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Somaclonal variation of the mitochondrial ATPase subunit 6 gene region in regenerated triticale shoots and full-grown plants

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Abstract Comparative hybridization analyses of total DNA from fertile and cytoplasmic male-sterile (CMS) triticale plants which had been regenerated from embryogenic callus cultures revealed the organization and variation of the mitochondrial *atp6* gene region. In order to compare different developmental phases, we analysed mitochondrial DNA (mtDNA) from both the shoots and full-grown regenerants. Somaclonal variants were identified on the basis of differences in the mtDNA from fertile and CMS triticale. Several shoots as well as all of the full-grown plants analysed showed somaclonal variation. This phenomenon could be traced back to having primarily orginated from the influence of the nuclear background, which give rise to a stoichiometric increase in a rye-specific *orf25* gene copy, and a tissue culture-induced combination of fertile and CMS-specific mtDNA organization of the *atp6* gene area. The latter event is probably caused by the homologous recombination of repetitive sequences that may be accompanied by selective amplifications.

Key words Somaclonal variation · Cytoplasmic male sterility · Triticale · Regeneration · Mitochondrial *atp6* gene region

Introduction

The term somaclonal variation – introduced by Larkin and Scowcroft in 1981 – includes all of the changes in the nuclear, mitochondrial (mt) and chloroplast (cp) genomes of higher plants induced by tissue culture. This phenomenon has been demonstrated on the morphological (Stolarz and Lörz 1986), biochemical (Ryan and Scowcroft 1987) and molecular levels (Lapitan et al. 1988; Brown et al. 1991) in different regenerated plant species. Certain conditions like culture period or growth factors can influence the extent of somaclonal variation (Sibi 1981). Over the years a large number of somaclonal variants have been selected for and are presently been used in breeding programmes (Krishnamurthi and Tlaskal 1974; Evans and Sharp 1983).

Several authors have reported the existence of somaclonal variation in particular for the mitochondrial genome of plant regenerants (Kemble and Shepard 1984; Shirzadegan et al. 1988; Hartmann et al. 1992). The mitochondrial genome of higher plants is assumed to consist of a population of circular molecules. Homologous recombination events initiated on direct and inverted repeats are responsible for the forming of subcircles, which could explain the high degree of heterogeneity observed in mtDNA (Quetier et al. 1985).

In the study presented here, comparative hybridization analyses were carried out on fertile and cytoplasmic male-sterile (CMS) triticale regnerants. The CMS phenomenon seems to be caused by a nuclear-mitochondrial incompatibility that results in pollen sterility (Lonsdale 1987). The use of CMS plants as maternal parents has proven suitable for the production of desired crossing combinations that can be of economical value by nuclear-induced fertility restoration. In both wheat and in triticale the CMS system was established by introducing the cytoplasm from *Triticum timopheevi* (Wilson and Ross 1962).

Hybridization analyses on the organization of the *atp6* gene region revealed large differences between fertile and CMS triticale regenerants (Pfeil et al. 1994). Further analyses of regenerated triticale shoots showed a high degree of somaclonal variation with respect to this gene region, especially in CMS plants (Weigel et al. 1995). In order to obtain more detailed information we took a closer look at this gene region by extending our investigations to full-grown triticale regenerants.

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Materials and methods

Plant material

Molecular analyses were carried out on regenerated triticale shoots and fully mature regenerants (*T. durum* cytoplasm) and CMS (*T. timopheevi* cytoplasm) triticale material. All of the triticale material was of the primary type and had the cross numbers 221, 228 and 745. More details on this material and the corresponding crossing diagram can be obtained from Weigel et al. (1995).

Heterologous probing

Probes were kindly provided by Prof. U. Kück (Ruhr-Universität Bochum, Insitut für Botanik). Table 1 gives a general view of the applied recombinant plasmid probes. Preparation of total DNA, restriction endonuclease digestion using *Eco*RI and *Hin*dIII, agarose gel electrophoresis, Southern blotting, Southern hybridization with digoxigenin (DIG)-labeled probes and immunological detection were performed as previously described (Pfeil et al. 1994).

Stripping and reprobing of membranes

Stripping and reprobing of membranes were carried out according to the DIG system user's guide of Boehringer (1989). For removal of the colour precipitate the nylon membranes were incubated in heated dimethylformamide (50°–60°C) until the blue colour precipitate was removed from the filter. For removal of the probe the membranes were rinsed thoroughly in water, incubated in 0.2 *M* NaOH, 0.1% SDS at 37°C for 30 min and rinsed in 2 × SSC buffer. The filter could then be dried or used directly for rehybridization.

