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Gametoclonal variation detected in the nuclear ribosomal DNA from doubled haploid lines of a spring wheat (*Triticum aestivum* L., cv. 'César')

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Summary. The organization of the nuclear ribosomal DNA from a parental line of wheat (*Triticum aestivum* L., cv. 'César') and its anther-derived first cycle and second cycle doubled haploid lines has been analyzed by DNA-DNA molecular hybridization. Restricted DNA has been probed by three subclones of wheat nuclear ribosomal DNA covering the entire repeat unit. No significant difference was detected in the extent of methylation of ribosomal DNA of the doubled haploid lines with respect to the parental line. On the other hand, a variation has been found in the organization of the nontranscribed spacer region of ribosomal DNA of the first cycle doubled haploid line. This variation remains stable after a second cycle of in vitro androgenesis. However, one out of five second cycle doubled haploid lines so far tested showed an additional hybridization band present in the parental line but lacking in the first cycle doubled haploid line.

Key words: Wheat – Anther-derived doubled haploids – Gametoclonal variation – Ribosomal DNA

Introduction

Somaclonal variation (Larkin and Scowcroft 1981) may be defined as the appearance of phenotypic variants in plants regenerated from in vitro cultures of somatic cells. When regenerant plants are obtained from in vitro cultures of gametophytic cells (pollen or embryo sac), it seems more convenient to use the term "gametoclonal variation" (Evans et al. 1984) and, therefore, to clearly distinguish the cellular origin of regenerants.

Indeed, somatic and gametophytic cells cultured in vitro may respectively induce different responses with respect to genotypic variation at the level of the regenerants: wheat (Rode et al. 1985) and triticale (Charmet et al. 1985) doubled haploid lines obtained from pollen cultures did not show variation in their mitochondrial DNA organization whereas, for instance, mitochondrial DNA variation was detected in potato plants regenerated from protoplasts (Kemble and Shepard 1984). Several years ago, de Paepe et al. (1982) showed that nuclear DNA of doubled haploids of *Nicotiana sylvestris* resulting from consecutive androgenetic cycles contained, on the average, increasing amounts of total DNA and increasing proportions of highly repeated sequences. More recently, Landsmann and Uhrig (1985), using a 25 S rDNA probe, found a 70% reduction in 25 S rRNA genes in some of the *Solanum tuberosum* plants regenerated from protoplasts, emphasizing the fact that rDNA probes – as well as other repetitive DNA probes – provided a suitable tool for testing variability in somacloned plants. Plant rDNA consists of a tandemly repeated unit sequence composed of a gene for each rRNA (25 S, 5.8 S and 18 S), a transcribed spacer region separating 25 S from 18 S rRNA genes (containing the 5.8 S rRNA gene) and a nontranscribed spacer region. The *Triticum aestivum* nuclear rDNA repeat unit has been shown to be heterogeneous in length, due to the nontranscribed spacer region (Gerlach and Bedbrook 1979; Appels and Dvorak 1982).

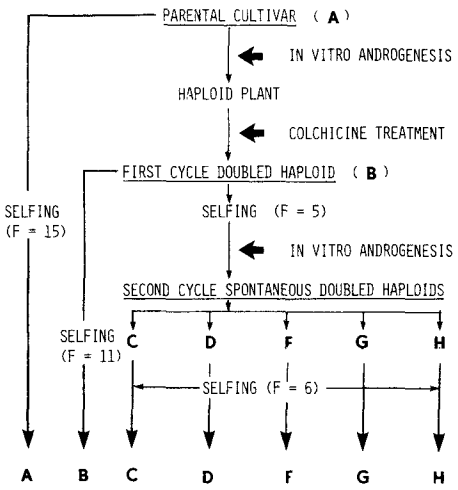
Herein we report on the modification of the nuclear genome organization in wheat doubled haploid regenerants obtained from pollen culture, by using three probes covering the entire wheat nuclear rDNA unit sequence. Taking into account the results obtained by Landsmann and Uhrig (1985) on somaclonal variation, this study may contribute to a comparison between somaclonal and gametoclonal variations with regard to the organization of a highly repeated DNA sequence.

