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Creation of an initiation codon by RNA editing in the *coxI* transcript from tomato mitochondria

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Abstract Nucleotide-sequence analysis showed that the gene for cytochrome oxidase subunit I (*coxI*) from tomato mitochondrial DNA has an ACG codon at a conserved position corresponding to an ATG initiation codon in other higher-plant *coxI* genes. cDNA-sequence analysis of the *coxI* transcripts showed that 15 positions in the genomic DNA were converted from C to U in the transcripts by RNA editing. One of the editing events is observed at the indicated ACG codon, producing an ATG initiation codon. The nucleotide sequences of 37 cDNA clones showed that the initiation codon was created in 32 out of the 37 clones, while nucleotide positions 254 and 11 were edited in 37 and 34 of the 37 clones examined, respectively, suggesting that creation of the initiation codon is a post-transcriptional event. The *Bam*HI site at nucleotide position 757–762 within the *coxI* genomic DNA was altered in all 97 cDNA clones examined, demonstrating that RNA editing at this site in the transcripts is very common. RNA editing takes place to a lesser extent at the initiation codon, compared with editing at internal position 254. This indicates that editing is either a random process or that it involves a mechanism favoring less RNA editing in the initiation codon than in internal sites.

Key words RNA editing · Tomato mitochondria · *coxI* gene · Initiation codon

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Introduction

RNA editing in plant mitochondria alters the nucleotide sequence of gene transcripts and often causes amino-acid substitution to a better conserved polypeptide sequence (Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989). RNA editing is a post-transcriptional modification and partially edited, as well as fully edited, mRNA molecules have been identified in various gene transcripts of different plant species (for a review see Gray et al. 1992).

Among several hundred editing sites identified, some of the most drastic effects on genetic information caused by RNA editing involve the creation of initiation or termination codons. Such substitutions have been reported in several plant mitochondrial genes. The creation of a termination codon was reported for the ATPase subunit-9 (*atp9*) gene of wheat (Bégu et al. 1990; Nowak and Kück 1990) and *Oenothera* (Schuster and Brennicke 1990), and for the ATPase subunit-6 gene of sorghum (Kempken et al. 1991) and *Oenothera* (Schuster and Brennicke 1991 a). The creation of an initiation codon by RNA editing has been proposed for the wheat NADH dehydrogenase subunit-I gene (Chapdelaine and Bonen 1991), and the same position is edited in *Oenothera* (Wissinger et al. 1991).

Temporal relationships of editing sites have been examined using spliced and unspliced cDNAs (Gualberto et al. 1991; Sutton et al. 1991; Yang and Mulligan 1991), suggesting that RNA editing in plant mitochondria is a post-transcriptional event. However, in spite of the importance of editing for gene expression, nothing is known about the temporal order of RNA editing between the creation of the initiation codon and substitutions at other editing sites. In the case of the creation of a termination codon, the extent of RNA editing has been examined; however, transcripts partially edited for the termination codon were not identified in the cDNAs from the *atp9* of wheat (Bégu et al. 1990), petunia (Wintz and Hanson 1991), *Oenothera* (Schuster and Brennicke 1990) and rice (Ishikawa and Kadowaki 1993). All of the analyzed cDNA clones of sor-

ghum *atp9*, except for one (Salazar et al. 1991), were edited to generate a termination codon. These results suggest that transcripts are edited with high efficiency at the termination codon in the pool of transcripts.

DNA sequences of the cytochrome *c* oxidase subunit-I (*coxI*) gene have been reported in maize (Isaac et al. 1985), *Oenothera* (Hiesel et al. 1987), pea (Kemmerer et al. 1989), rice (Kadowaki et al. 1989), sorghum (Bailey-Serres et al. 1986), soybean (Grabau 1986) and wheat (Bonen et al. 1987). The nucleotide position of the initiation codon is conserved in all these species, and the N-terminal peptide sequence of COXI is also highly conserved. Examination of RNA editing in *coxI* transcripts has been reported in three plant species; wheat (Gualberto et al. 1989), *Oenothera* (Schuster and Brennicke 1991 b) and a conifer (Glaubitz and Carlson 1992), but in no case was the entire cDNA sequence included. Computer-aided prediction of RNA editing sites for *coxI*, using a registered nucleotide sequence, has also been tried (Covello and Gray 1990). In spite of these efforts, the complete *coxI* cDNA sequence is still unknown.