Results and discussion

Organization of the *atp6* gene region in fertile and CMS triticale

The *atp6* gene is situated on a 1.4-kb repeat element that is able to undergo homologous recombination. In the mtDNA of fertile plants four different arrangements of the *atp6* gene region have been discovered. In *T. aestivum*, Bonen (1987) showed that two of these are *atp6* gene copies, called *atp6-1* and *atp6-2*; the other two copies probably arise from recombination processes between the first two (Bonen and Bird 1988). In constrast, in sterile cytoplasms (*T. timopheevi*) only one single-copy gene exists. Due to this difference in organization between fertile and CMS cytoplasms, a relationship between the *atp6* gene arrangements and the phenomenon of CMS was taken into consideration (Mohr et al. 1993). As the studies of Pfeil et al. (1994) and Weigel et al. (1995) as well as our own investigations did not reveal any differences between *T. aestivum* and *T. durum* cytoplasm with respect to the mitochondrial *atp6* gene region, we refer to the mtDMA organization known from *T. aestivum*. Figure 1 gives a general view of the organization of the mitochondrial *atp6* gene region in fertile and sterile cytoplasms in combination with the hybridization sites of the heterologous probes applied.

Table 1 Mitochondrial gene probes

Probe	insert
pTae8	*coxIII/atp6/rps13*
pTATP6-2	*atp6/rps13*
pTATP6-4	*atp6*

Comparative hybridization analysis of *Eco*RI-digested DMA

*Eco*RI-digested DNA from fertile full-grown regenerants was hybridized with the heterologous probe pTae8. This probe contains the 11.0-kb *Eco*RI fragment from *T. aestivum* on which can be found the cytochrome C oxidase subunit 3 gene (*coxIII*), the ATPase subunit 6 gene (*atp6-1*) and a part of 10.0-kb and 9.0-kb segments that contain the four *atp6* gene copies described above (Fig. 2, lane a). The hybridization pattern achieved did not reveal somaclonal variation. Weigel et al. (1995) also did not detect somaclonal variants within fertile regenerated triticale shoots using this restriction enzyme/probe combination.

When the sterile cytoplasm type was examined, bands of 12.2 kb and 3.0 kb appeared in the hybridization pattern. On the 12.2-kb fragment the *orf25* gene and the *coxIII* gene are involved, while the 3.0-kb fragment contains the *atp6* and *rps13* gene (Fig. 2, lane b). All of the regenerated full-grown CMS plants showed somaclonal variation in the form of an additional 11.0-kb band that has also been reported, to a certain extent, in the seedling stage of the same material (Weigel et al. 1995). We assume that this band probably can be traced back to a rye-specific *orf25* gene copy that is homologous to many sequences of the 12.2-kb-sized *coxIII/orf25 Eco*RI fragment from *T. timopheevi* cytoplasm (Fig. 1). Recently, hybridization analyses from Laser (1994) revealed the presence of a rye-specific gene copy in triticale plants. Applying the polymerase chain reaction (PCR) Laser denied the possibility of a paternal hereditary, however, she assumed the existence of this gene copy in a substoichiometric quantity in the cytoplasm of the maternal wheat lines. The stoichiometry is probably regulated by nuclear genes. On account of the fairly large band intensities that have been found in our material we accept the premise of a selective amplification of this *orf25* gene copy.

Comparative hybridization analysis of *Hin*dIII-digested DNA

For subsequent investigations of the mitochondrial *atp6* gene region we chose *Hin*dIII as a restriction enzyme and applied other molecular probes: the 3.0-kb insert from probe pTATP6-2 includes the *atp6* gene and a part of the *rps13* gene; the 1.6-kb insert from probe pTATP6-4 carried the *atp6* gene (Fig. 1). The complex hybridization patterns of *Hin*dIII-digested total DNA

