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Mitochondrial DNA of cytoplasmic male-sterile *Triticum timopheevi*: rearrangement of upstream sequences of the *atp6* and *orf25* genes

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Summary. The organization of mitochondrial DNA (mtDNA) and transcript patterns of the *atp6* and *orf25* genes were examined in cytoplasmic male-sterile (CMS) and fertile *Triticum* lines. Major differences are observed between CMS *T. timopheevi* and fertile *T. aestivum* for both mitochondrial genes. The *T. aestivum* mt genome carries two *atp6* gene copies, whereas only a single copy of the *atp6* gene is present in *T. timopheevi* mtDNA. Sequence data suggest that identical sequences upstream of the *atp6* gene and the *orf25* gene are involved in homologous recombination in both cytoplasms. The differences in the upstream sequences of the *atp6* or the *orf25* genes affect transcript sizes in both cytoplasms. Transcription initiation may occur at conserved promoter elements located at variable distances upstream of the aminoacid coding sequences. The correlation between the gene rearrangements and the CMS phenomenon in *T. timopheevi* is discussed.

Key words: Wheat mitochondrial DNA – *atp6* and *orf25* genes – Cytoplasmic male sterility – *timopheevi* cytoplasm

Introduction

Male sterility of higher plants can be conditioned by either nuclear or cytoplasmic genes. Male sterility due to nuclear mutants has been characterized by molecular approaches only in *Arabidopsis* and *Nicotiana* (Moffatt and Somerville 1988; Mariani et al. 1990). In cases when the sterility is inherited in a non-Mendelian fashion, it is designated cytoplasmic male sterility (CMS) (reviewed by Newton 1988; Fragoso et al. 1989; Levings 1991).

Although CMS has been reported in about 200 plant species (Kaul 1988), the molecular basis of the trait is not fully understood. However, the mitochondrial genome, rather than the chloroplast genome, has been implicated as the organelle responsible for the CMS phenomenon. In maize and petunia, two extensively studied CMS systems, novel chimeric mitochondrial reading frames, *urf13-T* and *pcf*, have been found in CMS lines (Dewey et al. 1986; Young and Hanson 1987). In both cases, the gene products of the chimeric genes are associated with the male-sterile phenotype. The chimeric reading frames are believed to have arisen by homologous recombination between repeated DNA sequences in the mitochondrial genome. Thus, mtDNA rearrangements play an important role in the CMS phenomenon. Whereas *urf13-T* and *pcf* are novel genes, mutations and rearrangements of essential mitochondrial genes may also be associated with CMS (Bailey-Serres et al. 1986; Makaroff et al. 1989; Kadowaki et al. 1990).

In this study, we have analyzed rearrangements in the mitochondrial genome from male-sterile wheat. With the discovery of the *Triticum timopheevi* CMS cytoplasm, described by Wilson and Ross (1962), commercial hybrid wheat production was visualized. In wheat breeding programs the most widely applied CMS system still uses the *T. timopheevi* cytoplasm. Although not fully satisfactory, it has important advantages over a wide array of common wheat genotypes. Sterility is complete and stable over a range of environments and highly effective restorer lines are available (Wilson and Driscoll 1983). Additionally, undesirable agronomic and qualitative traits

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due to nucleo-cytoplasmic interactions are insignificant, or less pronounced, compared to common cytoplasms such as *Aegilops* spp. (Panayotov 1980).

In contrast to the numerous molecular investigations of the mitochondrial DNA from the *Triticum aestivum* cytoplasm, few examinations of mitochondrial DNA derived from the *T. timopheevi* cytoplasm have been reported (Begu et al. 1989a, b). We, have therefore, begun a comparative molecular investigation of mitochondrial DNA from fertile and sterile cytoplasms of *T. aestivum* or *T. timopheevi* respectively. We report differences in the organization and transcriptional expression of the mitochondrial genes *atp6* and *orf25* that may be associated with the CMS phenotype acquired from the *T. timopheevi* cytoplasm.

Materials and methods

Plant material

We used *T. aestivum* var. Jubilar with a *T. aestivum* nuclear genotype and *T. durum* D30 with a *T. durum* nuclear genotype as fertile wheat lines. The male-sterile *T. timopheevi* line was achieved by interspecific crosses between *T. timopheevi* × *T. durum* (D30). The resulting F_1 generation shows a sterile phenotype. Subsequent backcrosses with *T. durum* (D30) were performed to replace the *T. timopheevi* nuclear genome to near completion.

Oligonucleotides

The following olignucleotides were employed:

No.	Sequence	Position
72	5′AAT AGG CCA TGG ACT TGG ATC TAC CCA ATG 3′	complementary to bases 576–604, Gualberto et al. 1990)
187	5′TGT AGA TCG GGA AGA CGG GA 3′	*atp6* coding region, position 84 to 104
197	5′GGC GGG GAG CAG CAA TGA TAC CGC 3′	5′ flanking region of the *atp6-1* gene copy from *T. aestivum*, position −172 to −148

DNA and RNA isolation

Total wheat DNA and RNA was isolated from 7-day-old etiolated seedlings, using a modification of a procedure described by Hoge et al. (1982). Approximately ten seedlings were ground in a mortar with liquid nitrogen and the powder dissolved in boiling lysis buffer (0.2 M boric acid, 30 mM EDTA, 1% SDS, pH 9.0 adjusted with sodium hydrooxide) and an equal volume of saturated phenol. After 10 min centrifugation at 12,000 g and 40 °C the supernatant was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1). The nucleic acids were precipitated with 0.3 M sodium acetate and the pellet dissolved in distilled water. The RNA was selectively precipitated by adding LiCl to a final concentration of 2 M and incubation for 2–3 h at 4 °C. After centrifugation, the RNA precipitate was washed twice with 70% ethanol and dissolved in distilled water. The DNA-containing supernatant was dialysed overnight against 0.1 × TE (50 mM Tris, 20 mM EDTA, pH 8.0) and precipitated with 0.3 M sodium acetate, washed twice with 70% ethanol and dissolved in distilled water.

