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Mitochondrial DNA variation in plants regenerated from embryogenic callus cultures of CMS triticale

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Abstract The mitochondrial DNA (mtDNA) organization of primary hexaploid cytoplasmic male-sterile (CMS) triticale regenerants containing *Triticum timopheevi* cytoplasm was analysed by hybridization experiments and compared with the mitochondrial genome organization of the corresponding regenerants with maintainer cytoplasm. Callus cultures had been derived from immature embryos, and 623 triticale plants were regenerated via somatic embryogenesis after three to four subcultures. The chondriome of 159 regenerants was investigated with regard to somaclonal variation. Six different mitochondrial gene probes and four different restriction enzymes were used for Southern blot analyses by the non-radioactive digoxigenin labeling technique. Alloplasmic regenerants showed a gain or loss of hybridization signals up to a high percentage, while euplasmic ones revealed only minor variability with respect to band stoichiometries. In 24 cases rearrangements in the mtDNA were proved. We suppose that recombination processes and selective amplification events are responsible for these findings.

Key words Mitochondrial DNA · Somaclonal variation · Cytoplasmic male sterility · *Triticum timopheevi* cytoplasm · Triticale

Introduction

In vitro culture is now commonly accepted as a tool by which to increase genetic variability among plant species with a reduced genetic heterogeneity due to extensive cultivation (Bajaj 1990). Variation induced during tissue culture is termed "somaclonal variation" (Larkin and Scowcroft 1981) and has been detected on the morphological (Mohmand and Nabors 1990) and biochemical levels (Brettel et al. 1986). Investigations on the genome of in vitro culture-derived plants have revealed genomic, chromosomal and point mutations, the type and extent of the genetic variation depending on the culture conditions and on the kind of explant used (Sibi 1981; Morère-Le Paven et al. 1992a). Although somaclonal variation has been found in many plant species (Bajaj 1990), the origin and molecular mechanism evoking this phenomenon are still not well-understood. The fundamental process of cell dedifferentiation and redifferentiation during in vitro culture is supposed to be responsible for this kind of genetic variation (Ball et al. 1990).

The plant mitochondrial (mt) genome has been proven to be a proper model for investigations on somaclonal variation. It is limited in size and, in contrast to the chloroplast genome characterized by a high degree of heterogeneity (Kemble and Shepard 1984) that results from homologous recombination along repeated sequences. Formerly, the chondriome was expected to represent a circular master chromosome. The model of a mastercircle which could be converted into subgenomic circular molecules by homologous recombination seems to be a very useful concept (Bonen and Brown 1993). However, it has not as yet been possible to find such master chromosomes, neither by electron microscopy nor by electrophoretic analyses. The present presumption is that in genomes containing multiple repeats, a single circular molecule carrying the complete genetic information may never actually exist in vivo (André et al. 1992). Rather, the chondriome is assumed to consist of a multipartite population of molecules (see Bonen and Brown 1993).

In wheat somaclonal variation has been found by several investigators in the form of mtDNA rearrangements (Hartmann et al. 1987, 1992, 1994; Rode et al. 1987; Chowdhury et al. 1994; Morère-Le Paven et al. 1992 a,b). In most cases the observed rearrangements take place at non-coding "hypervariable regions" that contain repeated sequences allowing homologous recombination events. Falconet et al. (1984) published a model for this mechanism. Comparative Southern hybridization analyses have

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revealed altered mtDNA configurations such as variable band intensities and the loss or gain of hybridization bands (Chowdhury et al. 1994; De Verno et al. 1994).

Triticale is known to contain the cytoplasmic components of its maternal crossparent wheat. Results from investigations on somaclonal variation in wheat should therefore be applicable to triticale. The cytoplasmic male-sterile (CMS) trait is widely spread among higher plant species, often occurring spontaneously. In hybrid breeding programmes it has turned out to be of great value to plant breeders because laborious hand-emasculation is avoided. CMS in triticale can be traced back to a system established for wheat by Wilson and Ross (1962) in which male sterility is induced by the introduction of the *Triticum timopheevi* cytoplasm. In many cases CMS is linked with rearrangements in the mtDNA (Newton 1988). Mutations arising by recombinational events may become stabilized and selectively amplified. It is also suggested that changes in mitochondrial gene expression occur because the "foreign" nucleus is unable to correctly regulate these nuclear mitochondrial interactions (Leaver et al. 1989). However, our understanding of the molecular mechanisms connected with CMS is very limited.

In the study presented here comparative hybridization analyses were carried out on allo- and euplasmic triticale material regenerated via somatic embryogenesis. Mitochondrial gene probes were non-radioactively labeled with digoxigenin and hybridized to total DNA. The hybridization patterns of alloplasmic plants were compared to those of euplasmic plants. It was our intention to determine whether CMS triticale containing *T. timopheevi* cytoplasm in a *T. durum* or *turgidum* nuclear background shows a different response to in vitro culture conditions than the corresponding maintainer plants.

