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RNA editing of *atp6* transcripts from male-sterile and normal cytoplasms of rapeseed (*Brassica napus* L.)

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The complete cDNA sequence corresponding to the rapeseed *atp6* gene transcript (coding for subunit 6 of F_0-ATPase) has been determined by a method involving cDNA synthesis, using specific oligonucleotides as primers, followed by PCR amplification, cloning and sequencing of the amplification products. Only one modification, a C-to-U conversion, has been found when compared to the genomic mitochondrial DNA sequence. Comparison of the extent and frequency of RNA editing of the *pol* cytoplasmic male sterile (cms) *atp6* transcript with those of normal *atp6* transcript indicates that there is no variation between the editing status of the *atp6* transcripts from *pol* cms and normal cytoplasms.

RNA editing; Mitochondrial *atp6*; Cytoplasmic male sterility; Rapeseed

1. INTRODUCTION

The mRNAs of plant mitochondria have recently been shown to be modified; at specific locations, cytidine residues on the genomic sequence are converted to uridines on the mRNA, resulting in polypeptides different from those predicted by the genomic DNA [1–3], as well as in the emergence of novel initiation and termination codons [4,5]. The number of nucleotides altered differs, however, between plant species and also between individual genes within a mitochondrion.

This RNA editing event is essential for the correct expression of plant mitochondrial protein genes. The reasons for the existence of RNA editing in plant mitochondria is not clear, but it appears that it has played a role in the conservation of protein sequences during evolution. Therefore, it is likely that RNA editing confers functional advantages as a modulator of gene expression. The basis for the specificity of the editing system of plant mitochondria is still not clear, but recent results in the study of the editing process occurring in the mitochondria of *Leshmania tarentolae* [6] suggest that anti-sense RNA ('guide' RNA) molecules, which were transcribed from maxicircle or minicircle mitochondrial (mt) DNA, might be involved in the RNA editing recognition process in kinetoplastid mitochondria.

RNA editing could regulate gene expression, and it could be both tissue specific and dependent on the developmental stage. The mechanism of cytoplasmic male sterility (cms) is of agronomic importance, and the role of RNA editing has yet to be established. To investigate a correlation between RNA editing and expression of cms, cDNAs of rapeseed *atp6* transcripts were sequenced and the extent of RNA editing was determined in the *atp6* transcripts from both normal and *pol* cms cytoplasms.

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2. MATERIALS AND METHODS

mtDNA and mtRNA were prepared from 8-week-old plants as previously described [7]. cDNA synthesis and PCR amplification were performed essentially according to the procedure described [8]. DNase-treated mtRNA (30 μg) was mixed with 12.5 pmol of primer, ATP6REV (a 27-mer oligonucleotide complementary to the 3′ end of the *atp6* mRNA, which has the sequence, GGGCTGTGTAACTGCAGTAAATAACAC). After denaturation at 80°C for 5 min, the reaction mixture was incubated for 2 h at 50°C for primer hybridization. First strand synthesis was initiated by the addition of M-MLV reverse transcriptase (Gibco BRL, MD, USA).

The resulting cDNAs were amplified by PCR using the primers, ATP6REV and ATP6FOR (a 25-mer oligonucleotide whose sequence was derived from the 5′ end of the mRNA, and which has the sequence CGGGAAGTGAGAATTCCGCTTTTCT). The primers ATP6REV and ATP6FOR contained mismatches as compared to the DNA sequence in order to create *Pst*I and *Eco*RI sites, respectively, at the ends of the DNA fragments amplified by PCR (Fig. 1). Amplification was obtained by 60 cycles of the following steps: denaturation (92°C, 2 min), annealing (47°C, 2 min) and DNA polymerization (72°C, 6 min), followed by a final incubation at 72°C for 6 min. Amplified cDNAs were cloned into pBluescript II and sequenced. Primers, ATP6INT1 and ATP6INT2 (17-mer oligonucleotides which have the sequences, ACTAAAAAGGGAGGAGG corresponding to positions 246–262, and TAGTCCAAGCGAACCCA complementary to positions 711–695, respectively) were used for internal sequence priming.