Construction of gene libraries

One hundred micrograms of total wheat DNA from male-sterile *T. timopheevi* or mitochondrial DNA from fertile *T. aestivum*, were partially digested using variable units of the endonuclease *Eco*RI. Four micrograms of restriction fragments in the range of 15–25 kb were ligated into lambda replacement vector EMBL4 as recommended by the manufacturer (Stratagene, Heidelberg, Germany). Approximately 2 µg of the ligated DNA was packed using commercial packaging extracts (Gigapack Plus from Stratagene, Heidelberg, Germany) and transfected into *E. coli* strain K803 (Federoff 1983). The resulting gene libraries were amplified as described by Philips and coworkers (1985). Gene library LT1 contains mitochondrial DNA from *T. aestivum*, whereas gene library LT2 contains total DNA from male-sterile *T. timopheevi*.

Screening of gene libraries

E. coli strain K803 was infected with aliquots of the gene libraries. Plaque filters were hybridized according to Benton and Davis (1977), using pOV *ATP6* from *Oenothera* (Schuster and Brennicke 1987), a 1.3-kb *Bgl*II subfragment of plasmid pTae8 (see Fig. 5, probe C), or oligonucleotide 187 and 197, as radiolabeled hybridization probes. The dsDNA fragments were labeled by oligonucleotide-primed labeling (Feinberg and Vogelstein 1983), and oligonucleotides were end-labeled as described in Maniatis et al. (1982).

Northern analyses

Total RNA was isolated from 7-day-old etiolated seedlings. Approximately 20 µg were fractionated in 2.2 M formaldehyde and 1.5% agarose in 1 × MOPS buffer (20 mM MOPS, 5 mM sodium-acetate, 1 mM EDTA, pH 7.0), transferred to Hybond N membrane (Amersham, Braunschweig, Germany) and bound by UV cross-linking or baking at 80 °C in vacuum. Northern experiments were conducted in 5 × SSPE (0.18 M sodium chloride, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 0.2% SDS, 50% formamide and 0.1 mg/ml salmon sperm DNA for dsDNA probes at 37 °C overnight followed by washes with 5 × SSPE, 0.2% SDS at 60 °C. Probe B (see Fig. 5) was synthesized by PCR amplification with oligonucleotide 197 and the reverse sequencing primer (Pharmacia, Freiburg, Germany) using plasmid p*ATP6-5* as a template. Plasmid p*ATP6-5* contains a 2.1-kb *Hin*dIII subfragment of LT2-1 which was cloned into vector pUC19 (Yanish-Perron et al. 1985). Oligonucleotide 72 was used as a *coxIII* gene-specific probe.

DNA amplification by the polymerase chain reaction (PCR)

In this technique, modified after Mullis and Faloona (1987), 0.01 µg of plasmid DNA in a total volume of 50 µl (containing 67 mM Tris-HCL pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.17 mg/ml BSA and 33 µM dNTPs) was amplified with 0.3 µg of each primer and two units of Replitherm DNA polymerase (Biozym, Hameln, Germany). Prior to amplification the samples were denatured 5 min at 95 °C. The amplification reaction involved denaturation at 92 °C for 2 min, annealing at 60 °C for 2 min, and polymerization at 72 °C for 2 min, for 30 cycles.

Nucleotide sequence analysis

The dideoxynucleotide chain-termination method was used for DNA sequencing (Sanger et al. 1977). Restriction fragments were subcloned into plasmid pUC19 (Yanish-Perron et al. 1985) and sequenced with a T7 polymerase sequencing kit (Pharmacia Freiburg, Germany). The universal and reverse sequencing

primers (Pharmacia, Freiburg, Germany) and oligonucleotides 187 and 197 were used for sequence determination. Sequence data were compiled and analyzed with programs from Lipman and Pearson (1985). The nucleotide sequence data reported will appear in the EMBL Sequence Databases under the accession number X62092 (*atp6* gene from *T. timopheevi*) and X62094 (*orf25* gene from *T. timopheevi*).

Results

The copy number of the atp6 genes is different in fertile and sterile wheat lines

Southern-blot hybridizations of total DNA from *T. aestivum*, *T. durum* and CMS *T. timopheevi* with mitochondrial gene probes from wheat (Schulte-Kappert and Mohr, unpublished) were performed to analyze the differences between the fertile and sterile cytoplasms. By RFLP analyses and Northern hybridization, no variation of mtDNA organization and transcript pattern were seen for the mitochondrial *coxIII*, *nad5* and *rps13* genes. The *cob*, *coxI*, *coxII*, *atp9* and *atpA* genes have a different organization in sterile compared to fertile wheat. However, the sizes of the transcripts are identical in all cytoplasms investigated, suggesting no major differences in the transcribed regions exist. The only exception detected involved the *atp6* and *orf25* genes, which exhibit a different mtDNA organization as well as a variant transcript pattern in *T. timopheevi* compared with *T. aestivum* or *T. durum*. Because rearrangements of the mitochondrial genome are believed to be associated with the CMS phenotype, we chose the *atp6* and *orf25* mtDNA regions from *T. timopheevi* for comparison with the appropriate genes from fertile *T. aestivum*. Since *T. aestivum* and *T. durum* did not show any differences in mitochondrial gene structure and the transcript patterns of mitochondrial RNAs, further comparative characterizations were carried out exclusively with material from *T. aestivum*.