Material and methods

Plant material

A series of doubled haploid lines of soft spring wheat (*Triticum aestivum* cv. 'César') was obtained according to the

following scheme:



Scheme 1. How the different doubled haploid lines used throughout this work were obtained

The parental cultivar (line A) was maintained by selfing. The first cycle doubled haploid (line B) was studied at its eleventh generation of selfing. The second cycle doubled haploids (lines C, D, F, G and H) were studied at their sixth generation of selfing. Parental and doubled haploid lines had 2n=42 chromosomes. Some of these doubled haploid lines have been shown to exhibit a differential response to several wheat pathogens, if compared to the parental line (Parisi and Picard 1986). In the same way, variations in some phenotypic traits, as well as in some agronomical characteristics, have also been detected (Picard et al. 1986).

Plant DNA preparation

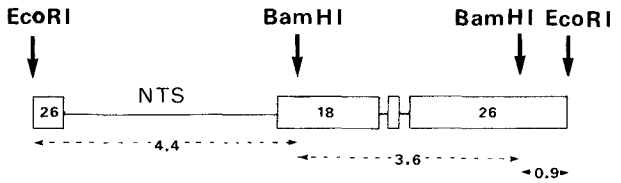
Seedlings were grown under complete darkness at 20°–22 °C and coleoptiles were harvested when about 8–10 cm long. The material was surface-sterilized with sodium hypochlorite (5% (v/v), 5 min), rinsed several times with distilled water and cooled at 2 °C. DNA was prepared according to Dellaporta et al. (1983) with several modifications. After extraction, nucleic acids were treated with ribonuclease A (200 µg/ml, 1 h, 37 °C) followed by several deproteinization steps including phenol (v/v), phenol-chloroforme (1/1, v/v) and diethylether (v/v, 65 °C, 5 mn). DNA was subsequently ethanol precipited (–20 °C, 2 h), pelleted, dried and dissolved in TE buffer so as to obtain a concentration close to 500 µg/ml.

DNA restriction and agarose gel electrophoresis

About 3 to 5 µg of total DNA were digested to completion in the presence of 4 mM spermidine in a total volume of 30 µl. According to the experiments, the restriction endonucleases used were BamHI+EcoRI or MspI+EcoRI and HpaII+EcoRI. The restriction fragments were separated by electrophoresis on 0.75% or 1.5% agarose vertical slab gels. After electrophoresis, gels were stained with ethidium bromide (1 µg/ml) and photographed under UV light.

rDNA probes

A wheat rDNA probe (gift of Dr. R. B. Flavell, plasmid pTA 71 isolated by Gerlach and Bedbrook (1979)) containing the entire rDNA repeat unit defined by the EcoRI site inside the 26 S rDNA gene has been used to prepare three subclones by restricting the rDNA insert with EcoRI+BamHI and cloning in pUC 19. The three subclones obtained correspond respectively to the plasmids pTA 250-1 (4.4 kb EcoRI–BamHI fragment, containing the entire nontranscribed spacer region and a small fraction of 26 and 18 S rRNA genes), pTA 250-2 (3.6 kb BamHI–BamHI fragment, containing the major part of 18 and 26 S rRNA genes, the transcribed spacer and the 5,8 S rRNA gene) and pTA 250-3 (0.9 kb BamHI–EcoRI fragment, containing part of the 26 S rDNA gene) already obtained by Gerlach and Bedbrook (1979). The localization of these three fragments within the rDNA repeat unit is shown in the following scheme:



Scheme 2. Schematic representation of the wheat rDNA unit showing the localization of the different probes used in this work. 26, 18: localization of the genes coding for 26 S and 18 S rRNAs. NTS: nontranscribed spacer region (from Appels and Dvorak 1982)

