Curr Genet (1998) 34: 79–87

ORIGINAL PAPER

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The radish (*Raphanus sativus* L.) mitochondrial *cox2* gene contains an ACG at the predicted translation initiation site

Received: 4 February / 3 May 1998

Abstract The mitochondrial *cox2* gene has been sequenced from radish (*Raphanus sativus* L.). The gene is interrupted by a 1346-bp group-II intron and contains an ACG codon as the predicted translation initiation site. Analysis of *cox2* cDNAs indicates that the ACG codon is not converted to an AUG codon in the mRNA, although 15 other RNA editing sites were identified. The *cox2* gene from *Raphanus raphanistrum*, and other varieties of *R. sativus*, also contain an ACG as the predicted start codon; plants in the closely related genus, *Brassica*, do not. Western-blot analyses indicate that *cox2* proteins in radish mitochondria are the same size as those found in *Brassica* mitochondria and different from *cox2* proteins in plants where *cox2* is nuclear-encoded. This finding, along with the observation that *cox2* sequences are not present in the nuclear genome of radish, suggests that ACG is utilized as the radish *cox2* initiation codon.

Key words ACG start codon · *cox2* Gene · Translation · RNA editing

Introduction

RNA editing is a common feature of higher-plant mitochondria. Almost all plant mitochondrial, protein-encoding RNAs examined to-date undergo the post-transcriptional conversion of specific cytidine residues to uridines (reviewed in Grienenberger 1993; Schuster and Brennicke 1994). While editing events are usually confined to the coding portions of the mRNAs, there are several examples of RNA editing in 5′ and 3′ flanking regions and introns. Most C-to-U changes result in amino-acid substitutions; however, some silent editing events are observed. The editing-induced changes in the sequence of messenger RNAs results in improved amino-acid conservation of the encoded proteins. In addition to restoring highly conserved amino acids, editing has also been shown to create functional start and stop codons in several proteins. Therefore, RNA editing plays a significant role in conserving protein structure and function in plant mitochondria.

RNA editing has been identified in all the major groups of land plants except liverworts (Gray 1996; Malek et al. 1996; Sper-Whitis et al. 1996). While there are numerous examples of species-specific RNA editing patterns, interspecific comparisons generally reveal a common set of edited bases. For example, eight editing sites are shared in *nad3* transcripts between monocots and most dicots, while up to 14 common editing positions are typically observed between *atp6* transcripts from the two groups of plants.

During the course of studies on mitochondrial genes and RNA editing patterns in radish, several examples were identified where either the analysis of genomic DNA sequences predicted the need for little or no editing, or cDNA sequencing directly revealed reduced or no editing in radish genes relative to other dicots. For example, based on the presence of radical amino-acid substitutions in *coxI* genes between plant and non-plant species, it has been predicted that *coxI* in dicots should be edited at up to eight positions (Covello and Gray 1990). Consistent with this prediction, *Oenothera coxI* transcripts are edited at seven positions (Wissinger et al. 1992). Radish *coxI* does not contain any of the radical amino-acid substitutions (Makaroff et al. 1991), but rather resembles the non-plant version of the gene, suggesting that it does not require editing. Likewise, three amino-acids are changed by editing in *Oenothera orfB* (Schuster and Brennicke 1991), while one and zero codons are changed by editing in Scarlet Knight and white radish cultivars, respectively (Krishnasamy and Makaroff 1994). The most dramatic example of reduced editing in a radish gene is *atp6*, which contains every base found to be edited in other species (Makaroff et al. 1989), including a change that generates a new stop codon in

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Communicated by K. J. Newton

Oenothera (Schuster and Brennicke 1991), *Petunia* (Lu and Hanson 1994) and sorghum (Kempken et al. 1991) *atp6*. Analysis of cDNA sequences demonstrated that *atp6* transcripts are not edited in the Scarlet Knight radish nuclear background, while one edited position was identified in *atp6* transcripts in the presence of several other radish nuclear backgrounds (Krishnasamy et al. 1994). The *atp6* gene in Scarlet Knight radish is the only example of a normally occurring mitochondrial gene that is not edited. The only other un-edited transcript identified in plant mitochondria is that of maize *T-urf13* (Ward and Levings 1991), which is unique to the CMS-T cytoplasm of maize (Dewey et al. 1986).