As shown by Bonen and Bird (1988), the *atp6* gene from *T. aestivum* is located within a repeated DNA element which can undergo homologous recombination, resulting in four genomic copies of the gene. We decided to construct lambda gene libraries in order to characterize extended mtDNA regions.

The lambda gene library LT1 was screened using the plasmid pOV*ATP6* from *Oenothera* (Schuster und Brennicke 1987). Four *Eco*RI fragments were found to contain the complete *atp6* coding sequence (Fig. 1). The fragments were subcloned into plasmid pBR328 (Soberon et al. 1980) and named pTae4, 5, 6 and 8. Two of the four fragments share identical upstream or downstream regions flanking the *atp6* gene as a consequence of homologous recombinations of the repeated DNA sequences (see Fig. 1). The *atp6* open reading frames and flanking regions represented in pTae8 and pTae6 will be further referred to as *atp6-1* and *atp6-2*, respectively.

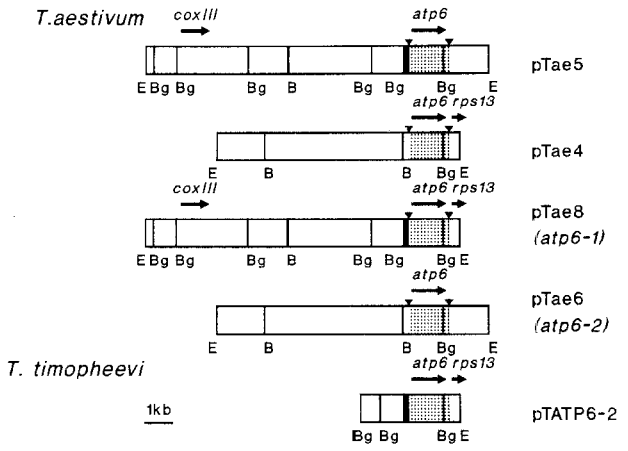

Fig. 1. Physical maps of the mtDNA *Eco*RI fragments carrying the *atp6* genes from fertile *T. aestivum* and male-sterile *T. timopheevi*. The location and transcription direction of the *atp6*, *coxIII* and *rps13* genes are indicated by *arrows*. Termini of duplicated fragments are marked by *triangles*; the homologous sequence in the *T. timopheevi* gene copy is shown by a *dotted pattern*. The *black box* indicates the 110-bp fragment present in the 5′ region of the *T. timopheevi atp6* gene as well as in two copies of the *atp6* gene from *T. aestivum*. Abbreviations of restriction sites; *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI

Hybridization experiments indicate that plasmids pTae4 and pTae8 carry the *rps13* gene downstream from the *atp6* coding region, as described by Bonen and Bird (1988). Upstream of the *atp6* gene, plasmids pTae5 and pTae8 contain the mitochondrial *coxIII* gene, as shown by hybridization and Northern experiments with oligonucleotide 72 (data not shown).

In the *T. timopheevi* cytoplasm, the *atp6* gene organization is completely different. Using a 1.3-kb *Bgl*II subfragment of plasmid pTae8 as a probe (see Fig. 5a, probe C), we isolated lambda clones carrying the *atp6* gene from the *T. timopheevi* cytoplasm. The *T. timopheevi atp6* gene is located on a 3.5-kb *Eco*RI fragment, as was determined by genomic DNA hybridization (data not shwon). The 3.5-kb *Eco*RI fragment was subcloned from recombinant lambda clone LT2-76, resulting in plasmid pTATP6-2 (Fig. 1).

The sequence that is repeated in *T. aestivum* is present in the *T. timopheevi* cytoplasm, but on a single-copy fragment (Fig. 1). The downstream organizations of the *T. timopheevi atp6* gene and the *T. aestivum atp6-1* gene copy are identical. As a consequence, the *rps13* gene is located downstream of the *atp6* genes in both cytoplasms. Total DNA hybridizations indicate that the similarity of the *atp6* 3′ flanking fragment with the *atp6-1* gene copy extends at least 12-kb further downstream (data not shown). Upstream of the *T. timopheevi atp6* gene, a small region of approximately 110 bp was detected that hybridizes to the upstream flanking regions of the *atp6-1* gene copy (indicated by black pattern in Fig. 1).

