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Is *Nor* region variability in wheat invariably caused by tissue culture?

A. Breiman [1], T. Felsenburg [2] and E. Galun [2]

[1] Department of Botany, George S. Wise Faculty of Life Science, Tel Aviv University, Tel Aviv 69978, Israel
[2] Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

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Summary. In a previous study we observed extensive *Nor* region variability in tissue-culture derived plants of only one out of three tested wheat cultivars. This finding prompted us to further question whether or not this variability was invariably caused by in vitro culture. In the present study, the upper halves of spikes from four source plants of the inbred cultivar 'ND7532' were removed 12 days after anthesis. The immature embryos from these halves were cultured and regenerated into plants. The lower halves of the same spikes were retained on the plants to obtain mature caryopses. DNA was extracted from seedlings, cut with *Taq*I endonuclease, run on agarose gels, and the respective Southern blots were probed with the plasmid pTA71 to reveal the *Nor* region patterns. The sexual progeny of regenerants from three out of four source plants derived from the immature embryos provided *Nor* region patterns which were exactly identical to the patterns obtained from seedlings which germinated from the caryopses matured on the respective source spikes. The regenerants from the fourth source plant provided variable *Nor* region patterns. Analyses of the *Nor* region patterns of 21 individual seedlings germinated from caryopses of this source plant showed that 18 had a three-fragment pattern (consisting of 3.0, 2.7 and 1.9 kb fragments) while three seedlings lacked one (2.7 of 1.9 kb) fragment. Furthermore, the next sexual progeny of the regenerants which had a three-fragment pattern further segregated into three- and two-fragment patterns. These results, in conjunction with previous reports on *Nor* region variability among tissue-culture derived plants, suggest that this variability is not invariably related to in vitro culture.

Key words: Wheat – Ribosomal DNA – Tissue culture – Somaclonal variation

Introduction

In a previous study (Breiman et al. 1987a), we analyzed the *Nor* loci in progenies of wheat plants which were regenerated from scutellar-calli. We observed no variability in the progenies of regenerants from two wheat cultivars ('Miriam' and 'Chinese Spring'), while cultivar 'ND7532' progenies showed extensive variability of the *Nor* loci. In spite of the apparent *Nor* region variability of the tissue-culture derived 'ND7532' plants, we did not observe variability of grain glutenin and gliadin profiles in these plants. It is technically impossible to analyze the *Nor* region of the very same immature embryo before and after in vitro culture. Our reference was, therefore, the analysis of pooled grains of the respective cultivar. The Southern blot hybridization of such a pooled DNA with an rDNA spacer probe could produce 'combined' patterns disguising two or more unique patterns from individual grains.

Wheat and barley rDNA spacer regions were reported to be preserved within species and cultivars, but variable among them (Appels and Dvorak 1982b, c; Saghai-Maroof et al. 1984; Breiman et al. 1987b). Variations in the *Nor* region of potato (Landsmann and Uhrig 1985), triticale (Brettell et al. 1986) and barley (Breiman et al. 1987b) were detected in tissue-culture derived plants as well as in wheat plants derived from anther culture (Rode et al. 1987). The variation of the *Nor* region thus seemed to be an appropriate tool for the evaluation of somaclonal variation. On the other hand, a detailed recent study on anther-culture derived wheat plants (Benslimane et al. 1988) revealed that, while the first cycle of haploidization-diploidization generated *Nor* region variability, a second cycle generated uniform androgenic plants. These and other observations prompted the latter authors to state that "the major part of the variations

observed after in vitro androgenesis could depend on events that are not related to the anther culture process per se". This doubt and the possibility that somaclonal variation is not the only interpretation of our own results (Breiman et al. 1987a) prompted us to re-evaluate the *Nor* region variability of scutellar-callus derived 'ND7532' plants. In this reevaluation, reference grains were obtained from the very same spikes from which the cultured immature embryos were removed. Furthermore, to evaluate possible inherent *Nor* region heterogeneity within this inbred cultivar, we analyzed plants germinated from single grains of sexually reproduced plants as well as individual germinated grains from self-pollinated, scutellar-callus derived plants.