During the course of DNA-sequence analysis, the tomato *coxI* gene was found to lack an initiation codon at the position conserved in other plants. Here we present the nucleotide sequences of genomic and the entire cDNA of the *coxI* gene from tomato. The initiation codon was created by RNA editing but less frequently than at other internal positions. The role of RNA editing in the creation of an initiation codon is discussed.

Materials and methods

Plant material. Tomato (*Lycopersicon esculentum* Mill. cv Ponderosa) seeds were obtained from Takii Seed Co., Ltd. (Kyoto, Japan) and grown in a greenhouse. Young leaves were used for nucleic-acid extraction. Mitochondrial DNA was isolated according to the method of Umbeck and Gengenback (1983). Mitochondrial RNA was isolated as described by Stern and Newton (1986). For preparation of cDNA, the isolated mitochondrial RNA was further purified by incubation with RNase-free DNase I according to the manufacturer's instructions (Takara Shuzo Co., Ltd., Kyoto, Japan).

Cloning of tomato coxI gene. Tomato mitochondrial DNA was digested by *Sac*I and the fragmented DNAs were cloned into a plasmid vector pBluescript SKII+ (Stratagene, Calif., USA). Colony hybridization was performed according to conventional methods (Sambrook et al. 1989) using a ^{32}P-labeled rice *coxI* gene (Kadowaki et al. 1989) as a probe.

Southern-blot analyses. Electrophoretic conditions and hybridization conditions were as described by Kadowaki et al. (1990). Approximately 3 μg of mitochondrial DNA was used in each lane for electrophoresis.

Reverse transcription and amplification of resultant cDNAs by the polymerase chain reaction. The oligonucleotide primers employed were synthesized using a 394 automated DNA synthesizer from Applied Biosystems (Calif., USA). The synthesized sequences were as follows: P4, 5′-CTCTGATAAGCTTGGAAACG-3′; P5, '5-GGCTAAGCTTAATAGGAGCA-3′; P6, 5′-GTTCTGTAAGCTTCTTAGTA-3′. The primers were designed according to the tomato genomic *coxI* sequence (this study), and modified to contain a *Hin*dIII recognition site, 5′-AAGCTT-3′, for cloning (underlined). The positions and orientations of these oligonucleotides are indicated in Fig. 1 and 2. Approximately 2.5 μg of isolated tomato mitochondrial RNA and 58 ng of the primer P5 or P6 were used for cDNA synthesis. Reverse transcription was carried out following the manufacturer's procedures (Pharmacia, Wis., USA). Resultant cDNAs were amplified at 92 °C for 1 min, 48 °C for 2 min, 72 °C for 2 min. Twenty-five and 30 cycles were carried out for the amplification of 1663- and 393-nucleotide cDNAs, respectively. Absence of DNA contamination in the RNA preparation was confirmed by control experiments in which water was used in place of reverse transcriptase.

DNA sequencing. A nested set of deletions of the *coxI* gene was constructed by exonuclease III and mung bean nuclease treatment. Single-stranded DNAs from selected deletion clones were purified from phage precipitated with polyethylene glycol. Sequencing reactions were performed on single-stranded templates using the fluorescent T7 primer according to the procedure of Applied Biosystems. Reaction products were separated by 6% polyacrylamide-gel electrophoresis and analyzed by an Applied Biosystems 373A apparatus. The computer software program GENETYX (Software Development Co., Ltd., Tokyo, Japan) was used for data analysis.

Results and discussion

Cloning and nucleotide sequence analysis of the tomato *coxI* gene

Tomato mitochondrial DNA was digested with *Sac*I and a DNA library was constructed using pBluescript vector. The tomato *coxI* gene was identified by colony hybridization using the rice *coxI* gene as a probe. The *coxI* gene was located on a 2.0-kb *Sac*I fragment in the tomato mitochondrial genome (Fig. 1).

The DNA sequence of the isolated *coxI* gene was determined (Fig. 2). The amino-acid sequence was derived from the tomato *coxI* gene nucleotide sequence and compared with the homologous sequence from other plants. The deduced tomato COXI sequence appeared to be 29 amino acids shorter at the N-terminus than those of other plants because the genomic sequence lacked an ATG initiation codon. An ACG codon was observed at a conserved position corresponding to an ATG initiation codon in other higher-plant *coxI* genes. An in-frame stop codon, TAA, was found 30 nucleotides upstream of the ACG codon and no in-frame ATG or GTG codons were evident between the ACG codon and the TAA codon. A possible initiation codon, ATG or GTG, was observed at nucleotide positions 88 and 85