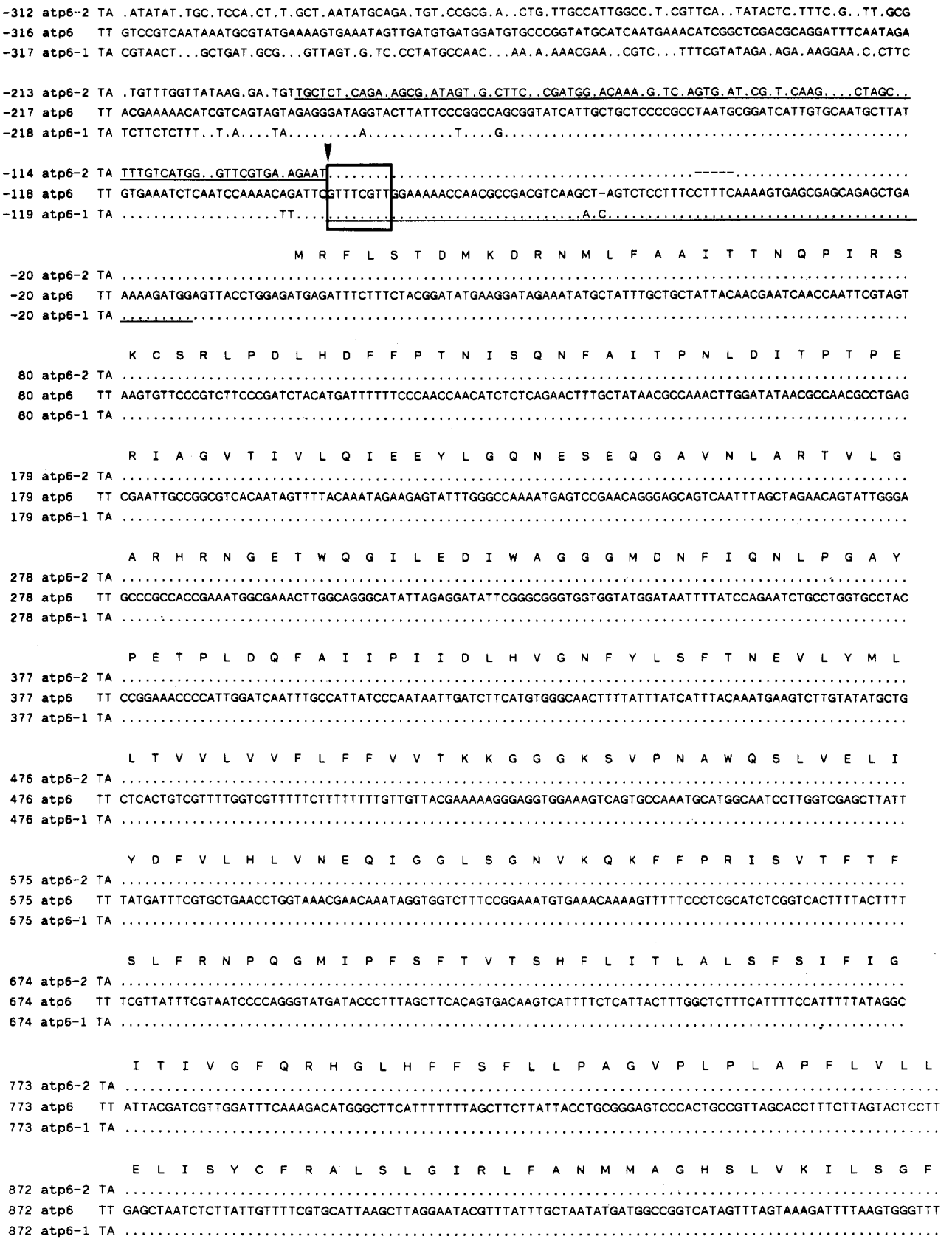

Fig. 2. Nucleotide sequence and derived amino-acid sequence of the *atp6* gene regions. The sequence of the singular *atp6* gene in *T. timopheevi* (*TT*) is shown in comparison to the two gene copies *atp6-1* and *atp6-2* from *T. aestivum* (*TA*). *Dots* indicate identical nucleotides; *dashes* were introduced to increase homology. *Arrowheads* delimit the recombinationally active repeated region in *T. aestivum*. The *box* marks a nucleotide sequence found upstream of several mitochondrial genes from cereals (see text for references). The *underlined sequence* is present 5′ of the *coxII* gene from *T. aestivum* (Bonen et al. 1984)

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                A W T M L F L N N I F Y F I G D L G P L F I V L A L T G L E L G V
971 atp6-2 TA ..................................................................
971 atp6   TT ACTTGGACTATGCTATTTCTGAATAATATTTTCTATTTCATAGGAGATCTTGGTCCCTTATTTATAGTTCTAGCATTAACCGGTCTGGAATTAGGTGTA
971 atp6-1 TA ..................................................................

                A I S Q A H V S T I S I C I Y L N D A T N L H Q N E S F H N *
1070 atp6-2 TA ..................................................................
1070 atp6   TT GCTATATCACAAGCTCATGTTTCTACGATCTCAATTTGTATTTACTTGAATGATGCTACAAATCTCCATCAAAATGAGTCATTTCATAATTGAATAAAA
1070 atp6-1 TA ..................................................................

1169 atp6-2 TA ..............................................................-----.....
1169 atp6   TT ACGAGGAGCCGAAGATTCTAGGGGGCTACAGCTGCGCTTTTGCAGCACTGAACATGGTTCCGGGTACTCAAGAAAGATATTGCGTTTGTGTTTGGAGAG
1169 atp6-1 TA ..................................................................

                ▼
1268 atp6-2 TA ........AGATAG.ACGTAGTCTTGCTCGATCAG.CC.CAAGC.AGG.AAG.GCAACCGCGCG
1268 atp6   TT GTGTAGATTGTAGATTCCAGCCGAGAAGACCAGGAAAAGATAAGGATAAAGAATGTCATATATC
1268 atp6-1 TA ..........................................................
                                                          --> rps13
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The 5′ adjacent region has no homology with any of the four *atp6* gene regions from *T. aestivum*.