Materials and methods

Plant material and regeneration of scutellar calli

The wheat (*Triticum aestivum* L.) cultivar 'ND7532', originally provided by Dr. A.C. Guenzi (Kansas State University), was maintained by self-pollination. One-mm-long immature caryopses were removed, about 12 days after anthesis, from surface-sterilized upper parts of spikes. The embryos were isolated from the caryopses and cultured on solidified medium with the scutellum facing up, as described by Gosch-Wackerle et al. (1979). The regeneration of wheat plants from the scutellar-calli was performed as reported previously (Breiman et al. 1987a).

Analysis of rDNA sequences

The analysis of rDNA from wheat seedlings was based on procedures reported by Appels and Dvorak (1982a) and modified as previously described (Breiman et al. 1987a). Briefly, either etiolated seedlings (1 g) or individual light-grown seedlings (2 g) were crushed in liquid nitrogen and homogenized with an isolation buffer. Total DNA was extracted, purified and dissolved in TE buffer ((0.01 N TRIS-HCl, pH 8.0; 0.001 *M* EDTA). The DNA was digested with *Taq*I endonuclease, fractionated on (1%) agarose slab-gels and Southern blot hybridization (Southern 1975) was performed by using the ^{32}P-labelled pTA71 plasmid (Gerlach and Bedbrook 1979; kindly provided by Drs. R. B. Flavell and M. O'Dell, IPSR, Cambridge, UK) as probes. Only the larger fragments (e.g. 3,0, 2.7 and 1.9 kb) which hybridized with pTA71 were considered for the *Nor* loci analysis, since the hybridization patterns consisting of the smaller fragments were identical in all the analyzed DNA samples. The former fragments were grouped in three types of patterns: (a) three fragments – 3.0, 2.7 and 1.9 kb; (b) two fragments – 3.0 and 2.7 kb; (c) two fragments – 3.0 and 1.9 kb. For brevity, these patterns will be designated as 3.0/2.7/1.9; 3.0/2.7 and 3.0/1.9, respectively.

Results

The experimental system

Since it is technically impossible to determine the rRNA gene organization of the very same genotype before and after in vitro culture, we improved the reliability of reference samples of the *Nor* region analyses of plants after in vitro culture, by devising an experimental system schematized in Fig. 1. Four randomly chosen seeds of the cultivar 'ND7532' were planted (plants I, II, III and IV) and designated as 'source' plants. The spikes of these source plants were tagged and, 12 days after the anthesis of each spike, the upper half was removed and its immature embryos were cultured and regenerated into plants. The grains matured on the lower halves of the source spikes were germinated and analyzed, serving as references to the culture-derived plants from the same source spikes. The regenerated plants (SC_1) were grown to maturity; part of the respective grains (SC_2) of each regenerant were germinated and the respective seedlings were pooled before analysis, while other SC_2 grains (sexual progeny) were germinated and analyzed individually.

Analysis of tissue-culture derivatives and comparison to reference samples

We first asked whether or not the source grains, representing random samples of four plants and several spikes on each of these plants, will provide uniform *Nor* region patterns. The Southern-blot hybridization patterns of *Taq*I-digested DNA, after probing with radio-labelled pTA71 (*Taq*I/pTA71 patterns) from several source spikes, are provided in Fig. 2. Additional source spike samples from plant IV (not shown) exhibited the same pattern as spike IV-1. It should be noted that the patterns of Fig. 2 resulted from pooled samples: ten seeds of each lower-half spike were germinated in the dark and the seedlings were homogenized together to provide the respective DNAs. The results showed clearly that the four samples from the spikes of plant I showed the 3.0/2.7/1.9 pattern. All spikes from plants II, III and IV displayed a different (3.0/1.9) pattern. Moreover, the labelling intensity of the three hybridized fragments varied among the samples from the four spikes of plant I. While a procedural artifact could not be excluded, a detailed comparison pointed to a more plausible possibility, indicating that the pooled samples from the four spikes of plant I indeed disguised different genotypes among the ten seedlings comprising each sample.