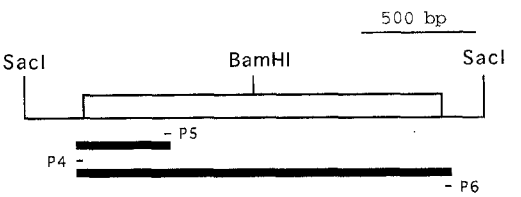

Fig. 1 Organization of the *coxI* locus in tomato mitochondria. Restriction enzyme sites are indicated in the figure. The regions cloned as cDNAs are indicated by two *thick lines* below the genomic arrangement. Positions of primers (*P4, P5 and P6*) are shown by *thin and short lines*

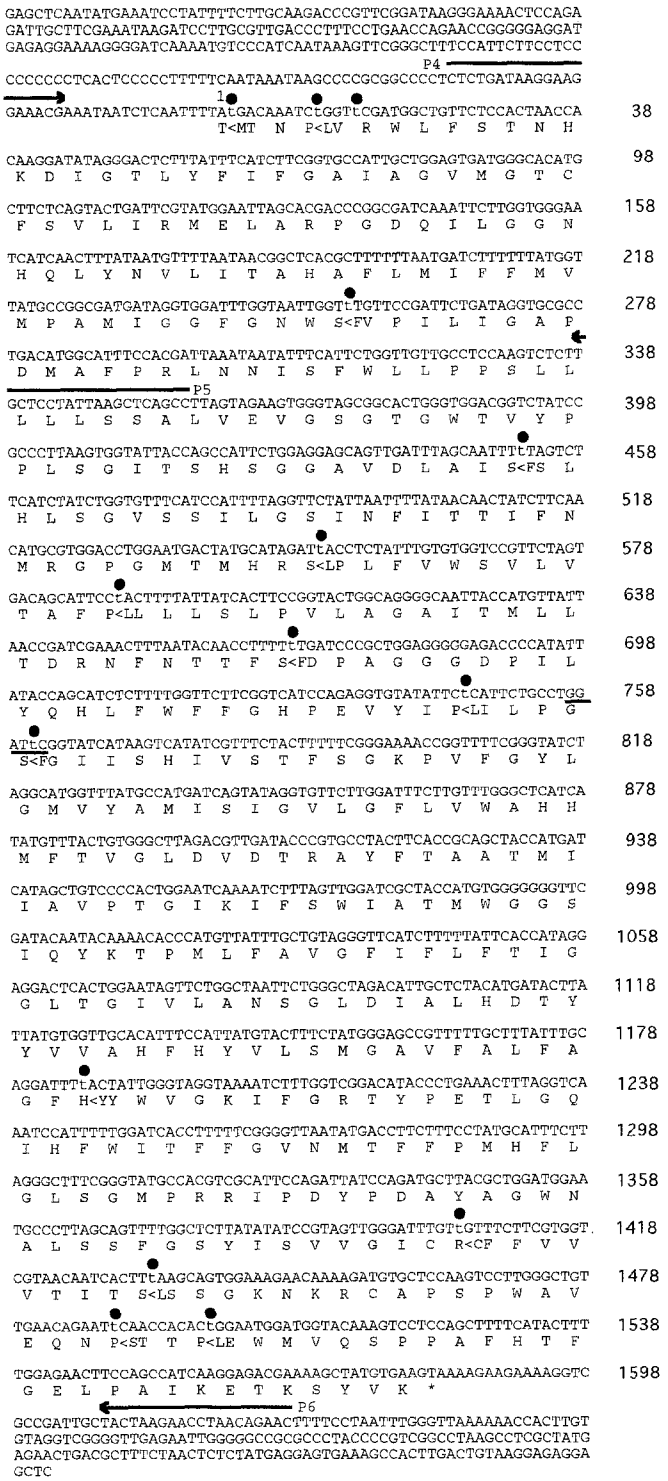

Fig. 2 Genomic and cDNA sequences of the *coxI* gene from tomato mitochondria. The predicted amino-acid sequence is shown using the *single letter* amino-acid abbreviation and indicated underneath the DNA sequence. The *Bam*HI site encoded by genomic DNA, 5′-GGATCC-3′, is *underlined*. The position and orientation of primers used for cDNA synthesis (*P5 and P6*) and DNA amplifications (*P4, P5 and P6*) are indicated by *arrows*. The C-to-U (C genomic DNA to T in cDNA) conversions caused by RNA editing are marked by *closed circles* with the edited nucleotide shown in lower case. The resulting amino-acid changes are shown on the right hand side. The A of the genomic-encoded ACG that is converted to the initiation codon ATG is numbered as 1. EMBL data accession number: X54 738