Materials and methods

Plant materials

Glasshouse-grown hexaploid primary triticale plants were used as source material. Eight triticale genotypes with *T. timopheevi* cytoplasm (CMS triticale) and 8 with *T. durum* or *turgidum* maintainer cytoplasm were investigated. Seeds were kindly provided by Dr. G. Oettler from the Landessaatzuchtanstalt, University of Hohenheim, where the different genotypes had been treated by crosses between tetraploid wheat containing the corresponding cytoplasm and eight different paternal rye inbred lines (Table 1). In comparative hybridization analyses between somaclones, 7-day-old etiolated seedlings of each genotype served as control plants.

Somatic tissue culture

Immature seeds were harvested 12–16 days after anthesis and surface-sterilized for 1 min in 70% EtOH, 6 min in 3% NaOCl, followed by rinsing 3×5 min in bidistilled water. The immature embryos were excised under a dissecting microscope, and 4–6 embryos were then placed onto callus induction medium in 5-cm Ø petri dishes. The basic medium contained the Murashige and Skoog (1962) inorganic salts supplemented according to Nakamura and Keller (1982) and Stolarz and Lörz (1986). Organic components were modified as recommended by Dormann (1992). Callus cultures were maintained at 24°C without illumination. Primary calli obtained after 5 or 6 weeks were subcultured three or four times in intervals of 4 weeks.

Regeneration

Calli showing embryogenic structures were placed on regeneration medium in baby-food jars, 4–6 per jar and held at 24°C under artificial light maintaining a day/night rhythm of 16/8 h. The MS-based regeneration medium was supplemented according to Dormann (1992).

Preparation of total DNA

Shoot and leaf tissue (200–600 mg) were ground in a mortar with liquid nitrogen. The resulting powder was transferred into a 2-ml reaction tube, dissolved in 700 µl lysis buffer (150 m*M* NaCl, 100 m*M* EDTA, 0.5% SDS) and homogenized vigorously on a Vortex; 500 µl of saturated phenol was then added, followed by repeated homogenization. After centrifugation for 15 min at 8000 rpm and 4°C the supernatant was extracted twice with chloroform/isoamylalcohol (24:1) and again centrifuged. The supernatant was dialysed overnight in 5 l TES buffer (30 m*M* TRIS-HCl, 50 m*M* NaCl, 0.5 m*M* EDTA, 3.5% PEG 4000) using Visking dialysis tubes type 8/32 (Roth, Karlsruhe). Usually 100–300 µl of DNA solution was obtained. DNA concentrations ranged between 0.5 and 2 µg/µl.

Molecular probes

Hybridization analyses were carried out using two plasmid probes and four lambda probes. Probes were kindly provided by Prof. U. Kück (Ruhr-Universität, Bochum). Probes and coding regions are shown in Table 2. Lambda probes contained additional 5′- and 3′-flanking regions.

Table 1 Crossing diagram and description of the triticale source material

Rye parent	Wheat parent							
	Alloplasmic				Euplasmic			
	cms D8	cms D30	cms D40	cms T34	D8	D30	D40	T34
L25	–	–	–	cms 652	–	–	–	652
L70	–	–	cms 745	–	–	–	745	–
L138	–	–	–	cms 658	–	–	–	658
L145	cms 221	–	–	–	221	–	–	–
L155	–	–	–	cms 661	–	–	–	661
L283	cms 224	–	–	–	224	–	–	–
L301	–	cms 733	–	–	–	733	–	–
L305	cms 228	–	–	–	228	–	–	–

Table 2 Mitochondrial gene probes and corresponding inserts

Plasmid probes	Insert	Lambda probes	Insert
pTae8	*cox III, atp6, rps13*	LT1-38	*cox II* [exon b]
pTae1	*atpA, atp9*	LT1-85	*nad5*
		LT1-95	*cox I*
		LT1-173	*nad1* [exon b and c]

Southern blot analyses

Restriction endonuclease digestion of total DNA using *Eco*RI, *Hin*dIII, *Bam*HI and *Bgl*II, agarose gel electrophoresis, Southern blotting, DNA labeling with digoxigenin (DIG) and immunological detection of DNA hybridization products were performed as previously described (Pfeil et al. 1994).

Results

Comparative hybridization analysis of euplasmic and alloplasmic triticale

The sensitivity of the non-radioactive labeling and detection method turned out to be sufficient for our purposes. The following results were obtained by comparative hybridization analysis of the control plants and non-variant regenerants. There were no differences in hybridization patterns among the 8 alloplasmic triticale genotypes; the different euplasmic triticale genotypes also showed no differences. With four out of the six probes differences were detected between alloplasmic and euplasmic triticales of the same paternal genotype (Table 3).