Despite conserved amino-acid coding sequences, the 5′ flanking noncoding region of the *atp6* genes are rearranged in the sterile wheat cytoplasm. To determine the extent of sequence homology between the *atp6* gene copies from *T. aestivum* and *T. timopheevi* precisely, the *atp6-1* (located on plasmid pTae8) and *atp6-2* (located on plasmid pTae6) gene copies were sequenced in addition to the *atp6* gene from *T. timopheevi*. No differences were seen in the amino-acid coding region of all three genes determined (Fig. 2). The sequence of the repeated element containing the *atp6* gene in *T. aestivum* is 99.8% identical in both copies. Separate five-nucleotide insertions occur 5′ and 3′ to the *atp6-1* copy. These insertions were also found in *T. timopheevi*. The insertion at the 3′ end consists of the sequence 5′ TGCGT 3′ (Fig. 2), whereas Bonen and Bird (1988) report an insertion of the sequence 5′ TTGCT 3′. The insertion near the 5′ end of the repeated element (5′ CTTTC 3′) differs from the sequence variations reported by Bonen and Bird (1988), which is most likely due to the different wheat varieties used in the two studies.

The *atp6-1* sequence from *T. aestivum* and the *atp6* sequence from *T. timopheevi* differ by 2 bp within the repeated region of *T. aestivum*. The similarity of the two sequences extends further 5′ beyond the recombination breakpoint determined for *T. aestivum* (Bonen and Bird 1988). In *T. timopheevi* no equivalent to the *atp6-2* gene copy from *T. aestivum* was detected. The *orf25* gene from *T. timopheevi* contains a 162-bp upstream sequence with 97% identity to the singular *atp6* gene copy that is present in the male-sterile line. Both the *atp6* and *orf25* genes from *T. timopheevi* carry the 5-bp insertion described above.

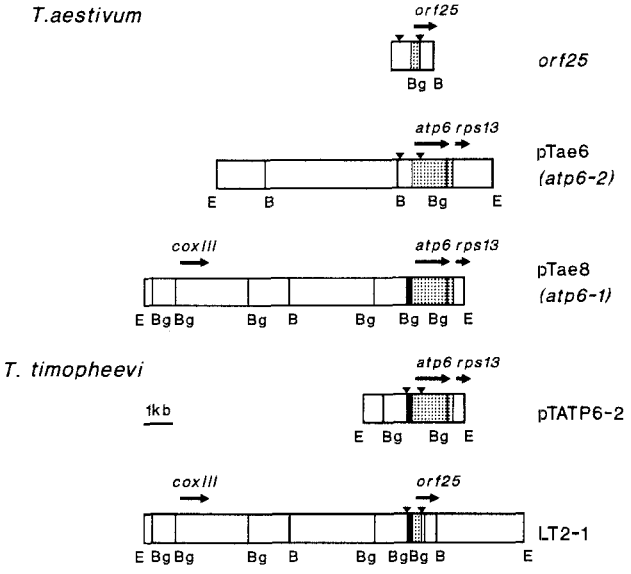

Fig. 3. Physical-genetic maps of the *orf25* gene regions from *T. aestivum* and *T. timopheevi* in comparison to the organization of the *atp6* genes. In *T. aestivum*, only two of the four *atp6* recombination products are shown. The data for the *orf25* gene from *T. aestivum* are taken from Bonen et al. (1990). Homologous sequences are shown by the same pattern and mark repetitive sequences either in the *T. aestivum* or the *T. timopheevi* mtDNA. Abbreviations are explained in the legend of Fig. 1

The sequence data in Fig. 2 demonstrate that the *atp6* gene region of male-sterile *T. timopheevi* has a different organization than found in fertile *T. aestivum*. The differences concern the copy number of the *atp6* genes and the sequences in the 5′ flanking regions. The effect of these 5′ sequence deviations on transcription initiation will be discussed later.