Fig. 3 Alignment of *coxI* nucleotide sequences from dicotyledonous plants. Published sequences are cited as follows; tomato (this study), soybean (Grabau 1986), pea (Kemmerer et al. 1989) and *Oenothera* (Hiesel et al. 1987). The positions of the ACG codon of tomato and the corresponding ATG initiation codon from other plants are *framed*. Only nucleotides deviating from the tomato *coxI* gene are shown

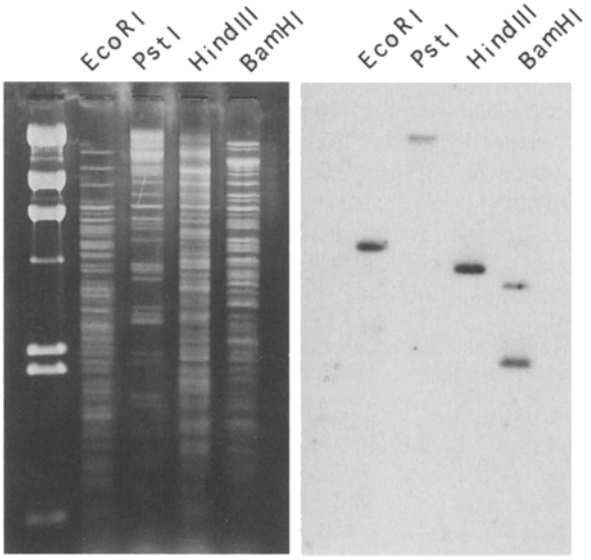

Fig. 4 Agarose-gel electrophoretic patterns of restricted mitochondrial DNA from tomato. Ethidium bromide-stained gel (left) and Southern-blot analysis with a ^{32}P-labeled rice *coxI* gene (right). The restriction enzymes used are indicated above the figure. Lambda DNA *Hin*dIII digestion was used as the molecular-weight size marker

downstream from the ACG codon, respectively. Initiation at position 85, however, predicted a large deviation of the deduced peptide sequence of tomato COXI from those of other plants. Alignment of the *coxI* genes from various plants showed high nucleotide-sequence conservation around the initiation codon and in the following sequence (Fig. 3). The high sequence homology (41 out of 44 nucleotides) also extended to the 5′ flanking region of the *coxI* gene between tomato and soybean or pea.

Three possible explanations are proposed to account for this nucleotide-sequence deviation at the initiation codon.

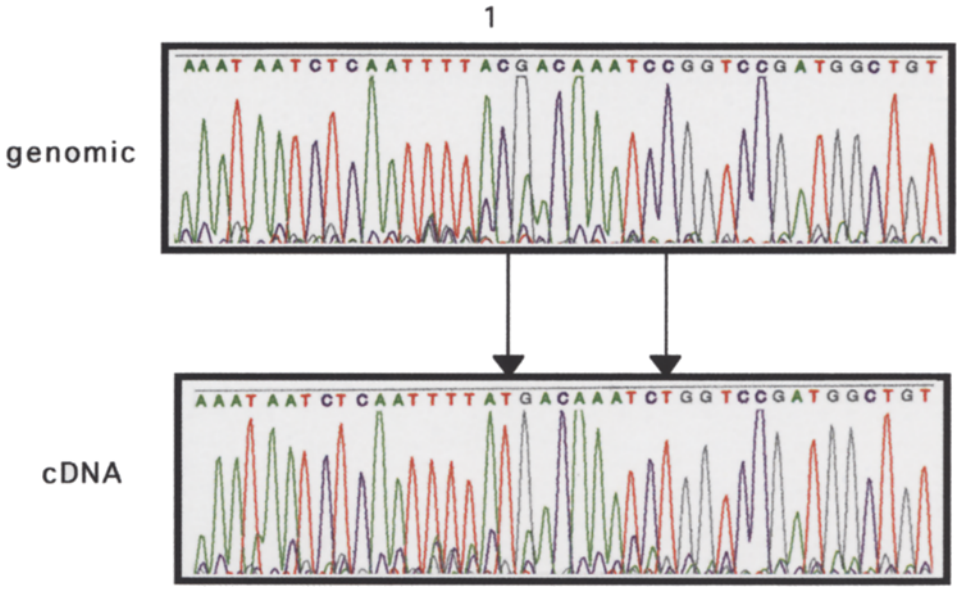

Fig. 5 Creation of the initiation codon in the tomato *coxI* transcript by RNA editing. Nucleotide sequences of the tomato *coxI* gene from genomic DNA (top) and cDNA (bottom) are compared. *Arrows* show nucleotide alterations