The most likely explaination for the lack of editing in radish *atp6* transcripts is that at some point in the evolution of modern radish a cDNA representing an edited *atp6* RNA became integrated into the mitochondrial genome of a radish progenitor, thereby eliminating the need for the editing of *atp6* transcripts. The presence of edited versions of *cox2* in the nucleus of cowpea (Nugent and Palmer 1991) and mungbean (Covello and Gray 1992), along with data suggesting that loss of introns two and three from the *nad4* gene of lettuce involved homologous recombination of an edited RNA intermediate (Geiss et al. 1994), provides support for this hypothesis. However, the observation that other radish mitochondrial genes also require little to no RNA editing suggests that reduced editing patterns may be a common feature in radish mitochondria.

As part of our studies to further investigate RNA editing patterns in radish, and possibly gain further support for the theory that homologous recombination of cDNA sequences can eliminate the need for RNA editing, we have characterized the radish *cox2* gene and its RNA editing patterns. In the present report we show that, like turnip, radish *cox2* contains the *cox2*i2 intron and lacks *cox2*i1 intron. In contrast to several other radish genes, *cox2* transcripts are edited in a fashion similar to that observed in other plants; no correlation between intron loss and editing patterns were observed. The most striking result from these studies is the finding that radish *cox2* appears to utilize an ACG for the initiation of translation.

Materials and methods

Plant material. The following plant materials, used in this study, were obtained from commercial sources: turnip (*Brassica campestris* L. cv Purple top white globe 372), radish *(Raphanus sativus* L. cv Scarlet Knight), cowpea (*Vigna unguiculata* L.), soybean (*Glycine max* L.), sweet potato (*Ipomoea batatas* L.) and mungbean (*Vigna radiana* L.). Seeds for *Brassica juncea* (CrGC89) and the rapid-cycling radish line CrGC15 were obtained from the Crucifer Genetics Cooperative. Seeds for *Brassica nigra (16 A)* and *Brassica oleracea* (4722B) were generously provided by Dr. Lisa Earle. Seeds for *Raphanus raphanistrum*, and two California wild radish cultivars (# 5260 and # 5262), were a gift from Dr. Maureen Stanton. In general, seeds were germinated in the dark and etiolated seedlings were harvested and used for the isolation of mitochondria. However, in some instances mitochondria were isolated from whole greenhouse-grown plants.

Mitochondrial DNA cloning and sequencing. The radish mtDNA clone (pUC8-*Sal*I 8.3) used in this study has previously been described (Makaroff and Palmer 1988). Standard techniques were used in the preparation, isolation, and analysis of plasmid DNA in *Escherichia coli* (Sambrook et al. 1989). Double-stranded plasmid DNAs were sequenced using the dideoxynucleotide chain-termination method (Sanger et al. 1977) as modified by the Sequenase Version 2.0 kit from United States Biochemical Corporation (Cleveland, Ohio, USA). Sequencing was conducted using a series of *cox2*-specific primers that were designed to generate an overlapping DNA sequence on both strands of the DNA. All of the sequences presented, except for approximately 200 bases at the 5′-end and 70 bases at the 3′-end, were sequenced on both strands. DNA sequences were analyzed on a Vax computer using the UWGCG package (Madison, Wis., USA). Oligonucleotides used for cDNA synthesis, PCR, and DNA sequence analysis, were designed using the program OLIGO (Rychlik and Rhoads 1989). In order to examine the putative *cox2* translation initiation site in related species, PCR was conducted on mtDNAs purified from *B. nigra*, *B. juncea*, *B. oleracea*, *R. raphanistrum*, rapid-cycling radish CrGC15 and California wild radish isolates 5260 and 5262 using primers FD8B and FD7A (Fig. 1). Amplified fragments were purified, cloned into the pGEM-T vector (Promega, Madison, Wis., USA) and sequenced.