Variability in the chondriome of triticale regenerants

Regenerants showed variability in the mitochondrial genome organization after in vitro culture whereas coding regions were preserved, with corresponding bands of the latter being stable in intensity and presence. Reproducible differences in the mtDNA organization were found in 24 CMS somaclones from a total of 159 regenerants investigated (15.1%). Genetic variation was detected by pTae8 and LT1-173 which contain the *atp6, cox III, rps13* region and *nad1* (exons b and c), respectively. The only cases considered were those showing a loss or gain of hybridization signals or distinctly different band intensities. Two examples of somaclonal variation were chosen to demonstrate different possibilities in the realization of mtDNA organization. Each shows the divergent hybridization patterns detected when regenerants having the same alloplasmic genotype were compared. Hybridization patterns of 11 somaclones probed with pTae8 revealed a gain of one (11.0 kb) or two (11.0 kb and 10.0 kb) bands (Fig. 1). When probe LT1-173 was applied, 13 regenerants showed a significant reduction in a 7.0-kb band (Fig. 2). The hybridization results shown in Figs. 1 and 2 resemble the banding

Table 3 Band sizes of *Eco*RI or *Hin*dIII restriction fragments obtained from six probes hybridized to total DNA of fertile (f) and cytoplasmic male-sterile (s) triticale control plants and non-variant regenerants. *Bold figures* refer to bands having no equivalent when the two cytoplasm types are compared.

Probe (insert)	Restriction endonuclease	Cytoplasm type	Band sizes in kb
pTae 1 (*atp6*)	*Eco*RI	f	7,3–6,7–3,8–2,6
		s	7,3–6,7–**6,0**–3,8–2,6
pTae 8 (*atpA*)	*Eco*RI	f	**12,0–11,0–10,0–9,0**
		s	**12,2–3,0**
LT1-38 (*cox II*)	*Eco*RI	f	4,7–4,0–2,6–1,6
		s	4,7–4,0–2,6–1,6
LT1-85 (*nad5*)	*Eco*RI	f	12,5–3,0–2,5–1,7
		s	12,5–3,0–2,5–1,7
	*Hin*dIII	f	9,1–5,7–4,7–3,4–1,3–1,2
		s	9,1–5,7–4,7–3,4–1,3–1,2
LT1-95 (*cox I*)	*Eco*RI	f	13,5–7,8–3,1–**2,2**
		s	13,5–7,8–**4,0**–3,1
	*Hin*dIII	f	9,1–**5,9**–2,6–2,4–1,9–1,8
		s	9,1–**6,2**–2,6–2,4–1,9–1,8
LT1-173 (*nad1*)	*Eco*RI	f	6,8–4,3–2,6–1,9
		s	6,8–**6,0**–4,3–2,6–**2,1**–1,9
	*Hin*dIII	f	9,3–**8,0**–7,0–3,5–2,0–1,9–**1,4**–1,3–1,1
		s	**13,0**–9,3–7,0–3,5–2,0–1,9–1,3–1,1

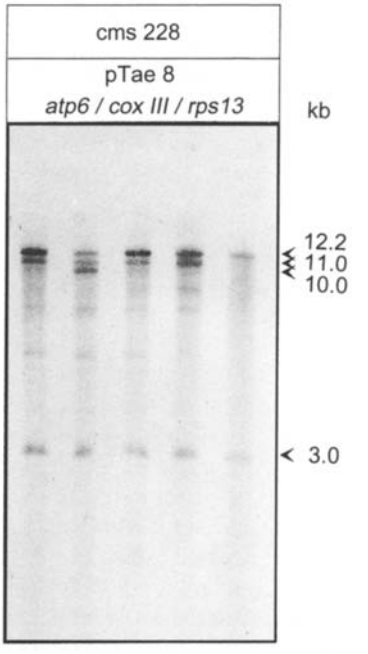

Fig. 1 Southern blot hybridization analyses of total genomic DNA from five different cms 228 triticale regenerants with mtDNA probes: blot of *Eco*RI digests hybridized to the pTae8 probe consisting of the *atp6*, *cox III* and *rps13* genes. Only the regenerant in *lane 5* (*right*) shows a non-variant hybridization pattern similiar to that of the corresponding control plants. *Lanes 1, 3* and *4* show one additional band of 11 kb; *lane 2* shows two additional bands of 10 and 11 kb