First, the cloned *coxI* gene may be a pseudogene and there is a functional *coxI* gene located elsewhere in the tomato mitochondrial genome. Secondly, RNA editing or intron splicing occurs at the ACG codon and results in the production of an ATG initiation codon. Thirdly, a functional *coxI* gene could be encoded by the nuclear genome with the protein product transported into the mitochondria. In order to examine the first possibility, we determined the *coxI* gene copy number in the tomato mitochondrial genome.

Determination of *coxI* gene copy number in the tomato mitochondrial genome

The *coxI* gene copy number in the tomato mitochondrial genome was determined using Southern hybridization and a rice *coxI* gene as a probe. A single hybridization signal was observed when the DNA was digested with *Eco*RI, *Pst*I and *Hin*dIII, suggesting a single-copy tomato *coxI* gene (Fig. 4). Two hybridization signals were observed in *Bam*HI-digested DNA. The genomic DNA sequence shows the presence of a *Bam*HI site in the tomato *coxI* gene at nucleotide position 757–762 (Fig. 2). This result indicates that *Bam*HI splits the *coxI* gene and results in two *Bam*HI fragments containing parts of the same *coxI* gene. Therefore, the *coxI* gene is a single-copy gene in the tomato mitochondrial genome.

Determination of cDNA sequence of tomato *cox*I transcripts

RNA editing in tomato *coxI* transcripts was examined. cDNAs containing the entire *coxI* gene were synthesized by reverse transcription with a P6 primer, and were amplified by the polymerase chain reaction using primers P4 and P6. Amplified products had the expected size of 1663 bp and they were subsequently cloned into the plasmid vector. The cDNA sequences were determined and compared with the genomic sequence. Fifteen sites deviated from the corresponding genomic sequence by C-to-U transitions and hence were concluded to be editing sites (Fig. 2). This result also confirmed that the tomato *coxI* gene was transcribed. No U-to-C change was found in the cDNAs examined. Deduction of the amino-acid sequence showed that one of these substitutions was silent whereas the other 14 positions led to changes in the amino-acid sequence (Fig. 2).

RNA editing at nucleotide position 2 converted the ACG codon to an AUG codon in the tomato *coxI* mRNA (Fig. 5), creating an initiation codon at the position that is conserved in other *coxI* genes. This also suggests that intron splicing is unlikely to be a mechanism for initiation codon production. A nucleotide-sequence comparison shows that positions 11, 254, 452, 551, 590, 668, 761 and 1186 are occupied by C in dicotyledonous plants, tomato, soybean, pea and *Oenothera*. These nucleotides are edited to U in tomato transcripts. On the other hand, these positions are already occupied by T in monocotyledonous plants, rice, wheat, maize, and sorghum. Although many nucleotides edited to U in dicotyledonous plants are already encoded by T in monocotyledonous plants, the possibility of RNA editing still exists at positions 1405, 1433 and 1489. Sequence analysis of a 712-base portion of *coxI* cDNAs from a conifer (a gymnosperm), showed 26 RNA editing events (Glaubitz and Carlson 1992). This number is larger than the 15 RNA editing events found in the 1663-base region of tomato *coxI* transcripts and probably also exceeds the number of RNA editing events in *coxI* transcripts of monocots. The positions of RNA editing sites were different between conifer and tomato but the deduced