We then analyzed the *Taq*I/pTA71 patterns of the DNA from the tissue-culture derived plants. The grains from each of the latter (SC_1) regenerants were germinated and the DNA of these seedlings (SC_2) was analyzed (Figs. 3 and 4). The seedlings obtained from regenerants of plant II (Fig. 4), as well as from plants III and IV (not shown), all provided the same two-fragment pattern (3.0/1.9). This indicated that no *Nor* region variability could be detected following a tissue-culture cycle. The analysis of the sexual progeny of regenerants obtained from plant I provided a different result. Three types of patterns were observed (Fig. 3): a three-fragment pattern (3.0/2.7/1.9) or either of the two-fragment patterns (3.0/2.7 or 3.0/1.9). Moreover, two different patterns re-

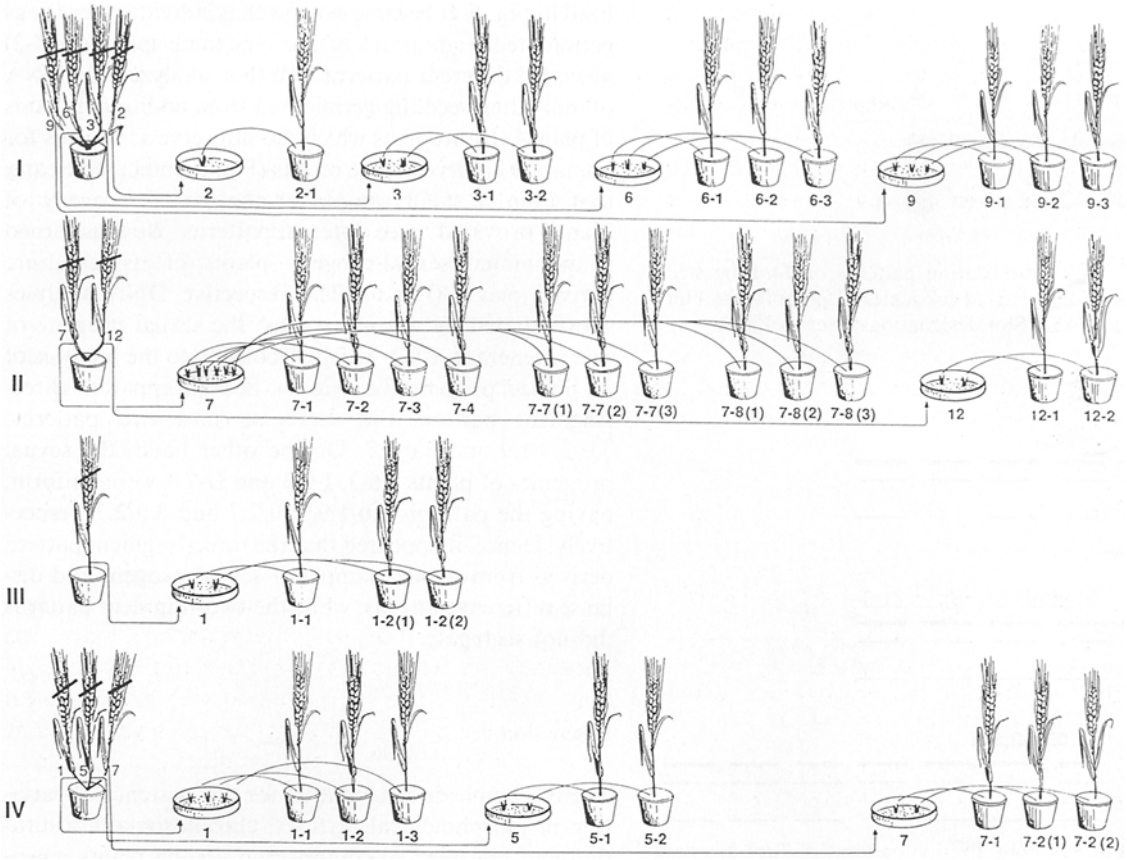

Fig. 1. Scheme of experimental system

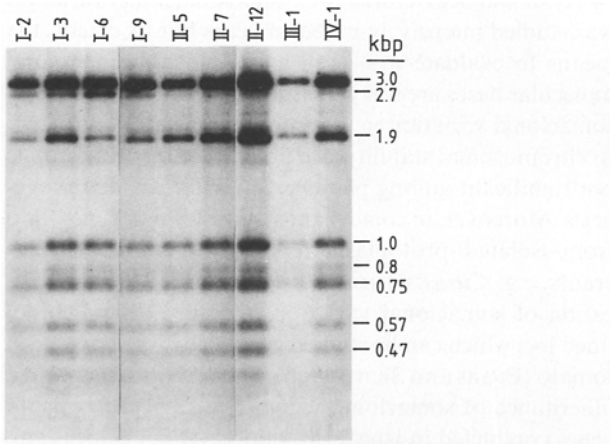

Fig. 2. Southern blot hybridization patterns of *Taq*I-digested DNA from seedlings germinated from grains of source spikes; the blot was probed with radio-labelled pTA71. Slot designations refer to Fig. 1. (Spike II-5 is not presented in Fig. 1; regenerants from its embryos did not attain sexual maturity)