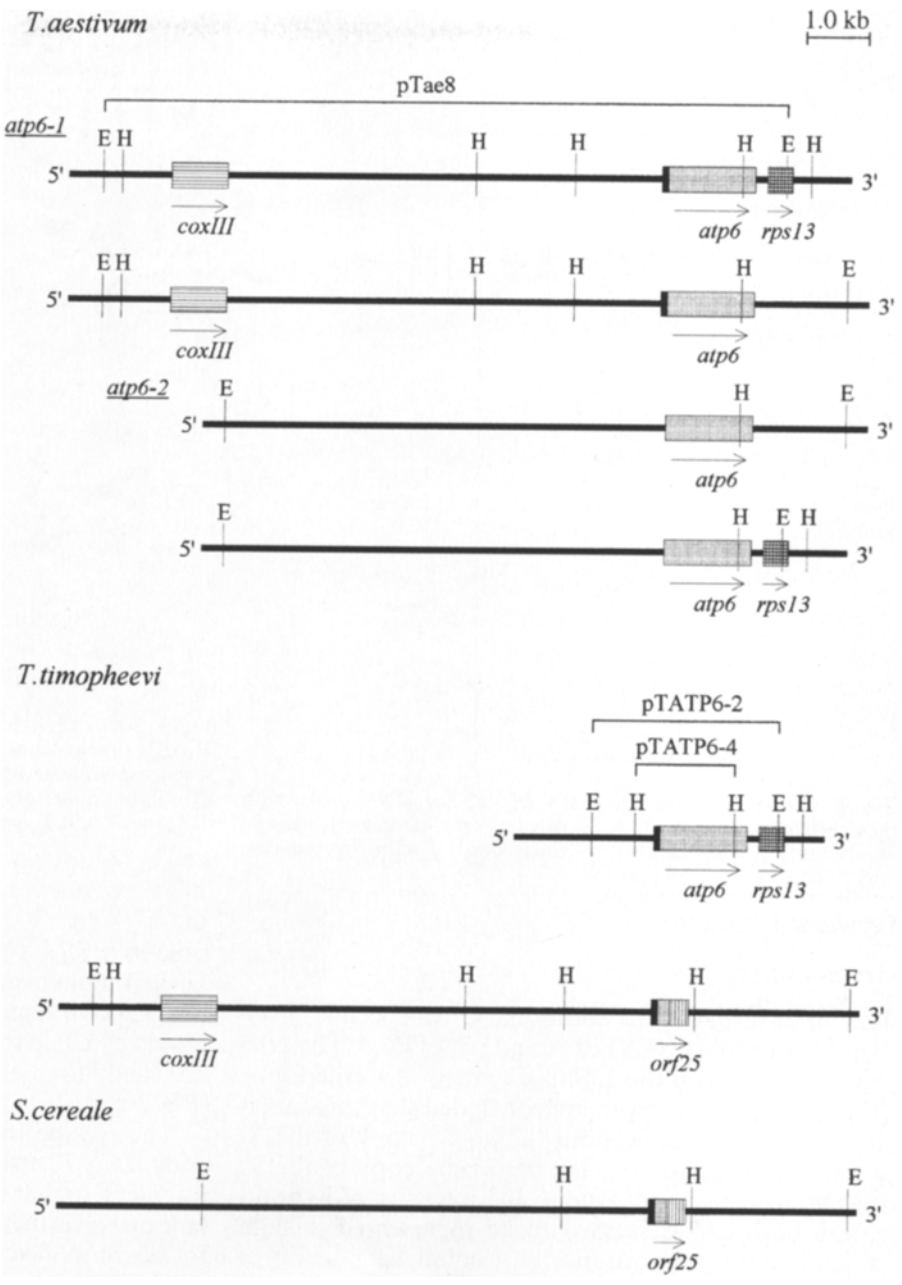

Fig. 1 Restriction maps of the *atp6* gene region in *T. aestivum* and *T. timopheevi* and of the *orf25* gene region in *T. timopheevi* and *Secale cereale*. Direction of gene location and transcription is indicated by *arrows*. Homologous sequences are marked by the same pattern. In addition, the positions of the probes employed are shown. *E EcoRI*, *H HindIII*

again confirmed the large organization differences of the *atp6* gene region between fertile and CMS triticale (Fig. 3).

In fertile cytoplasm the *coxIII* gene is integrated in a 5.7-kb *Hin*dIII fragment (Gualberto et al. 1990), the *atp6-1* gene copy in a 2.7-kb fragment and the *rps13* gene in a 1.1-kb fragment (Bonen 1987). A low-hybridizing band of 9.2 kb contains the *coxII* gene, which has sequences that are homologous to the *atp6-1* gene copy (Mohr et al. 1993). On account of this information and on the basis of our hybridization analysis, we could decode, to a certain extent, the complexity of the hybridization pattern that we achieved with the three mitochondrial probes. The 1.6-kb *Hin*dIII fragment corresponds to the mtDNA region downstream of the *coxIII* gene and upstream of the *atp6-1* gene (Fig. 1), whereas the 8.0-kb band detected (hybridized with pTae8, pTATP6-2 and pTATP6-4) probably carries the *atp6-2* gene copy; additional bands of 5.5 kb (pTATP6-2, pTATP6-4), 5.0 kb (pTATP6-2), 6.5 kb (pTae8) and 4.6 kb (pTae8) had to be attributed to non-definable DNA regions with sequences that are homologous to the specific probes (Fig. 3, lane a).