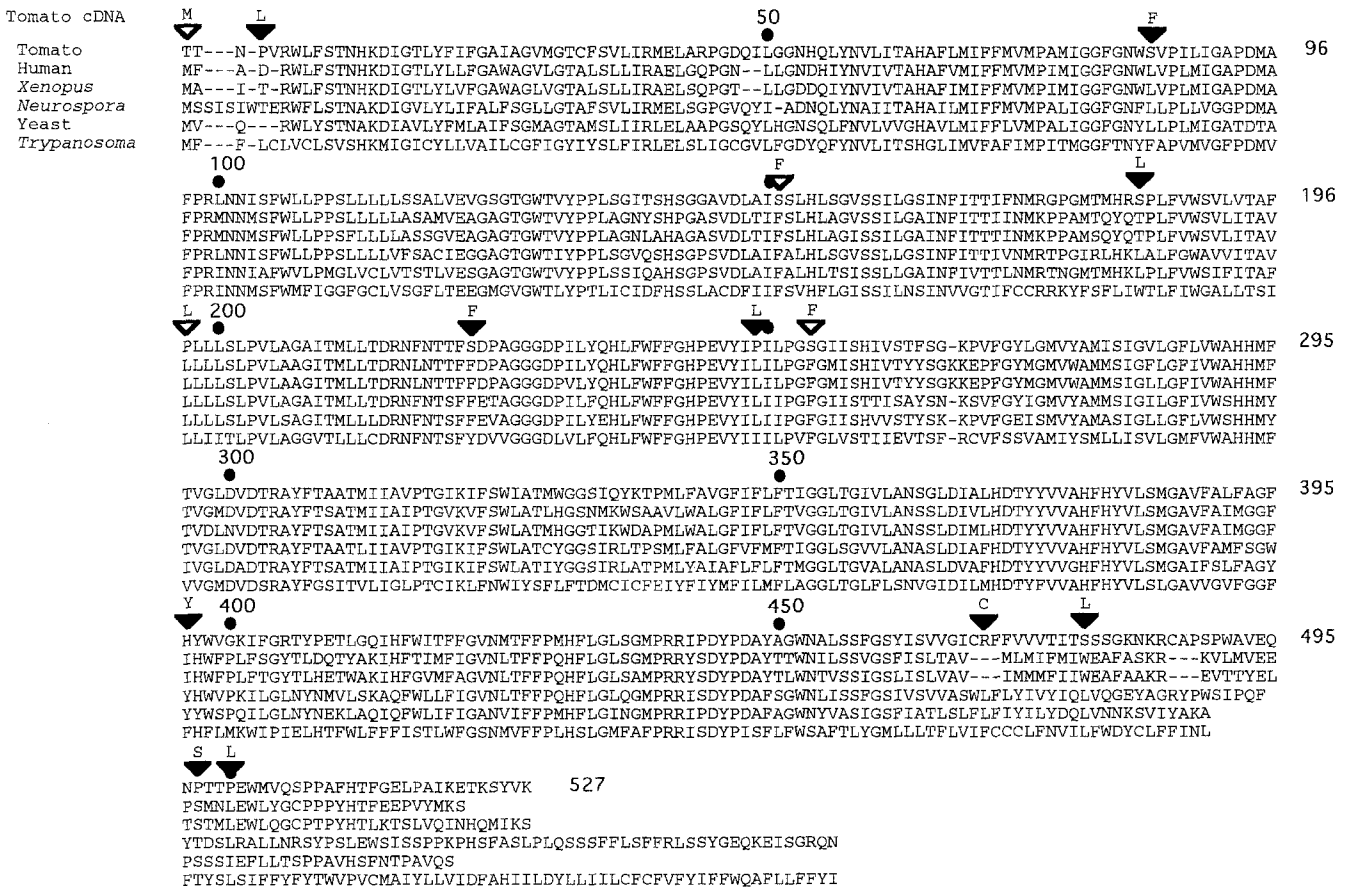

Fig. 6 Alignments of the amino-acid sequences deduced from the genomic and cDNA sequences. Amino acids that could be altered by RNA editing are shown in the top row above *open and closed arrowheads*. The *open arrowheads* show 100% conservation for amino-acid residues among six species. The sequences are cited as follows: tomato (this study), human (Anderson et al. 1981), *Xenopus* (Roe et al. 1985), *Neurospora* (Burger et al. 1982), yeast (Bonitz et al. 1980) and *Trypanosoma* (Hensgens et al. 1984)

amino-acid sequences of *coxI* were similar. A comparatively higher degree of RNA editing is also observed for cytochrome oxidase subunit-III transcripts of *Ginkgo biloba*, *Cycas revoluta* and *Picea abies* than for *cox3* transcripts of other plant species, indicating that RNA editing is more frequent in gymnosperms than in angiosperms (Hiesel et al. 1994). The above results imply that there is a general tendency to lose RNA editing during the evolution of higher plants. Covello and Gray (1990) have predicted possible sites of RNA editing in plant *coxI* genes by comparing the registered amino-acid sequences between plants and non-plants. They predicted 11 positions of amino-acid alteration in dicotyledons and eight of these can be explained by C-to-U conversion. Our results directly demonstrate the occurrence of RNA editing at 15 positions in tomato *coxI* transcripts and show that the prediction of RNA editing sites by comparing amino-acid sequences between plants and non-plants (Covello and Gray 1990) is effective in some cases.