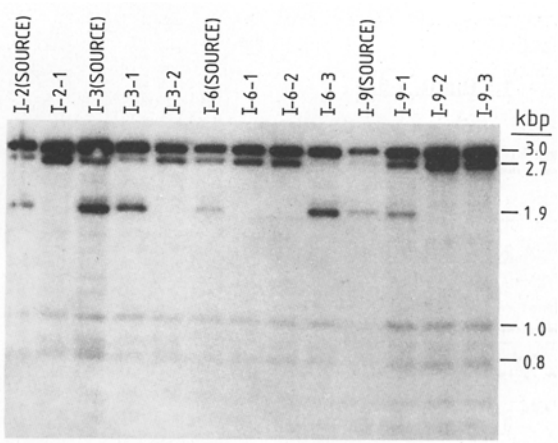

Fig. 3. Southern blot hybridization patterns of *Taq*I-digested DNA of scutellar-callus derived progenies of plant I. The blot was probed with radio-labelled pTA71. Slot designations refer to Fig. 1

sulted from the SC_2 generation of each of three spikes (spikes I-3, I-6 and I-9). This result could indicate a very extensive variability induced by the in vitro culture or could mean that the genotypes of the individual immature embryos from which the regenerants were derived differed in their respecitive *Nor* region organization.

To further evaluate these possibilities, we returned to the grains which matured (without a tissue-culture cycle) on source plant I. We germinated individual grains from the lower halves of spikes I-2, I-3, I-6 and I-9, extracted the DNA of each seedling and obtained the respective *Taq*I/pTA71 *Nor* region patterns. The results are schema-

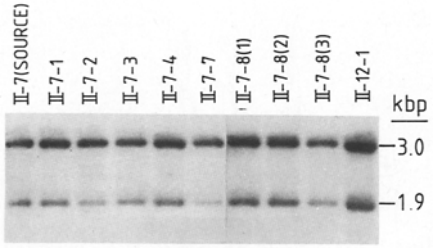

Fig. 4. Southern blot hybridization patterns of *Taq*I-digested DNA of scutellar-callus derived progenies of plant II; the blot was probed with pTA71. Slot designations refer to Fig. 1