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          ●  ●
          GAATTC
  1 CGGGAAGTGACAATACCGGCTTTTCTTCAGCACATTTTGGATGATTTGAGCGAAAACGGAG  60

 61 TACAAAGTTCAGCCTTTAAGGAGGCTATGAATCAAATAGGGCTGGTGGCGCAGTCCCCAC 120
                               M  N  Q  I  G  L  V  A  Q  S  P

121 TTGACCAATTTGAGATTGTCCCATTGATTCCTATGAATATCGGAAACTTCTATTTCTCAT 180
    L  D  Q  F  E  I  V  P  L  I  P  M  N  I  G  N  F  Y  F  S

             S
            TCA
181 TCACAAATCCATCTTTGTTCATGCTGCTAACTCTGAGTTTTTTCCTACTTCTGATTCATT 240
    F  T  N [P] S  L  F  M  L  L  T  L  S  F  F  L  L  L  I  H

241 TTATTACTAAAAAGGGAGGAGGAAACTTAGTCCCAAATGCTTGGCAATCCTTGGTAGAGC 300
    F  I  T  K  K  G  G  G  N  L  V  P  N  A  W  Q  S  L  V  E

301 TTCTTTATGATTTCGTGCTGAACCTGGTAAAGGAACAAATAGGTGGTCTTTCCGGAAATG 360
    L  L  Y  D  F  V  L  N  L  V  K  E  Q  I  G  G  L  S  G  N

361 TGAAACAAATGTTTTTCCCTTGCATCTTGGTCACTTTTCTTTTTTTGTTATTTTGTAATC 420
    V  K  Q  M  F  F  P  C  I  L  V  T  F  L  F  L  L  F  C  N

421 TTCAGGGTATGATACCTTATAGCTTCACAGTGACAAGTCATTTTCTCATTACTTTGGCTC 480
    L  Q  G  M  I  P  Y  S  F  T  V  T  S  H  F  L  I  T  L  A

481 TCTCATTTTCTATTTTTATTGGCATTACTATAGTGGGATTTCAAAGACATGGGCTTCATT 540
    L  S  F  S  I  F  I  G  I  T  I  V  G  F  Q  R  H  G  L  H

541 TTTTCAGCTTTTTATTACCCGCAGGAGTCCCACTGCCCTTAGCACCTTTTTTAGTACTCC 600
    F  F  S  F  L  L  P  A  G  V  P  L  P  L  A  P  F  L  V  L

601 TTGAGCTAATTTCTTATTGTTTTCGCGCATTAAGCTTAGGAATACGTTTATTTGCTAATA 660
    L  E  L  I  S  Y  C  F  R  A  L  S  L  G  I  R  L  F  A  N

661 TGATGGCCGGTCATAGTTTAGTAAAGATTTTAAGTGGGTTCGCTTGGACTATGCTATGTA 720
    M  M  A  G  H  S  L  V  K  I  L  S  G  F  A  W  T  M  L  C

721 TGAATGAGATTTTCTATTTTATAGGGGCTCTTGGTCCTTTATTTATAGTTCTTGCATTAA 780
    M  N  E  I  F  Y  F  I  G  A  L  G  P  L  F  I  V  L  A  L

781 CCGGTCTGGAATTAGGTGTAGCTATATTACAAGCTTATGTTTTTACGATCTTAATCTGTA 840
    T  G  L  E  L  G  V  A  I  L  Q  A  Y  V  F  T  I  L  I  C

841 TTTACTTGAATGATGCTATAAATCTCCATTAAAGTTCTTCTTTCTTTTATTTAGATTTAT 900
    I  Y  L  N  D  A  I  N  L  H  *
                                             ●●
                                             CTGCAG
901 AATTGAACAAAAGCCGAGGGATGAGAGTAGTGTTATTTAGAGCAGTTACACAGCCC     955
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Fig. 1. Comparison of rapeseed genomic and cDNA *atp6* sequences. The upper sequence is obtained from *atp6* cDNA clones, while the lower sequence is the corresponding genomic DNA sequence. Where the two are identical only the genomic sequence is shown. The sequences corresponding to oligonucleotides ATP6REV, ATP6FOR, ATP6INT1 and ATP6INT2, used for cDNA amplification by PCR and internal sequence priming, are underlined. The amino acid sequence deduced from the genomic sequence using the universal genetic code is presented. The codon modified by editing is boxed, with the corresponding amino acid modification indicated above the cDNA sequence. Dots indicate nucleotide mismatches introduced in the oligonucleotide sequences in order to create *Pst*I and *Eco*RI sites.