Comparison of the two full-length tomato *coxI* cDNA sequences showed identity except for position 15. Pre-edited codon GTC and edited codon GTT at position 15 both encode Val and do not affect the polypeptide sequence of tomato COXI. Subsequent sequence analysis showed that nucleotide position 15 was edited in a total of 10 out of 37 clones; thus, editing at this position is infrequent and is only seen in fully edited transcripts. Strong selection to maintain RNA editing at position 15 appears to be unlikely because both pre-edited and edited codons result in the same amino acid. This may represent a transition state of losing or gaining of an RNA editing event at position 15.

Amino-acid sequences deduced from tomato genomic and cDNA sequences were compared with the respective proteins of other species (Fig. 6). This alignment shows that RNA editing improves the peptide sequence similarity of tomato COXI with COXI from different species.

The possibility of an active *coxI* gene present in the tomato nuclear genome and targeting of the protein product to mitochondria has not been examined. However, the creation of the initiation codon by RNA editing at the conserved position in the *coxI* transcripts suggests that the *coxI* gene we have cloned is functional in tomato mitochondria. The next question is whether or not RNA editing at the initiation codon site plays a role in translational control. If the initiation codon is created by RNA editing prior to editing events in the other sites, some regulation is needed to control translation of partially edited transcripts, otherwise polypeptides with heterologous sequences will be trans-

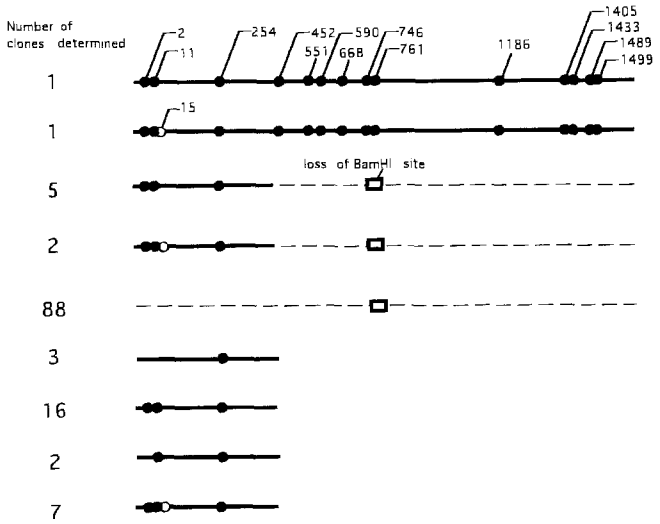

Fig. 7 Overview of RNA editing sites in the transcripts of tomato mitochondrial *coxI* gene. The nucleotide sequence of cDNA was determined and compared with the genomic DNA sequence. The length of bars (*bold, bold and broken, and broken*) show the cDNA sequence prepared by reverse transcription and subsequent amplification by the polymerase chain reaction. *Bold lines* show the DNA sequences analyzed. *Broken lines* show regions where the DNA sequences have not yet been examined. The number of clones analyzed is shown on the left. *Black circles* show changes from C-to-U that cause amino-acid alterations. *White circles* show changes of C-to-U which are silent with respect to the encoded amino acid. *White squares* show loss of the *Bam*HI site (nucleotide position 757 to 762) analyzed by *Bam*HI digestion analysis

lated. If the initiation codon is edited subsequent to other editing sites, it would favor recruiting more completely edited transcripts for translation.