DNA-DNA hybridizations

Gels were treated as described by Southern (1975) and transferred onto nitrocellulose filters (Schleicher and Schüll, BA 85) by the "sandwich" method (Ketner and Kelly 1976). Probes were prepared by a boiling method (Holmes and Quigley 1981) followed by CsCl-ethidium bromide ultracentrifugation and labelled by nick-translation (Rigby et al. 1977). For DNA-DNA hybridizations, nitrocellulose filters were preincubated in a 2×SSC Denhardt solution and allowed to hybridize for 14–16 h in a 2×SSC – 45% formamide (v/v) – 100 µg/ml salmon sperm carrier DNA mixture containing the nick-translated rDNA probe. After hybridization, filters were washed in 2×SSC (6×15 min at room temperature) then in 0.01×SSC (2×15 min at room temperature) and dried. Autoradiographs were at –80 °C during 1 to 3 days, using X-Omat AR 5 Kodak films and an intensifying screen. Filters were dehybridized by gentle soaking in boiling distilled water for 8–10 min, dried and checked for the efficiency of dehybridization.

Results

1) rDNA length variability

Figure 1 shows the hybridization patterns obtained with the total DNA of parental line (A) and first (B) and second (C, D, F, G, H) cycle doubled haploid lines restricted with EcoRI+BamHI and hybridized against the three labelled rDNA probes. Hybridization with the labelled 0.9 kb rDNA fragment (part of 26 S) visualizes, in the parental and doubled haploid lines, the expected corresponding 1.0 kb fragment and a DNA band of length 4.6 kb (Fig. 1a). As the labelled 3.6 kb

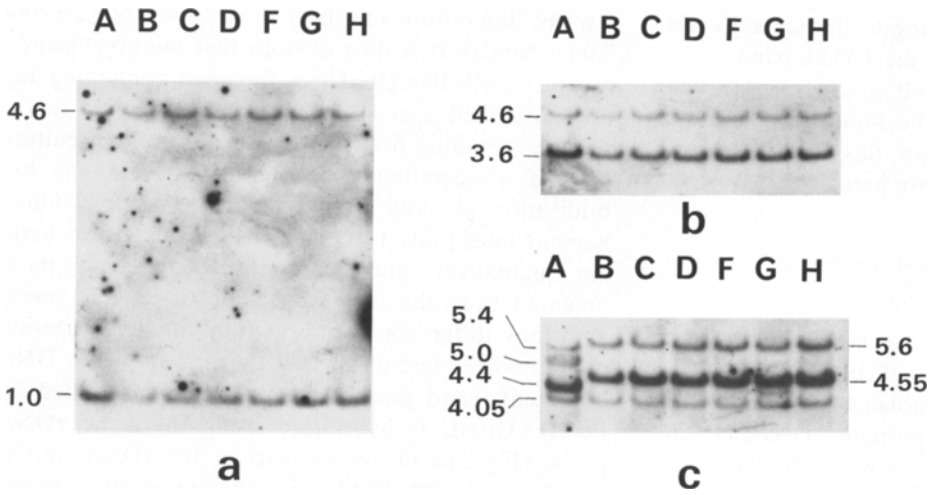


Fig. 1. EcoRI + BamHI double digest patterns of DNA *T. aestivum* hybridized with (**a**) 0.9 kb rDNA subclone; (**b**) 3.6 kb rDNA subclone; (**c**) 4.4 kb rDNA subclone. For each set of experiments: *lane A* DNA of parental line; *lane B* DNA of the first cycle doubled haploid line; *lanes C, D, F, G, H* DNA of second cycle doubled haploid lines C, D, F, G and H. Electrophoresis on 0.75% agarose was run at 1.4 mA/cm for 12 h in TAE buffer (0.04 M Tris-Acetate, pH 8, 0.002 M EDTA). The lengths of the hybridization bands are indicated in kb: "1 kb ladder" (BRL) was used as a molecular weight marker

fragment (part of 18 S and 26 S) is visualized in addition to the same DNA band of length 4.6 kb (Fig. 1 b), it is expected that this 4.6 kb band results from the nondigestibility of the BamHI recognition site separating the 3.6 kb fragment from the 1.0 kb fragment (see scheme 2). Since we always detected this 4.6 kb hybridization band, whatever the conditions of enzyme excess, we believe that this band does not correspond to a partial digest but likely arises from a point mutation or a methylation occurring within the BamHI site of a part of the rDNA repeat units. Other than this observation, no noticeable difference was detected, with both 3.6 kb and 0.9 kb rDNA probes, between the hybridization patterns of parental and doubled haploid lines. It thus turns out that the coding regions of the rDNA repeat unit do not exhibit any restriction fragment length polymorphism in response to in vitro androgenesis.