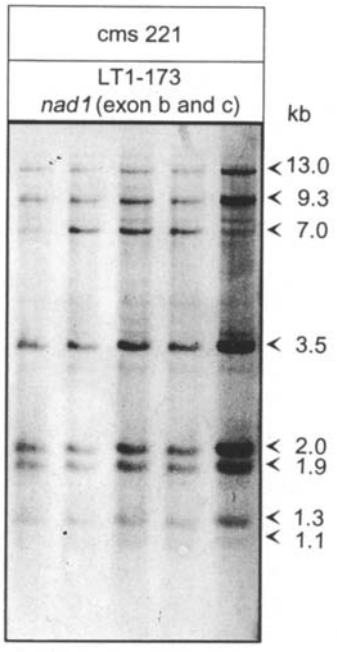

Fig. 2 Southern blot hybridization analyses of total genomic DNA from five different cms 221 triticale regenerants with mtDNA probes: blot of *Hin*dIII digests probed with LT1-173 consisting of the *nad1* exons b, c and flanking regions. Hybridization patterns of the regenerants in the *lanes 2–4* do not differ from plants grown from seed. In *lanes 1* and *5* the usually distinct 7-kb signal shows lower intensity

patterns of somaclones presented elsewhere (Kück et al. 1995), although the present data derive from different regenerants. This fact indicates that while somaclonal variation is a common phenomenon in CMS triticale after in vitro culture, certain specific mtDNA rearrangements are predominantly realized. In regenerants derived from fertile triticale only a slight variability with respect to band intensities was detected.

Discussion

In triticale the mitochondrial genomes derived from either tetraploid wheat cytoplasm donor *T. durum* or from *T. turgidum* appear to be quite similar. The nuclear rye genome seems to have little influence on chondriomic configurations: among genotypes containing the same cytoplasm type but various rye genomes no differences were detected. Similar results were reported by Song and Hedgcoth (1994) for the *orf256-cox I* gene region: no differences in mtDNA organization were revealed through different nuclear backgrounds but variable transcription of the *orf256-cox I* gene region.

In comparison to euplasmic plant material mtDNA organization in triticale with *T. timopheevi* cytoplasm proved to be different when four out of six probes were applied. These results are in accordance with those found by Breiman (1987) in wheat. It is not known if changes in the chondriome of alloplasmic triticale result from rearrangements corresponding to cytoplasmic male sterility or if they preexist in the cytoplasm donor. The latter possibility seems to be verified by Rathburn et al. (1993), who showed that CMS in *T. aestivum* is not associated with rearrangements of mtDNA in the gene regions for *cob, cox I* and *cox II*, and Mohr et al. (1993), who found different mtDNA configurations with respect to the *atp6* gene region between fertile *T. aestivum* and CMS *T. timopheevi*.

Our results demonstrate the conservation of the investigated coding regions in triticale regenerants. While obvious changes in band stoichiometries occurred and additional bands appeared, bands representing mitochondrial genes remained totally stable when compared to the hybridization bands of plants grown from seed.

Somaclonal variation observed during this study is supposed to originate in recombinational processes. The wheat mitochondrial genome is known to contain about ten repeats (Quetier et al. 1989) that allow the occurrence of homologous recombination. One of those is part of the *atp6* gene region that is included in pTae8; this repeat is known to be recombinationally active (Mohr et al. 1993). Thus, the additional bands detected with pTae8 could be the result of homologous recombination and selective amplification of former substoichiometric molecules emerging from the stress of in vitro culture. Investigations by quantitative polymerase chain reaction are providing abundant evidence for this theory (Laser 1994). The appearance of additional hybridization bands has also been previously reported by Hartmann et al. (1994) for a wheat regenerant and by Shirzadegan et al. (1991) for cultured cells of *Brassica campestris*.

Homologous recombination and selective amplification processes can also lead to changes in band stoichiometries such as those detected with LT1-173 (*nad1* exons b and c). For a better understanding of this particular type of variation further investigations must take a closer look on the 3′-flanking region of the *nad1* gene, which is not yet well-characterized. Sequence analysis of the upstream gene region rules out the presence of a recombinationally active repeat (Bonen 1987). Similar variability in the stoichiometry of hybridization bands has been previously reported by De Verno et al. (1994) for *Larix* and by Chowdhury et al. (1994) for *T. aestivum*.

In our study each of the obvious cases of somaclonal variation was detected in regenerants from CMS plants. The heterogeneity of their mitochondrial genome seems to increase during in vitro culture while the mtDNA of regenerants derived from maintainer plants seems to remain more stable. No comparable results have as yet been reported elsewhere. We suggest that in alloplasmic triticale a nuclear-cytoplasmic incompatibility introduces a higher range of variability in the mitochondrial genome than occurs in that of euplasmic triticale where nuclear-cytoplasmic interactions are better coordinated. The mechanisms leading to these observations are not yet well-understood. There is possibly a relation between events resulting in the structural variability of the mtDNA and those resulting in cytoplasmic male sterility. Further investigations on so-

maclonal variation detected in the mtDNA should extend our knowledge on the reasons for and mechanisms of the chondriomic heterogeneity. Simultaneously, a great step towards our understanding of the CMS trait may result from these efforts.

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