Among the fertile shoots one somaclonal variant was detected that showed a hybridization pattern that was a combination of the fertile and sterile cytoplasms (Fig. 3,

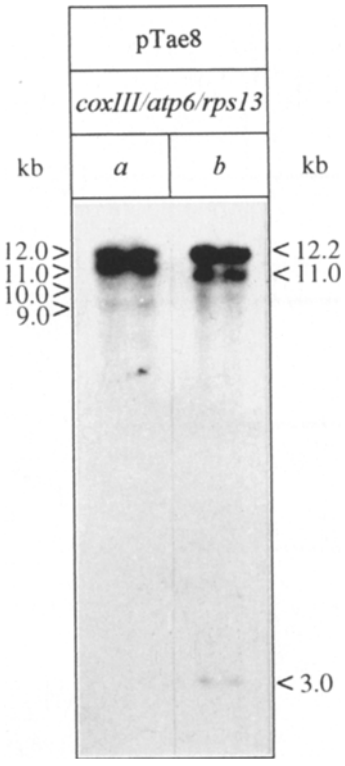

Fig. 2 Southern hybridization of a Dig-labeled DNA probe with *Eco*RI-digested total DNA isolated from full-grown triticale regenerants of cross number 221 (*lane a* with *T. durum* cytoplasm, *lane b* with *T. timopheevi* cytoplasm)

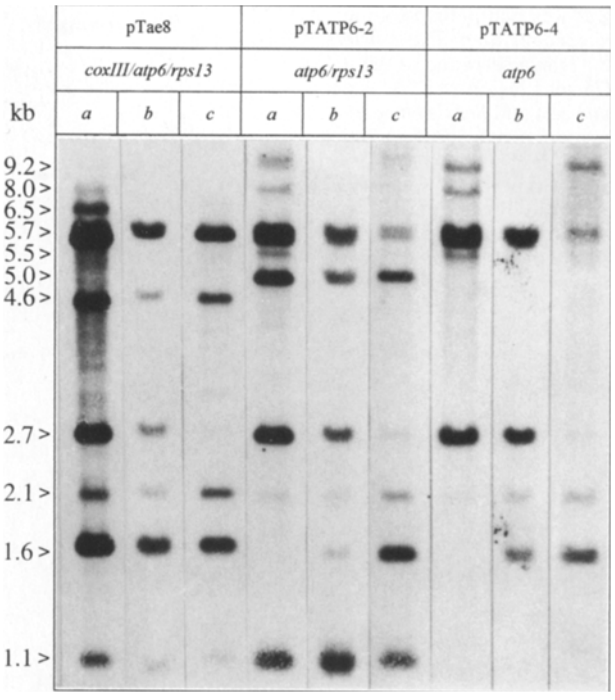

Fig. 3 Southern hybridization of Dig-labeled DNA probes with *Hin*dIII-digested total DNA isolated from full-grown triticale regenerants (*lane a* with *T. durum* cytoplasm, *lane c* with *T. timopheevi* cytoplasm) and from regenerated triticle shoots of cross number 745 (*lane b* with *T. durum* cytoplasm)

lane b). It revealed one additional CMS-specific 1.6-kb band with probes pTATP6-2 and pTATP6-4. The *atp6* gene is located on the 1.6-kb fragment of sterile forms (Fig. 3, lane c). Therefore, we concluded that this somaclonal variant showed both the *atp6-1* copy of the *T. aestivum* cytoplasm and the *atp6* gene copy of the *T. timopheevi* cytoplasm. Due to the intensity of hybridization both copies seemed to be represented in high stoichiometric proportions. This could be a result of amplification, preferably of the *atp6* gene, which is specific for the CMS trait (Fig. 3, lane b).