As shown in Fig. 1 c, the nontranscribed region of the rDNA repeat unit displays modifications. In this experiment, total DNA of the parental line (A), first cycle doubled haploid line (B) and second cycle doubled haploid lines (C, D, F, G, H) has been restricted by EcoRI + BamHI. Hybridization has been carried out with the 4.4 kb rDNA probe which contains the entire nontranscribed spacer region. The hybridization pattern relative to the parental line (A) exhibits four bands at 4.05 kb, 4.4 kb, 5.0 kb and 5.4 kb whereas the hybridization bands corresponding to the doubled haploid lines are located at 4.05 kb, 4.55 kb, 5.0 kb (very faint band) and 5.6 kb. In addition, the second cycle doubled haploid line G possesses a faint band at 4.4 kb, similar to the major band detected in the parental line. With the exception of this particular feature, the length of each of the two larger hybridization bands is about 1 kb more than each of the two smaller ones. It is then highly probable that the 5.0 kb band arises from the nondigestibility of some EcoRI sites separating the 4.05 kb band from the 1.0 kb band. Likewise, the 5.4 kb band (parental line) and the 5.6 kb band (doubled haploid lines) probably arise from the nondigestibility of some EcoRI sites separating the 4.4 kb band and the 4.55 kb band from the 1.0 kb band. Thus, one may assume that the DNA fragment containing the nontranscribed spacer region displays a length polymorphism, as already discussed by several authors (Gerlach and Bedbrook 1979; Fodor and Beridze 1980; Appels and Dvorak 1982; Kato et al. 1982; Siegel and Kolacz 1983; Waldron et al. 1983; Yakura et al. 1984). Two families of EcoRI–BamHI nontranscribed spacer fragments differing by their length (4.05 kb and 4.4 kb) are found in the nuclear DNA of the parental line, within the limits of detection provided by the method. Two such families differing by their lengths (4.05 kb and 4.55 kb) are also detected in doubled haploid lines B, C, D, F and H: a minor family whose length is identical to that of the minor family found in parental line (4.05 kb) and a major family (4.55 kb) 150 bp longer than the corresponding family detected in the parental line. Only the doubled haploid line G possesses three families differing by their length, two of them being identical to parental line. Consequently, in vitro androgenesis by itself – or the subsequent regeneration step – induces a change in the organization of the 4.4 kb rDNA family especially at the level of the first cycle doubled haploid B. Indeed, the hy-

bridization patterns corresponding to the second cycle doubled haploid lines all exhibit the 4.55 kb band.

The entire rDNA repeat unit used as a labelled probe and hybridized against the same blots as those used with the rDNA subclones has confirmed the results described above (not shown here).