Isolation and characterization of mtRNA. Mitochondrial RNAs were isolated in the presence of aurintricarboxylic acid from radish mitochondria purified by differential centrifugation (Stern and Newton 1986). The RNAs were then purified by LiCl-precipitation and treated with DNAse I to remove contaminating DNA prior to their use. Northern-blot hybridization of radish mtRNAs followed standard procedures (Sambrook et al. 1989). Specifically, mtRNAs were separated in a 1.5% formaldehyde, agarose denaturing gel, transferred to a Nylon membrane and cross-linked under UV light. The blot was hybridized with a radish cDNA probe that was labeled by random-priming. To analyze RNA editing patterns, radish cDNAs were prepared by reverse transcription (Kawasaki 1990) of 2.5 μg of mtRNA isolated from etiolated seedlings using primer FD7A (see Fig. 1). PCR-amplification (30 cycles) was performed on 0.125 μg of first-strand cDNA using standard conditions (Kawasaki 1990; D'Aquila et al. 1991) and primers FD8B and FD7A (see Fig. 1). Amplified fragments were purified using the Wizard PCR prep DNA purification system (Promega, Madison, Wis., USA) and cloned into pGEM-T. The cDNA clones were sequenced using the dideoxynucleotide chain-termination method. Purified RT-PCR products were also used directly as templates in sequencing reactions. S1 nuclease mapping of the radish *cox2* 5′ and 3′ transcript termini was carried out using the methods described by Sambrook et al. (1989). Double-stranded DNA probes were prepared by PCR-amplification of the radish mitochondrial *Sal*I 8.3 plasmid using primer pairs FD37 and FD30 for 3′-end mapping and AA and FD31 for 5′-end mapping (Fig. 1). Purified fragments were either 3′ end-labeled using ^{32}P-dATP and terminal deoxynucleotidyl transferase (United States Biochemical, Cleveland, Ohio, USA) or 5′ end-labeled using ^{32}P-ATP and polynucleotide kinase. Labeled DNAs (approximately 0.1 μg) were denatured and annealed to 50 μg of radish mitochondrial RNAs or *E. coli* tRNA (control) in hybridization buffer (0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA, 80% formamide) at 52°C overnight. After hybridization, nine volumes of S1 nuclease reaction buffer (50 mM NaOAc, 0.15 M NaCl, 0.5 mM ZnSO4, pH 4.75) containing 50 or 100 units of S1 nuclease enzyme (BRL) were added to the hybridization buffer. The solution was mixed and incubated at 37°C for 1 h. The sample was extracted twice with Tris-saturated phenol, ethanol-precipitated and re-suspended in 1× sequence loading buffer. The size of the protected DNA was determined by analysis on an 8% polyacrylamide sequencing gel.

Western-blot analyses. Total mitochondrial proteins from turnip, radish, sweet potato, soybean, cowpea, and mungbean were examined by Western-blot analysis. One-hundred microliters of mitochondria purified by differential centrifugation were mixed with 50 μl of 8 M Urea/10% SDS and then heated at 95°C for 5 min. Thirty microliters of supernatant, collected by centrifugation for 5 min at 12 000 g,

were loaded and run on a 12% SDS-PAGE gel. The proteins were transferred to nitrocellulose membrane using a semi-dry apparatus and then treated with a monoclonal antibody to yeast COX2 (1:100 dilution, prepared from Hybridoma cell line CCO-6, a gift from J. D. Palmer, Indiana University). The COX2 primary antibody was detected with goat anti-mouse IgG (H+L) alkaline phosphatase-conjugated secondary antibody (1:5000) using NBT and BCIP as substrates.

Results

Mitochondrial *cox2* gene in radish

Radish *cox2*, previously localized to an 8.3-kbp *Sal*I fragment (Makaroff and Palmer 1988), was used as the source of *cox2* for this study. A total of 4.2 kbp of DNA sequence was determined around radish *cox2*, and a portion of the sequence is presented in Fig. 1. The *cox2* coding region is interrupted by a 1346-bp intron. This intron is in the same position as, and shares high sequence similarity with, introns found in *cox2* genes from turnip (99.70%, Dong et al. 1998), *Arabidopsis* (97.45%, Unseld et al. 1997) and carrot (94.66%, Lippok et al. 1992).