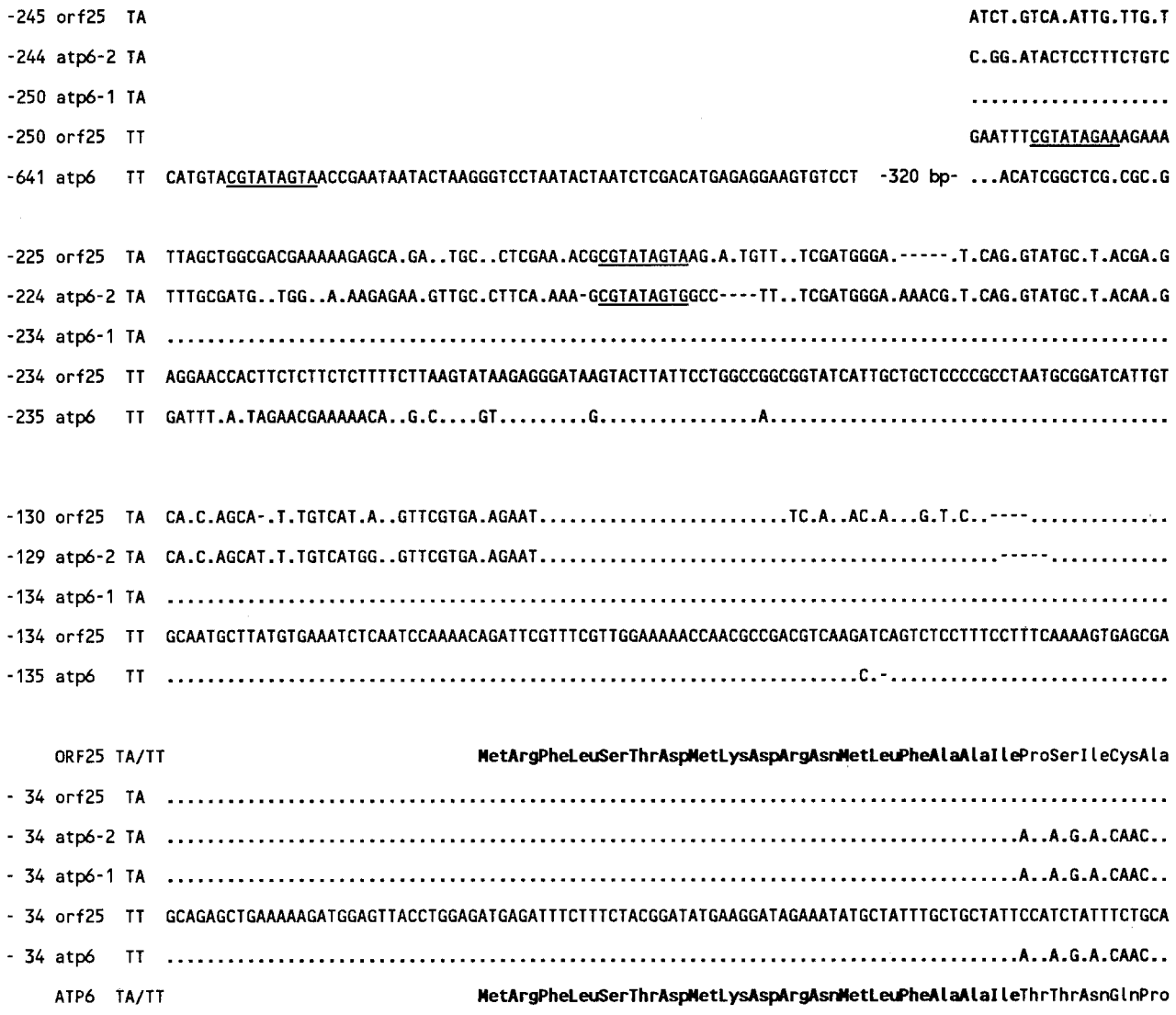

Fig. 4. Nucleotide sequences and derived amino-acid sequences of the amino-termini and upstream regions of the *atp6* and *orf25* genes from *T. timopheevi* (*TT*) and *T. aestivum* (*TA*). All sequences were compared to the *orf25* gene from TT, identical nucleotides are shown by dots. *Dashes* are introduced to increase homology. Amino acids identical between the *ORF25* and *ATP6* translation products are typed in *bold letters*. Putative promoter sequences are *underlined*, according to Covello and Gray (1991)

Sequence similarity between the atp6 and orf25 genes from fertile and sterile wheat cytoplasms

A sequence block of approximately 150 bp from the upstream region of the *atp6* gene is duplicated in the *T. aestivum* (Bonen et al. 1990) and *T. timopheevi* cytoplasms. Using a 1.3-kb *Bgl*II subfragment of plasmid pTae8 as a probe (see Fig. 5a, probe C), we isolated lambda clones from the *T. timopheevi* library. Restriction and hybridization analyses identified a 0.4-kb *Bgl*II fragment which shows similarity with the 5′ terminus of the *atp6* gene from *T. aestivum*, but does not contain the complete open reading frame (Fig. 3, LT2-1). Sequence analysis revealed that the duplicated fragment is part of the plant-specific *orf25* gene, as shown by Bonen et al. (1990). The duplication includes the N-terminal 18 amino acids from the *atp6* and *orf25* coding regions (see Fig. 4, amino acids typed in bold letters). The amino-acid coding region of the *orf25* gene from *T. timopheevi* (data not shown) is completely identical to the *orf25* sequence from *T. aestivum* (Bonen et al. 1990).

The duplicated 150-bp sequence block is shared by both the *atp6* gene copies from *T. aestivum* and the *atp6* gene from *T. timopheevi* as well as the *orf25* genes from both cytoplasms. The 5′ flanking sequences of the 150-bp duplicated region of the two *orf25* genes differ remarkably between *T. aestivum* and *T. timopheevi* (Fig. 3). The *orf25* gene from *T. aestivum* shows sequence identity with