2) Extent of methylation of the nontranscribed spacer region

As plant DNA is known to be highly methylated (Gruenbaum et al. 1981; Scott et al. 1984) the extent of methylation of rDNA from doubled haploid lines has been investigated. For this purpose, DNA of the parental and doubled haploid lines was restricted either with EcoRI + MspI or with EcoRI + HpaII and hybridized, after agarose electrophoresis and blotting, against the labelled 4.4 kb rDNA probe (Fig. 2). MspI and HpaII are isoschizomers which recognize the CCGG sequence. MspI cleaves whether or not the internal cytosine of the recognition sequence is methylated whereas HpaII does not cleave when the internal cytosine is methylated. As shown in Fig. 2a, the hybridization patterns corresponding to the DNA of parental line A and first cycle doubled haploid B restricted by EcoRI + MspI are qualitatively and quantitatively different. Indeed, some bands detected in parental line A DNA are absent in first cycle doubled haploid line B DNA (2.7 kb, 2.6 kb, 1.15 kb bands); the reverse is also true (0.85 kb band). Moreover, the same hybridization band detected in both lines may quantitatively differ from one line to the other (2.3 kb and 2.0 kb bands). It is then obvious that the organization of the EcoRI–BamHI rDNA fragment containing the nontranscribed spacer region undergoes some modifications resulting from the first in vitro anther culture (or the regeneration process). As expected, the hybridization patterns relative to second cycle doubled haploid lines (only F, G and H lines are shown here) are qualitatively and quantitatively similar to those obtained from the first cycle doubled haploid line B and thus differ considerably from the hybridization pattern corresponding to parental line A. When DNA of parental and doubled haploid lines, restricted by EcoRI + HpaII, is hybridized using the same rDNA probe (Fig. 2b) the major part of the rDNA unit is shown to be methylated at its cytosine residues, in the parental line as well as the doubled haploid lines, without any significant quantitative difference between themselves. This result shows that in vitro androgenesis does not alter the extent of methylation of the nontranscribed spacer region of the rDNA of regenerant lines. The presence of restriction fragments smaller than the entire rDNA unit shows that a number of the cytosines of the nontranscribed spacer region are submethylated. Moreover, it confirms the occurrence of modifications in the nontranscribed spacer region consecutive to the first cycle of in vitro androgenesis (3.5 kb and 2.4 kb bands for parental line A and 3.6 kb and 2.6 kb bands for doubled haploid line B) in so far as the hybridization patterns of the second cycle doubled haploid lines (Fig. 2b) are similar to those ob-

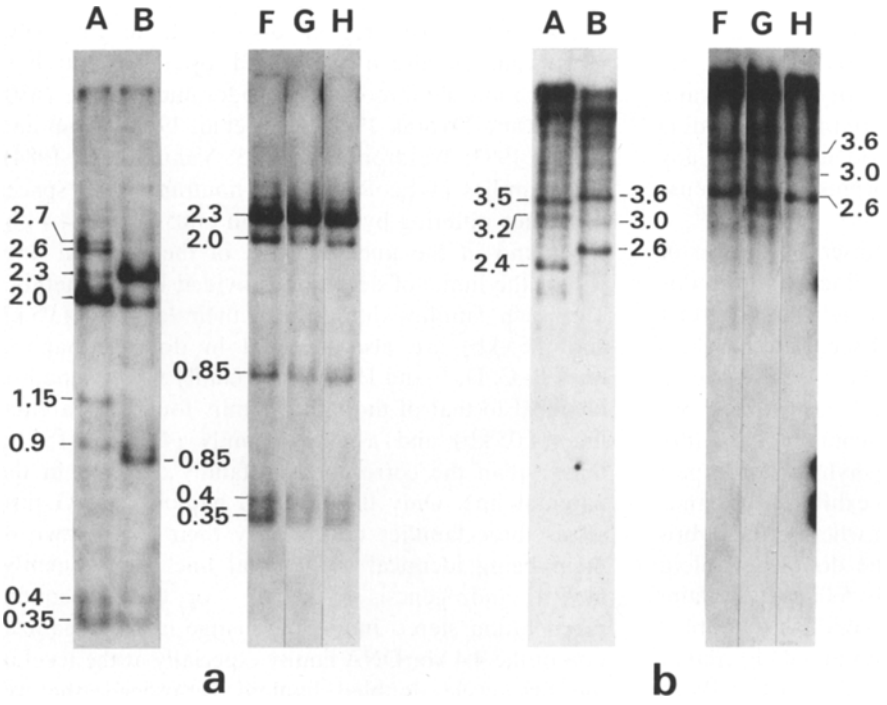


Fig. 2. EcoRI + MspI (**a**) and EcoRI + HpaII (**b**) double digest patterns of DNA from *T. aestivum* hybridized with the 4.4 kb rDNA subclone. For each set of experiments: *lane A* DNA of parental line; *lane B* DNA of the first cycle doubled haploid line; *lanes F, G, H* DNA of second cycle doubled haploid lines F, G and H. Electrophoresis on 1.5% agarose was run at 0.9 mA/cm in TAE buffer for 13 h (lines A and B) and for 10 h (lines F, G and H). The lengths of the hybridization bands are indicated in kb: "1 kb ladder" (BRL) was used as a molecular weight marker