3. RESULTS

3.1. *Cloning of* atp6 *cDNAs*

To synthesize and amplify *atp6* cDNA sequences, two oligonucleotides (ATP6REV and ATP6FOR) were prepared, with sequences in opposite orientations, corresponding to the extremities of the rapeseed *atp6* mRNA. Before cDNA synthesis, mtDNA had been eliminated by DNase treatment since it acts as a contaminant of mtRNA preparations and it would also be amplified during the PCR reaction. ATP6REV, complementary to the 3′ end of the *atp6* mRNA, was used to prime cDNA synthesis. The primer extension products were subsequently amplified by PCR. The 1 kb amplified fragment was cloned into *Pst*I and *Eco*RI sites of pBluescript II vector.

In the 25 clones sequenced we found 22 nucleotide changes which were attributed to mis-incorporations of nucleotide by the *Taq* DNA polymerase. None of those modifications was found at the same position in more than one of the clones. Therefore, we only considered the nucleotides which were different from that in the genomic DNA sequence but were found in more than one cDNA clone, and which resulted from an RNA editing event. The rate of the mis-incorporations under our PCR condition was 0.88 per clone (22/25 clones), and 9.2×10^{-4} per nucleotide (0.88/955 nucleotides).

3.2. *Editing sites of the rapeseed* atp6 *cDNAs*

Sequencing of cDNA clones revealed only one C-to-U transcript editing event within the *atp6* open reading frame. The edited site and the corresponding genomic sequence with the amino acid sequence is shown in Fig. 1. This C-to-U editing at nucleotide 189 within the coding region resulted in a proline-to-serine change (Fig. 2). The frequency of this editing event was very high; eight cDNA clones derived from normal cytoplasm were examined for this RNA editing site, and seven of these had an edited transcript (87.5%). No modification was found in the untranslated flanking region.

Comparison of the amino acid sequence deduced from the rapeseed cDNA sequence with respective polypeptides from *Oenothera* and sorghum cDNA sequences [9,10] revealed strong sequence homologies between them (Fig. 3). These data show that the resultant polypeptide was very similar in all three species, as has been generally observed after the editing process. As an example, homology between the core sequences of the rapeseed ATP6 protein and the *Oenothera*, and rapeseed and sorghum ATP6 proteins would increase from 85.4 to 92.5% and from 83.4 to 89.7%, respectively.

3.3. *No variation of RNA editing observed between normal and* pol *cms cytoplasms*

To explore the extent of RNA editing within the *atp6* coding sequence of *pol* cms mitochondria, analogous cDNA clones were constructed from mtRNA of *pol* cms cytoplasm. Only one RNA editing event occurred in the *pol* cms transcript at the same position as in the normal transcript. The frequency of this editing event was also very high and was similar to the normal transcript: sixteen of the seventeen clones analysed had an edited transcript (94.1%). Thus the C-to-U editing event at position 189 is a common phenomenon in both mitochondrial *atp6* transcripts from normal and *pol* cms cytoplasms and its frequency is similar in both cases.

4. DISCUSSION

The investigation of RNA editing in the *atp6* mRNAs of rapeseed reported here showed that only one editing event occurs in the *atp6* coding region (Fig. 1). Comparative analyses of the extent of RNA editing in plant *atp6* transcripts indicate substantial variation: *Oenothera* [9] and sorghum [10] represent extreme examples, with 21 and 19 editing events within the core polypeptide, compared to 1 in rapeseed. In rapeseed, most of the amino

Fig. 2. Comparison of *atp6* cDNA and the corresponding genomic DNA sequences. Only parts of the sequences are presented. The position where the C-to-U modification is found is indicated by the arrowheads.

acids which are specified only *after* editing in the mitochondria of the other two species are already encoded by the mitochondrial genomic sequence (Fig. 3). As far as *atp6* is concerned, rapeseed already seems to have an 'edited' sequence on its mitochondrial genome, while *Oenothera* and sorghum have a 'pre-editing' sequence which needs extensive RNA editing to produce the mature mRNA. Such an extreme divergence of editing patterns between different higher plant species is very interesting, considering the requirements of RNA editing in plant mitochondria for the conservation of protein sequences. The edited genomic sequence found in rapeseed mitochondria implies that, during evolution, edited sequences have been integrated into the mitochondrial genome. There is also another alternative, namely that the genomic sequence of rapeseed has remained in its primitive and original form, and therefore would not show the mutations seen in the other species.