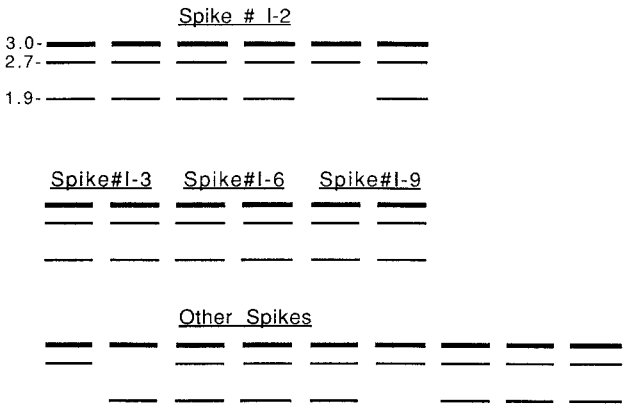

Fig. 5. Schemes of blot hybridization patterns of *Taq*I-digested DNA from individual seedling derived from source spikes and other spikes of plant I; the blots were probed with pTA71

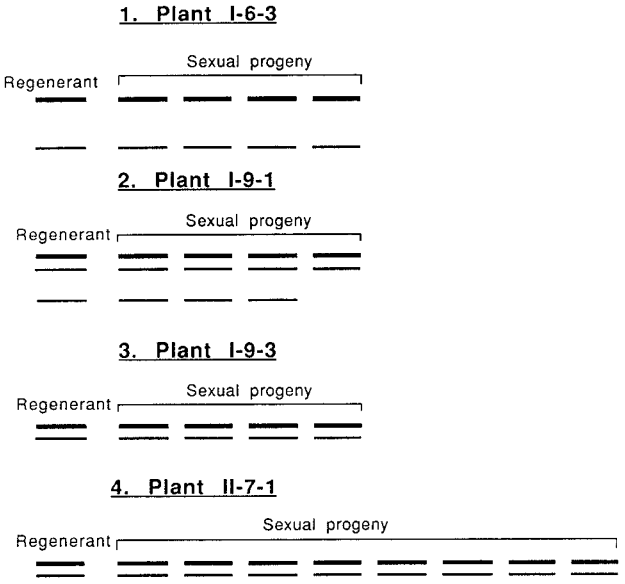

Fig. 6. Schemes of blot hybridization patterns of *Taq*I-digested DNA. The DNA was either from pooled seedlings of regenerated plants (regenerants) or of individual seedlings resulting from self-pollination of the regenerated plants (sexual progeny); the blots were probed with pTA71

tized in Fig. 5. It became evident that individual seedlings germinated from grains of the very same spike (e.g. I-2) provided different patterns. We thus analyzed the DNA of individual seedling germinated from additional grains of plant I (from spikes which did not serve as sources for immature embryos). The results (Fig. 5) indicated clearly that even a small sample of the sexual progeny of Plant I provided three different patterns. We thus turned to individual sexual-progeny plants of tissue-culture derived plants (Fig. 6). The respective DNA analyses substantiated the former results: the sexual progeny of one regenerant, I-9-1 which, according to the analysis of its pooled progeny (i.e. Fig. 3), had an apparent three-fragment pattern, did segregate into two patterns: 3.0/2.7/1.9 and 3.0/2.7. On the other hand, the sexual progenies of plants I-6-3, I-9-3 and II-7-1 were uniform, having the patterns 3.0/1.9, 3.0/2.7 and 3.0/2.7, respectively. Hence, it appeared that the three-fragment pattern derived from pooled sample of sexual progeny did disguise different patterns, while the two-fragment patterns did not segregate.