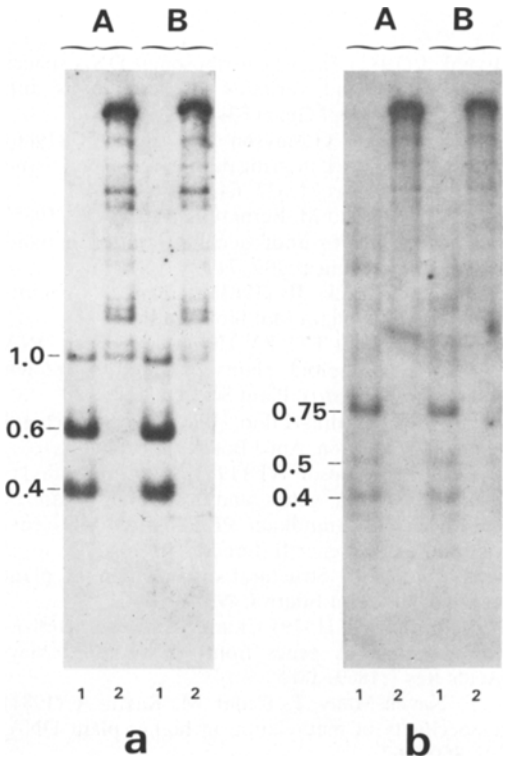


Fig. 3. EcoRI + MspI (1) and EcoRI + HpaII (2) double digest patterns DNA from *T. aestivum* hybridized with either the 0.9 kb rDNA subclone (**a**) or the 3.6 kb rDNA subclone (**b**). For each set of experiments, *A* refers to the parental line A and B to the first cycle doubled haploid line *B*. Electrophoresis on 1.5% agarose was run at 0.9 mA/cm in TAE buffer for 11 h. The lengths of the hybridization bands are indicated in kb: "1 kb ladder" (BRL) was used as a molecular weight marker

tained with the first cycle doubled haploid line B. The same experiment has been carried out using the labelled probes corresponding to the coding fractions of the rDNA repeat unit (Fig. 3). EcoRI + MspI and EcoRI + HpaII double digests of DNA from parental line A and first cycle doubled haploid B have been hybridized with either the labelled 0.9 kb fragment (Fig. 3 a) or 3.6 kb fragment (Fig. 3 b). The resulting hybridization patterns show a strict identity between parental line A and first cycle doubled haploid line B as well as for MspI digestion as for HpaII digestion. Consequently, the extent of methylation as well as the organization of the coding fraction of rDNA are identical for parental line A and androgenetic line B, confirming thereby the data obtained from EcoRI + BamHI digests. The results obtained with the second cycle doubled haploid lines C, D, F, G and H (not shown here) are identical to those found for lines A and B. A more detailed understanding of the distribution and extent of methylation in the nontranscribed spacer and coding regions of parental and doubled haploid lines rDNA would, however, require further experiments.

Discussion

Three subclones covering the totality of the wheat nuclear rDNA were used as probes for detecting some eventual DNA rearrangements in one first cycle and five second cycle wheat doubled haploid lines obtained from in vitro anther cultures. Digestions of total DNA with EcoRI + BamHI and subsequent molecular hybridizations with each of the three rDNA subclones have allowed us to detect a change, in the first cycle doubled haploid line, of the organization of the EcoRI–BamHI rDNA fragment containing the nontranscribed spacer region. This variation was maintained in the second cycle doubled haploid lines. However, one of them also exhibited a faint hybridization band whose length was similar to that of the parental line but absent in the other doubled haploid lines. It is now well known, as mentioned above, that rDNA length variability is almost exclusively confined to the nontranscribed (or large) spacer region whereas the length of the coding regions appears to be well conserved. Our results not only confirm this fact but also show that in vitro androgenesis by itself – or the regeneration process following androgenesis – may be able to induce such variations in the nontranscribed spacer region. It is also of interest to point out that only the first cycle of androgenesis seems to be capable of inducing such gross rearrangements which are, in turn, transmitted through further cycles of androgenesis.

