RI-*Bam*HI digest seems to be less sensitive than by the *Taq*I digest. Only the plant represented in lane 6 (Fig. 3) showed a clear diversion in hybridization pattern from the control (lane 5), the former plant lost the 4.4 kbp *Eco*RI-*Bam*HI fragment. It should be noted that a population of control plants of the 'ND7532' variety did not show any polymorphism for spacer length (data not shown). To examine the uniformity of 'ND7532' we analyzed its endosperm-proteins by SDS-PAGE. No variation in either glutenins or gliadins was revealed (data not shown). When the endosperm-proteins of grains from progeny-plants derived from regenerated plants (SC_2 seeds) were likewise analyzed, again no variability was found. This may indicate that *Nor* loci analysis provides a more sensitive assay for genetic variability than endosperm-protein analysis.

Discussion

Our study was aimed at furnishing an answer to the following question: is in vitro culture implicated in nuclear-DNA changes in regenerated wheat plants derived from scutellar calli? We focussed on the *Nor* loci since they were shown to be relatively uniform within cultivars but rather diversified between cultivars and between populations of wild species (e.g. Appels and Dvorak 1982; Dvorak and Appels 1982). Furthermore, recent methods furnish a simple assay for the detection of diversity in these loci. Albeit, posing a simple question and using efficient experimental tools, we did not come up with a simple answer, our results did indicate that in two cultivars ('Miriam' and 'Chinese Spring') in vitro culture either caused no changes in the *Nor* loci or that such changes occurred but were rare and therefore were not detected in the samples which were analyzed. But, in a third cultivar ('ND7532') extensive heritable variations (possibly deletions) did occur in the intergenic spacer-region of the rRNA coding genes localized on the *Nor* loci. Furthermore, our analysis of gliadins and glutenins in sibs derived from regenerated 'ND7532' plants did not reveal diversity from the standard storage-protein profile of this cultivar. This result is compatible with the recent report of Cooper et al. (1986) who analyzed 5586 regenerated 'ND7532' plants and concluded that gliadins were invariant in this cultivar with only rare exceptions; they found 4 families of scutellar-callus derived plants which had changes in their gliadin profiles.

While in most previous studies (see reviews by Larkin and Scowcroft 1981; Karp and Bright 1985; Scowcroft 1985) somaclonal variation was analyzed by its morphological and biochemical expression, there is one recent study in which the *Nor* loci (i.e. the diversity in nuclear DNA) was analyzed (Brettell et al. 1986). The latter authors examined plants regenerated from in vitro cultured scutellar calli of triticale. They found that, in general, the *Nor* loci were stable, except in one family, where a marked reduction of rDNA was observed.

Due to the limited sample sizes in our study we cannot quantitatively evaluate the rate of somaclonal *Nor* diversity in 'ND7532' as compared to the *Nor* diversities in 'Miriam' and 'Chinese Spring' (the two latter cultivars showed no such diversity in the analyzed samples). Limited evidence from several studies on somaclonal variation indicates that the genotype of the donor plant has a significant effect on the extent of variations generated during culture (e.g. Karp and Bright 1985; Brettell et al. 1986).

Several mechanisms may account for the variation in repeated sequences and the rapid fixation of such variants through evolution in natural populations. Tandemly arrayed repeats evolve together probably due to fre-

quent unequal crossing over and/or gene conversion (e.g. Whitehouse 1982). Mutations may spread in a population and increase in frequency as a result of one or more of the following mechanisms: natural selection, random drift, trasposition, unequal crossing over, biased gene conversion, concerted evolution and molecular drive (Arnheim 1983; Dover and Flavell 1984); The potential instability associated with the repeated DNA implies that mechanisms must have evolved to suppress or control variation. These mechanisms being under genetic and physiological control may become destabilized by stress (i.e. tissue culture, Flavell 1985).

The presented results demonstrate that heritable changes occur in repeated sequences (intergenic spacer) following a short period (a few weeks) of in vitro culture, while comparable events are expected to require many generations when no in vitro culture is involved (Appels and Dvorak 1982; Saghai-Maroof et al. 1984). We propose the possibility that a periodic destabilization imposed during the undifferentiated callus may lead to an increased mutation rate in somatic cells. Such mutations are transferred with a relatively high efficiency to the gametes of regenerated plants because these mutations do not pose deleterious effects.

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