Differences in the editing patterns of *atp9* and *cob* transcripts from normal and cms wheat lines have been observed [11]; however, no variation in the extent and frequency of RNA editing between *atp6* transcripts

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                       10        20        30        40        50        60
rapeseed cDNA  .........................S..................................
rapeseed       SPLDQFEIVPLIPMNIGNFYFSFTNPSLFMLLTLSFFLLLIHFITKKGGGNLVPNAWQSL
Oenothera      ...E..S.L.........L................LV...VN.V.......S........
sorghum        ......G.H.ILDL......L....L..S.....GLV...VFVV......KS........

                       70        80        90       100       110       120
rapeseed cDNA  ............................................................
rapeseed       VELLYDFVLNLVKEQIGGLSGNVKQMFFPCILVTFLFLLFCNLQGMIPYSFTVTSHFLIT
Oenothera      ...I....F.F.N.............K...R.S...T.S..R.F.................
sorghum        ...I....F...N.............K.....S...T.S..R.F.....F...........

                      130       140       150       160       170       180
rapeseed cDNA  ............................................................
rapeseed       LALSFSIFIGITIVGFQRHGLHFFSFLLPAGVPLPLAPFLVLLELISYCFRALSLGIRLF
Oenothera      .G................N....L..S...................PI......S.....
sorghum        ...............................................I......S.....

                      190       200       210       220       230       240
rapeseed cDNA  ............................................................
rapeseed       ANMMAGHSLVKILSGFAWTMLCMNEIFYFIGALGPLFIVLALTGLELGVAILQAYVFTIL
Oenothera      ........S...............DL.....D............F......S....S..S
sorghum        ........S............FL.N......D...................S..I.S..S

                      250       260       270       280       290
rapeseed cDNA  .............
rapeseed       ICIYLNDAINLH*
Oenothera      ........I...QSGWFFFIIEQKRKNQKIKEHRRERALHKPRRSLL*
sorghum        ........I...QNE*
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Fig. 3. Amino acid alignment of the core sequences of ATP6 polypeptides deduced from the nucleotide sequences of rapeseed, *Oenothera* and sorghum. The amino acid sequence deduced from the rapeseed *atp6* genomic sequence, using the universal genetic code, is compared with: (i) the amino acid sequence deduced from the rapeseed *atp6* cDNA sequence; (ii) the amino acid sequences deduced from two other plant mitochondrial *atp6* sequences. Identical amino acid residues are indicated by dots. Boxes indicate editing positions in the other two plant sequences, which result in the specification of the same amino acid as that specified by the rapeseed *atp6* genomic sequence as a result of RNA editing.

from normal and *pol* cms cytoplasms can be observed, which suggests that RNA editing is not a primary determinant for male-sterility induction in rapeseed.

Transcriptional alterations among normal, *pol* cms, and fertility-restored *pol* plants have been detected at this *atp6* locus [7,12]. The *atp6* gene of *pol* cms cytoplasm is co-transcribed with a novel open reading frame, *pol-urf*, into the same precursor RNA, and then mature transcripts can be made from the precursor by RNA processing. On the other hand, in normal cytoplasm the *atp6* gene is transcribed by itself. Nuclear restorer genes appear to specifically alter the transcription patterns of the *pol atp6* locus. We can suppose two possible relationships between these transcriptional alterations and RNA editing events. First, RNA editing could be affected by RNA maturation of the *atp6* transcript from the precursor. A correlation between RNA editing and RNA maturation has been found in the wheat *nad3-rps12* transcript [13]; RNA editing in plant mitochondria is a process with a temporal development correlating with transcript maturation. Therefore, it is possible that RNA editing patterns are different between precursors (*atp6-pol-urf* co-transcript) and mature RNA, and RNA maturation and RNA editing both take part in the induction of male sterility. Second, although we have had no evidence for RNA editing events within the upstream region of *atp6*, they might act as modulators of transcription, affecting secondary structures. RNA editing generally occurs in the coding region to improve conservation of the encoded protein product in evolution, however, RNA editing occurring in the non-coding region plays a crucial role in the *trans*-splicing of the *Oenothera nad1* gene [14]: it is essential for continuous paring of the stem structure of the intron sequences.

Additional work is required to clarify the relationship between RNA editing and regulation of gene expression, such as male-sterility induction, and to explain the role of RNA editing in the conservation of protein sequences.

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