Gene conversion (Petes and Fink 1982) and unequal crossing over (Szostak and Wu 1980) events are thought to be involved in maintaining sequence homogeneity within a family of repeated genes. Recently, Lassner and Dvorak (1986) showed, by sequencing DNA from the nontranscribed spacer of two wheat rDNA gene clones, that these clones differed in length by one 133 bp internal subrepeat. They propose these two clones are products of the unequal crossing over event in so far as they were located on the same chromosome. Another hypothesis, mentioned by these authors, involved insertions and excisions of transposable elements. Taking into account the high degree of reiteration of the wheat nuclear rDNA unit – several thousand copies per genome (Flavell et al. 1983) – it seems difficult and speculative to provide a suitable explanation for the total conversion of the 4.4 kb family (parental line) into the 4.55 kb family (doubled haploid lines) as the period of time (one generation) would be probably too short to obtain this change in rDNA structure. However, it must be emphasized that the difference in the lengths of parental (4.4 kb) and doubled haploid (4.55 kb) lines represents nearly the length of one unit subrepeat and could then correspond to the insertion of an additional subrepeat into the nontranscribed spacer. The question arises as to whether the occurrence of a translocation event in which every rDNA repeat unit would be concerned is a reliable hypothesis. Alternatively, an unequal crossing over event with the arrays out of frame by one

subrepeat would lead to nearly 4.25 kb and 4.55 kb fragments (n−1 and n + 1 subrepeats). This hypothetical 4.25 kb fragment has never been observed in our experiments, making the occurrence of a a simple unequal crossing over event unlikely. Nor can this rearrangement of rDNA structure be explained by simply a partial or complete chromosome alteration or loss, as may be the case when a definite DNA sequence undergoes only a quantitative variation (Brettell et al. 1986). If such a phenomenon was partly responsible for the qualitative changes detected in doubled haploid lines, we must suppose that both families (4.4 kb and 4.55 kb) were pre-existing in the parental germ cell, the 4.55 kb family being present only in trace amounts. Supposing a complete loss of the part of the chromosome bearing the 4.4 kb family, an amplification of the 4.55 kb family would have taken place simultaneously, leading to the observed hybridization patterns. The faint hybridization band detected at 4.4 kb in the second cycle doubled haploid line G might be explained by an amplification process, supposing that trace amounts of this DNA family were still present in the first cycle doubled haploid B.

However, it must not be deduced from the results that androgenesis in vitro and/or plant regeneration obligatorily induce qualitative DNA rearrangements. Indeed, we have analyzed first cycle doubled haploid lines derived from other cultivars of *T. aestivum* by using exactly the same approaches (results not shown here). No qualitative or quantitative rDNA changes were found for the cultivar 'Benoist' (21 samples) and only quantitative changes were detected for the cultivar 'Moisson' (3 samples). Moreover, it must be kept in mind that only one first cycle doubled haploid line was available in the experiments presented here. We cannot therefore exclude the possibility of various responses in the organization of the nontranscribed spacer region of the rDNA unit if several first cycle doubled haploid lines would have been available. It is then obvious that the occurrence of variations in DNA organization in response to in vitro androgenesis is not to be considered as a general rule. It probably depends on environmental factors as well as genetic features which so far are not controlled.

The question arises then as to whether androgenesis or in vitro culture by itself (or a combination of both) is responsible for the changes observed in the organization of the rDNA repeat unit. A similar work using callus cultures prepared from immature embryos of wheat and corresponding regenerant lines is currently under way. This will allow us to compare the organization of the rDNA repeat unit in response to in vitro culture of either gametophytic or somatic cells.

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