Only several fertile shoots but all of the analysed fertile mature plants showed somaclonal variation in the form of a 2.1-kb signal (Fig. 3, lanes a,b). This band corresponds to the rye-specific *orf25* gene. While the presence of this gene has already been demonstrated in *Eco*RI-digested DNA from sterile triticale regenerants, fertile regenerants analysed with *Eco*RI did not reveal this kind of amplification. The reason for this may be that one of the four *atp6* gene copies is part of an *Eco*RI fragment of approximately the same size as the 12.2-kb fragment that contains the rye-specific *orf25* gene copy (Fig. 1). Therefore, the two different fragments could not be discriminated.

In the cytoplasm of CMS triticale the *atp6* gene is incorporated in a 1.6-kb *Hin*dIII fragment, the *cox*III gene in a 5.7-kb *Hin*dIII fragment, the *rps13* gene in a 1.1-kb fragment and the *orf25* gene in a 2.1-kb fragment. The 9.2-kb fragment detected represents the *coxII* gene. Bands of 5.0 kb (pTATP6-2) and 4.6 kb (pTae8) may be attached to unknown homologous DNA regions (Fig. 3c).

The rye-specific *orf25* gene copy and the *orf25* gene copy from *T. timopheevi* were both located on a 2.1-kb fragment, so that they could not be discriminated (Fig. 3, lane c). Nevertheless, the rye-specific *orf25* gene seemed to be amplified in both sterile (detected with the *Eco*RI/pTae8 combination) and fertile forms.

A single hybridizing 2.7-kb band characterized all of the full-grown plants and several shoots as somaclonal variants (Fig. 3, lane c). We assume that this band corresponds to the *apt6-1* gene copy from fertile forms. Nevertheless, one should not forget that our results are based on the length of DNA fragments and that fragments of the same length need not always be equivalent, even though in all probability they are.

Conclusions

Except for somaclonal variants, no differences in the hybrdization patterns were found within the three fertile (*T. durum* cytoplasm) and the three CMS (*T. timopheevi* cytoplasm) genotypes. Furthermore, the ratio of soma-

clonal variation was not dependent on a specific genotype. Within the fertile genotypes used somaclonal variants seemed to occur at equal frequencies which was also the case for CMS genotypes. These results confirm former analyses of the *atp6* gene region (Weigel et al. 1995).

In maize, Brown et al. (1991) revealed a decrease in the variability of mtDNA regions during the course of regeneration: Discriminating between three developmental phases, callus, shoot, and grown-up, they found that cellular dedifferentiation and redifferentiation can lead to a shift in genetic material. Genetically altered cells may prevent further development during the step from callus to shoot or from shoot to grown-up. While our investigations of other mitochondrial gene regions (data not shown) confirmed the results of Brown et al. (1991), the detected somaclonal variation concerning the *atp6* gene region has been unexpected. The results achieved gives us reason to suggest either a positive, or at least a no negative, influence on vitality caused by the rye-specific *orf25* gene.

Also, the presence of fertile and CMS-specific mtDNA fragments in the same hybridization pattern has been surprising. Proceeding from the assumption that the differences detected between fertile and CMS triticale regenerants are correlated with CMS occurrence and that the latter goes along with rearrangements in mtDNA, one has to imagine a coexistence of fertile and sterile mtDNA organizations. Due to outer stress, e.g. induced by in vitro culture, one form of organization may occur at first in low stoichiometric proportions, then in equilibrium or even more. The existence of fertile revertants is already known from investigations on CMS maize regenerants, where fertility restoration was correlated with differences in mtDNA organization (Earle et al. 1986). According to the frequently observed instability of the CMS phenomenon a spontaneous fertility restoration seems to be explanable. It is still unknown whether stress-induced rearrangements of the mtDNA are combined with selection advantages for the better survival of certain phenotypes. One should not forget that correlations between mtDNA organization and the phenotypic behaviour of the plant have not yet been completely clarified.

On the basis of our hybridization analyses we conclude that, strengthened or even initiated by in vitro culture, homologous recombination of the mtDNA and selective amplification, partially induced by the nuclear background, can influence the organization of the *atp6* gene region. This may lead to a stoichiometric increase in the rye-specific *orf25* gene copy that is already present in a substoichiometric quantity in the maternal wheat lines. On the other hand, we have shown the formation of a fertility-specific mtDNA organization in CMS regenerants. It was also possible to get a more sterile organization in fertile regenerants. Based on the results achieved by hybridization analyses of full-grown triticale regenerants we suppose that this kind of somaclonal variation provides a positive influence on vitality. It is left to future investigations to elucidate whether these additional mtDNA arrangements are expressed and how they affect the biochemical and morphological levels.

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