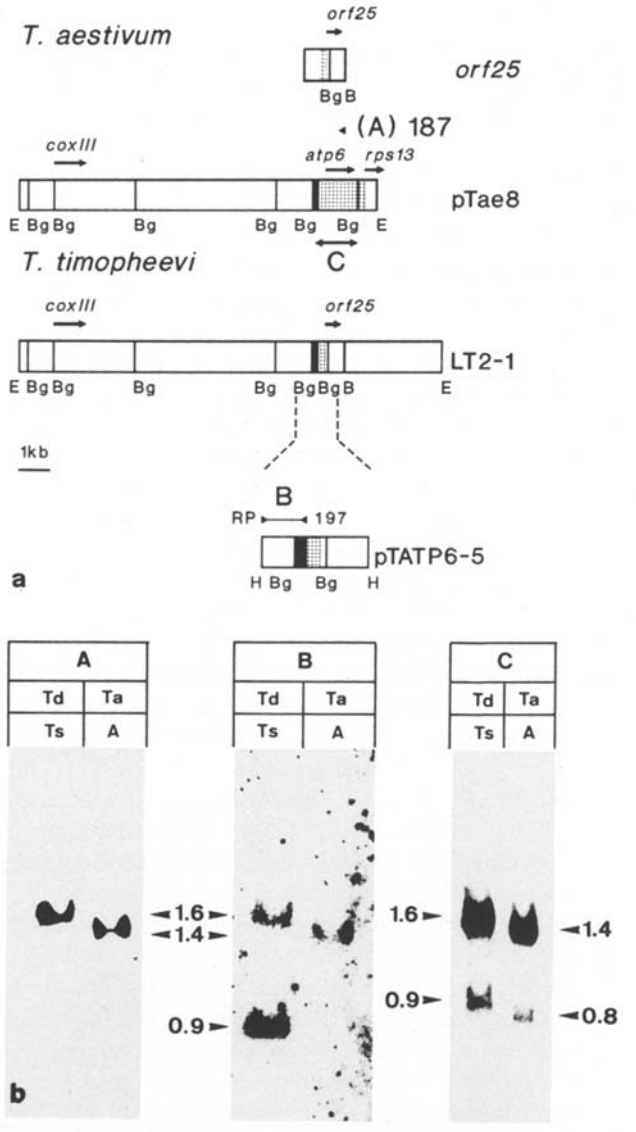

Fig. 5. Transcript analysis of the *orf25* and *atp6* gene regions in *T. aestivum* (*TA*) and *T. timopheevi* (*TT*). **a** Restriction maps of the *atp6* and *orf25* genes from TA and TT showing the position of probes used for transcription analysis. Patterns indicate similar fragments as described in the legend of Fig. 1. Probe *A* is ^{32}P-labeled oligonucleotide 187; its orientation is shown by the *triangle*, the sequence is given in Materials and methods. Probe *B* is an end-labeled DNA (see Materials and methods for synthesis) which detects transcripts derived from the *atp6* genes as well as from the *T. timopheevi orf25* gene. Probe *C* is a ^{32}P-labeled 1.3-kb restriction fragment of plasmid pTae8, containing coding and non-coding 5′ sequences of the *atp6-1* gene copy from *T. aestivum*. **b** Autoradiograph of the Northern hybridization analysis with gene probes as indicated. The headings indicate from top to bottom: the hybridization probes and the nuclear (*Td*, *T. durum*; *Ta*, *T. aestivum*) and cytoplasmic (*Ts*, Timopheevi, sterile; *A*, *T. aestivum*) genomes of lines used for investigation. The size of the transcripts is given in kb

the *atp6-2* gene (indicated by the dotted pattern in Fig. 3), whereas the upstream sequence of the *orf25* gene from *T. timopheevi* is similar to the 5′ flanking region of the *atp6-1* gene.

The *atp6* and *orf25* genes of both cytoplasms are transcribed. The maps and transcript analyses are shown in Fig. 5 a and b. Hybridization with oligonucleotide 187 (probe A in Fig. 5) detects *atp6* transcripts of approximately 1.4 and 1.6 kb in the *T. aestivum* and *T. timopheevi* cytoplasms, respectively. The hybridization with probe B demonstrates that the transcription initiation of the *orf25* gene from *T. timopheevi* must occur beyond the identical sequences of the gene in both cytoplasms.

A *Hin*dIII subclone of LT2-1 in pUC19 was used to amplify a fragment with labeled oligonucleotide 197 and the reverse sequencing primer. This fragment contains sequences similar to the *atp6-1* gene copy from *T. aestivum* and the *orf25* and *atp6* genes from *T. timopheevi*. Consequently *orf25* transcripts gene from *T.* aestivum were not detected with this probe. Using a 1.3-kb *Bgl*II subfragment of pTae8 (probe C), both the *atp6* and *orf25* transcripts in the *T. timopheevi* and *T. aestivum* cytoplasms were detected, since this probe contains the duplicated sequence that is shared by the *atp6* and *orf25* genes of both cytoplasms. In *T. aestivum*, *atp6* gene transcripts were also detected using an oligonucleotide specific for the *atp6-2* gene copy (data not shown). This demonstrates that both the *atp6* gene copies are transcribed in *T. aestivum*.

Discussion

Repeated sequences may be responsible for mitochondrial DNA rearrangements in fertile and sterile wheat

Only limited molecular data exists concerning the CMS phenotype in wheat. MtDNA hybridization analyses with fertile *T. timopheevi* were performed as a basis for characterizing the cytoplasms of *Triticum* and *Aegilops* species (Breiman 1987). The organization and sequence of the *atpA* and *atp9* genes have been determined for the male-sterile *timopheevi* cytoplasm (Bégu et al. 1989 a, b). Despite different gene organizations, transcripts from the CMS and fertile wheat lines are the same size (Schulte-Kappert and Mohr, unpublished). Of the eight genes examined to-date, only the *atp6* and *orf25* genes from the *T. aestivum* and *T. durum* cytoplasm reported in this study exhibit different transcript patterns, compared to those from male-sterile *T. timopheevi* wheat.

Rearrangements of the mitochondrial DNA primarily occur by homologous recombination between repeated regions of the mitochondrial genome (Newton 1988; Fauron et al. 1990). Recombinationally active homologous sequences have been reported for several

plants: *Petunia* (Folkerts and Hanson 1989), pea (Morikami and Nakamura 1987), *Sorghum* (Pring et al. 1988), maize (Fauron et al. 1990) and *Phaseolus* (Mackenzie et al. 1988).

The two gene regions analyzed in the present study differ in their organization, although extensive sequence simlarities have been detected through hybridization and sequence analysis. This may indicate that the sequences may have undergone several separate homologous recombinations. In *T. aestivum*, sequences upstream of both *atp6* gene copies and of the *coxII* gene (Bonen et al. 1984, underlined in Fig. 3) are 85% identical. This sequence is also present upstream of the *orf25* gene (Bonen et al. 1990).