Frequency of RNA editing at the initiation codon of tomato *coxI* transcripts

In order to examine the frequency of RNA editing at the initiation codon and at internal editing sites, two kinds of cDNAs were used. One was initiated by the primer P6 and subsequently amplified by primers P4 and P6 as described previously. The other was initiated by primer P5 and amplified by primers P4 and P5, having an expected size of 393 bp. The resultant cDNA sequences were cloned into plasmid vectors. Nine clones including two full-length cDNAs, prepared by P4 and P6, and 28 clones containing only the 5′ portion of the *coxI* gene, prepared by P4 and P5, were analyzed. Nucleotide-sequence analysis showed that the initiation codon was created in 32 out of the 37 cDNA clones. Three clones retained ACG at the initiation site, while position 254 was edited in all 37 clones, demonstrating partial RNA editing (Fig. 7). Another two cDNA clones were shown to be partially edited in a different way, having edited sites at positions 11 and 254 but not at position 2. Along with the edited positions, additional nucleotide substitutions were found at positions –8 (T to C), 55 (C to T), 262 (A to G) and 328 (C to T) during analysis of the 37 cDNA sequences. Each substitution was a single event in only one cDNA clone, suggesting that these substitutions may result from a misincorporation of a nucleotide during reverse transcription or the amplification of cDNA by the polymerase chain reaction. The above possible misincorporation at position 262 was found in one of the three cDNA clones that had been edited only at position 254, while the remaining two other partially edited cDNA clones had no such substitution at position 262. This suggests that the three clones are not likely to be derived from the same transcript. The above results suggest that RNA editing at position 2 tends to be less frequent than at positions 11 and 254. Transcripts unedited at the initiation codon could be intermediates on the way to editing and translation. Alternatively, the transcripts lacking the functional initiation codon could remain untranslated and face degradation. Different extents of RNA editing have been observed in different kinds of gene transcripts, and they are thought to be intermediates in RNA maturation. Thus, the former interpretation seems to be more likely.

Vidal et al. (1990) showed that paramyxal viral transcripts are edited by a co-transcriptional polymerase stuttering. Gualberto et al. (1991), Sutton et al. (1991), and Yang and Mulligan (1991), have shown that spliced transcripts are more frequently edited than unspliced transcripts, and they suggested that RNA editing in plant mitochondrial transcripts is a post-transcriptional event. If plant mitochondria employ the same RNA editing mechanism for all types of transcripts, it is likely that the creation of an initiation codon by RNA editing in tomato *coxI* is a post-transcriptional event. However, we can not rule out the possibility that RNA editing takes place in a co-transcriptional manner in the tomato *coxI* case because we lack direct evidence.

The RNA editing event at position 761 changed the sequence of a *Bam*HI site 5′-GGATCC-3′ in the genomic DNA to 5′-GGATTC-3′ in the cDNA, resulting in the loss of the *Bam*HI site in the cDNA *coxI* sequence. This nucleotide alteration at the *Bam*HI site was used to measure the occurrence of RNA editing at position 761. Ninety-seven cDNA clones were tested for presence/absence of the *Bam*HI site by *Bam*HI digestion analysis. The cloning vector contains a *Bam*HI site in the multicloning site. Hence, if the *Bam*HI site of the *coxI* gene is retained in cDNA, two bands of approximately 0.75 and 3.95, or 0.95 and 3.75 kb, are expected to be produced. If RNA editing changes the DNA sequence at the *Bam*HI site of the cDNA, only one band of approximately 4.7 kb will be detected. All of the 97 clones analyzed by *Bam*HI digestion showed one 4.7 kb band, suggesting a lack of the *Bam*HI site in the cDNAs. This result, together with nucleotide-sequence analysis of the two full-length cDNA clones, suggests that RNA editing at the *Bam*HI site occurs with a high frequency.

These results imply that the creation of the initiation codon by RNA editing takes place with less frequency compared with the events at positions 11 and 254. The region analyzed, where partial editing is found, does not reflect the overall maturity of the RNA molecule. The relative

amount of editing between the initiation codon and all of the remaining positions was not examined, leaving open the possibility that editing at the initiation codon may be more frequent than at some other editing sites in the same transcript. Nevertheless, it is evident that a difference in the extent of editing occurred at the initiation codon. One interpretation for these results is that the presence of partially edited transcripts at the initiation codon in tomato *coxI* transcripts may be an indication of the control of translation by RNA editing. RNA editing at the initiation codon is probably a prerequisite for translation of the *coxI* transcript. Less frequent RNA editing at the initiation codon seems to be helpful for providing enought time for complete RNA editing to occur. Another interpretation is that this temporal order may have been produced at random. We can not determine which explanation is correct at this time.

Recently, the creation of an initiation codon was reported in the chloroplast transcript encoding NADH dehydrogenase subunit 4 (*ndhD*; Neckermann et al. 1994). This report is interesting because incomplete editing of this site was found in the same tobacco and spinach chloroplast RNA samples for which complete editing was identified in the initiation codon of *psbL* cDNA. Neckermann et al. (1994) discussed the possibility that the incomplete creation of the *ndhD* translational start site in *psaC-ndhD* cotranscripts may play a role in the down-regulation of the synthesis of *ndhD*-encoded polypeptide. Further study is necessary to elucidate the reasons why creation of an initiation codon by RNA editing is less frequent than RNA editing in some internal positions.

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