Discussion

There is ample documentation for the existence of variation in morphological features, chromosomal constitution and biochemical composition among plants regenerated from in vitro cultured cells and tissues (see review by Ryan and Scowcroft 1987). This somaclonal variation was studied intensively in recent years but its extent, the means to evaluate it as well as its morphogenetic and molecular bases are not yet settled. Vasil (1986) evaluated somaclonal variation in Gramineae, especially in respect to chromosomal stability. He noted that this variation is not significant among plants regenerated via embryogenesis. Moreover, in some plant systems even regeneration from isolated protoplasts resulted in uniform regenerants, e.g. *Citrus sinensis* (Kobayashi 1987). The verification of somaclonal variation in respect to single defined loci which can be studied genetically was possible in tomato (Evans and Sharp 1983). Similarly, studies on the inheritance of somaclonal variants in respect to gliadin genes conducted in wheat (Maddock 1986; Cooper et al. 1986) indicated that such heritable variants occurred at a very low frequency (less than 1%) in immature-embryo derived progenies.

Heritable somaclonal variation, by definition, is based on alterations in DNA sequences. Consequently, the detection of such alterations should serve as an appropriate criterion for the evaluation of heritable variability caused by in vitro culture. While negative selection may eliminate the detrimental changes in the coding sequences, such eliminations are much less expected in the non-coding sequences. This rationale and the avail-

able information on the molecular structure of rDNA spacer regions in Triticeae (Appels and Dvorak 1982a; Saghai-Maroof et al. 1984; May and Appels 1987) prompted other investigators (Brettell et al. 1986; Rode et al. 1987; Benslimane et al. 1988) as well as us (Breiman et al. 1987a, b) to utilize *Nor* region variability for the evaluation of somaclonal variation in wheat, triticale and barley. The latter studies did not provide evidence for extensive spacer-length variability caused by either anther-culture or immature-embryo culture. Variability was apparent in tissue-culture derived plants with respect to quantitative changes in certain *Nor* regions. In our previous study on *Nor* region variability in immature-embryo derived wheats (Breiman et al. 1987a), we observed no somaclonal variation in two out of three tested cultivars. Tissue – culture derived plants of the third cultivar ('ND7532') varied quantitatively in their *Taq*I/pTA71 patterns, but no spacer length polymorphism was revealed. In the present study we focussed on this latter cultivar.

The variability which we observed in the *Taq*I/pTA71 patterns among the scutellar-callus derived 'ND7532' plants could be interpreted in the simplest way by assuming an inherent heterozygosity residing even in inbred lines. Evidence for such heterozygosity was indeed found in *Triticum dicoccoides* plants (Flavell et al. 1986) and in some wheat cultivars (e.g. May and Appels 1987). Thus, *Taq*I/pTA71 pattern heterogeneity, as revealed among the sexual progeny of plant I, may reflect a heterozygous state of the source plant: in the pooled seed-sample from a source-plant, an apparent three-fragment pattern (3.0/2.7/1.9) could emerge from the addition of two patterns of two-fragments (3.0/2.7 and 3.0/1.9). The segregation of these patterns, following either sexual reproduction or culture of immature-embryos revealed, in the respective progenies, either the 3.0/2.7 pattern (Fig. 6, plant I-9-3) or the 3.0/1.9 pattern (Fig. 6, plant I-6-3). However, the heterozygotic state may be maintained in the tissue-culture derived plant and later segregate in it's sexual progeny (Fig. 6, plant I-9-1). This suggests that the apparent somaclonal variation observed among tissue-culture derived plants (as compared to the *Taq*I/pTA71 fragment pattern obtained from pooled seeds of 'ND7532') can be explained, at least partially, by perpetual *Nor* region heterozygosity. It should be noted that Saghai-Maroof et al. (1984), who studied the inheritance of rDNA clusters in barley, found that crossing two inbred lines, each with a different two-fragment pattern, caused the appearance of a four-fragment pattern in the F_1 plants; the latter pattern segregated as Mendelian alleles in the F_2 plants.

Finally, whether or not tissue-culture conditions have an additive role, either by facilitating rearrangement dynamics or through other mechanisms, in increasing *Nor* region variability could not be definitely decided by our study. The *Nor* region variability, which is an apparently appropriate tool for the evaluation of somaclonal variation on the molecular level, should be treated with caution. The evidence gathered from our study and from other investigations tends to answer in a negative way the question posed in the title of this publication.

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