The upstream sequence of the *coxII* gene contains the 5-bp insertion 5′ CTTTC 3′ as does the gene copy *atp6-1*, whereas these nucleotides are missing in the *atp6-2* gene copy and the *orf25* gene (see Fig. 4). Therefore, one can assume that recombination events occurred between the *atp6-1* gene copy and the *coxII* gene and between the *atp6-2* gene copy and the *orf25* gene. It remains to be determined whether or not the repeated sequences are recombinationally active. An indication that recombination does take place can be derived from recombinant lambda clones carrying the repeated sequences as part of recombined mtDNA fragments. On the contrary, the recombined fragments may have been deleted, as proposed for example for recombination intermediates in maize cms-T revertants (Fauron et al. 1990). Similarly, it was speculated that intermediates were lost during evolution in the related maize T and RU cytoplasms (Small et al. 1987).

The 5′ breakpoint of similarity between all sequences investigated in this paper is the 8-bp motif 5′ GTTTCGTT 3′ (box in Fig. 2). Interestingly, this sequence is commonly found at a defined distance upstream of several mitochondrial genes from cereals. For example, the sequence is present upstream of the *coxIII* gene from *T. aestivum* (Gualberto et al. 1990), the *coxIII* gene from maize (McCarty et al. 1988) and rice (Kaleikau et al. 1990), and the *coxII* gene from rice (Kao et al. 1984). This sequence appears to be located within the transcribed region and could be used as an internal transcription signal. An alternative function of the sequence may be a recombination signal. Short sequences that are most probably involved in homologous recombination events have already been described (for example (Kadowaki et al. 1990).

Different transcription units for the atp6 and orf25 genes in fertile and sterile wheat

The differences in the upstream sequences of the *orf25* and *atp6* genes involve putative promoter elements (Covello and Gray 1991) that are present at varied distances from the ATG codon. These elements are underlined in Fig. 4. If these elements are used as transcription initiation sites, the *orf25* transcript of the *T. timopheevi* cytoplasm must be 66 bp longer than the transcript from *T. aestivum*, provided that transcription termination is similar. Since downstream sequences of the *orf25* genes are identical in both cytoplasms, it is likely that the transcripts terminate at the same region. The Northern analysis (Fig. 5b) demonstrates that the *T. timopheevi* transcript is approximately 100 bp larger than the *T. aestivum* transcript, which corresponds well with the distance between the putative promoter elements and the ATG start codon. If these promoter sequences also function as transcription initiation sites for the *atp6* genes, the transcription of the *atp6-1* and *atp6-2* copy from *T. aestivum* starts 5′ of the repeated element. The transcripts have a size of approximately 1.4 kb and, therefore, transcription termination of the *atp6-1* and *atp6-2* genes must be within the repeated region that has a length of 1,354 and 1,360 bp, respectively. The proposed differences in the transcript sizes of the two gene copies, due to the different location of the promoter elements, are too minor to be identified effectively on agarose gels. The *atp6* gene from male-sterile *T. timopheevi* has a putative promoter sequence at position −624, which may result in a transcript 387-bp longer than the *atp6* transcript from *T. aestivum* (for comparison see Fig. 4). This difference in transcript length can be seen on Northern blots. The termination of the *atp6* gene from *T. timopheevi* presumably occurs at the same position as it does in *T. aestivum*, because the repeated *atp6* gene region from *T. aestivum* is completely conserved in *T. timopheevi*.

An interesting feature is that it is likely that both *orf25* genes are transcribed from putative promoter sequences that are also used for the transcription initiation of the *atp6* genes. A comparable situation has been found in the male-sterile CMS-T cytoplasm from maize. The *orf25* gene in CMS-T is located downstream from a chimeric reading frame, *urf13-T*, which is correlated with the CMS phenotype (Dewey et al. 1986). The co-transcription of both genes is initiated at a sequence which is used for the transcription of the *atp6* gene as well (Kennell and Pring 1989). The *atp6* promoters drive the *urf13-T* and *orf25* reading frames by virtue of the unique recombinational events of the T cytoplasms. Since no such situation exists in the maize cytoplasms C, S and N (Dewey et al. 1986; Stamper et al. 1987), similarities between the transcription initiation of the *atp6* and *orf25* genes do not seem to be a general phenomenon in higher plants.

It is not yet known whether a correlation exists between the CMS phenotype and the rearrangements of the mitochondrial genome in *T. timopheevi*. It can be speculated that both *atp6* genes are transcribed in fertile *T. aestivum*, but only the *atp6-2* gene copy is translated and processed. In male-sterile *T. timopheevi*, RNA processing

may be incomplete because factors of the alien nucleus prevent proper processing of transcripts derived from the singular *T. timopheevi atp6* gene. The isolation and characterization of nuclear genes which affect mitochondrial transcription and the translation of mitochondrial mRNAs has been demonstrated for yeast and filamentous fungi (see review by Tzagoloff and Myers 1986; Akins and Lambowitz 1987; Grivell 1989; Pel et al. 1992). So far, the isolation of analogous genes from higher plants has not been accomplished, but several reports have shown that variations in the nuclear background can influence the transcription of mitochondrial genes. For example, the *atpA* gene shows a different transcription pattern in male-fertile varieties of *Beta vulgaris* (Duchenne et al. 1989). Similarly, transcriptional regulation of mitochondrial genes from male-fertile and/or male-sterile lines dependent on the nuclear background has been observed in *Epilobium* (Schmitz and Michaelis 1988) and in *Nicotiana* lines (Hakansson and Glimelius 1991). For maize, it has been demonstrated that restorer genes control transcription in male-sterile cultivars (Dewey et al. 1986; Kennell et al. 1987; Walker et al. 1987).

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