Plant *cox2* genes, including their deduced amino-termini, are highly conserved. The *cox2* gene from most dicots, including turnip, *Arabidopsis*, *Oenothera*, *Petunia* and sugar beet, contain a conserved ATG codon that is predicted to be the translation initiation codon (Fig. 2). The carrot, pea and soybean *cox2* genes are predicted to initiate translation at ATG codons that are located either one codon upstream of or downstream from that found in most dicots. In the monocots, maize, wheat and rice, translation is predicted to initiate at an ATG codon that is two codons upstream of that found in turnip. Even the *cox2* gene from a very distantly related non-vascular land plant, *Marchantia*, contains an ATG that is two codons downstream from the predicted turnip start site. Alignment of the predicted radish *cox2* protein with other *cox2* coding sequences indicates that while it is essentially identical to turnip *cox2*; it does not contain an ATG codon in the region predicted to be the translation initiation site in other plants (Fig. 2). Instead, a T-to-C mutation in radish *cox2* generated an ACG codon at the position corresponding to the start codon in *cox2* from turnip and other dicots. In-frame ATG codons are found in the radish sequence 76 codons upstream of and 31 codons downstream from the position predicted to be the start codon in turnip (Fig. 1). The presence of an ACG codon at the position predicted to act as the *cox2* start codon in other plants raised several possibilities concerning the expression of radish *cox2*, the most likely possibility being that RNA editing is used to generate a functional start codon.

Radish *cox2* transcript and RNA-editing pattern analyses

In order to determine if in fact RNA editing is involved in the conversion of the ACG codon into a conventional AUG start codon, 39 radish *cox2* cDNA clones were isolated and sequenced in the region in question. None of the clones contained the predicted "C"-to-"T" change that would be necessary to convert the ACG codon into an AUG codon. This result was further confirmed by the direct sequencing of *cox2* RT-PCR products.

In addition to the 39 *cox2* cDNAs that were sequenced in the region surrounding the predicted start codon, eight cDNA clones were sequenced in their entirety. A total of 15 positions that exhibited C → T changes were identified. Twelve of the editing events result in amino-acid changes. Of the 15 editing sites, all but one were completely edited (codon 9, indicated by a single underline in Fig. 1). Two cDNA clones were found to be edited at this position while six others were not. The radish editing pattern is very similar to that observed for other plant mitochondrial *cox2* genes (Fig. 1). Of the 15 editing sites, 14 are also found in turnip *cox2* (Dong et al. 1998) and 12 are common to the maize (Covello and Gray 1989; Yang and Mulligan 1991), wheat (Covello and Gray 1989), *Oenothera* (Hiesel et al. 1990), Petunia (Sutton, et al. 1991) and pea (Covello and Gray 1989) *cox2* transcripts.

Therefore, results from the editing studies confirm that editing does occur in the radish *cox2* RNAs, but that it does not generate an AUG codon. This raised the possibility that: (1) another in-frame ATG may have been recruited to serve as the initiation codon, (2) the radish mitochondrial *cox2* gene is not active, rather the active form of the gene may have been transferred to the nucleus, or (3) an unconventional start codon, possibly the ACG codon, is used for translation initiation of radish *cox2*. To investigate these possibilities, we examined *cox2* expression patterns and determined the 5′ and 3′ transcript termini.

When a ^{32}P-labeled *cox2* cDNA probe was used to probe a Northern blot containing mtRNA isolated from radish roots, two transcripts were detected: an abundant RNA of approximately 1.3 kb and a large low abundance RNA (Fig. 3, lane 1). The larger transcript also hybridizes with an intron-specific probe (data not show), which indicates that the band represents a primary transcription product. The 1.3-kb transcript is the same size and is of the same relative abundance as the mature *cox2* transcript from turnip mitochondria (lane 2). This observation, along with the results from the cDNA analysis, indicates that *cox2* is expressed normally in radish mitochondria.

The 5′ and 3′ ends of the mature *cox2* transcript were mapped using end-labeled probes and S1-nuclease. Two 5′ termini were identified, centered around positions 940 and 998, which are 222 and 164 nucleotides upstream of the ACG codon, respectively (Fig. 1). The 5′ most in-frame AUG codon maps upstream of both transcript termini indicating that it is not a potential start codon. The 3′ transcript terminus was mapped approximately 307 nucleotides downstream from the predicted stop codon (Fig. 1). Based on these results, mature transcripts are expected to be of 1256–1314 nucleotides, which corresponds well with the 1300 nt size predicted by Northern-blot analysis. The positions of the radish 5′ and 3′ transcript termini map to the same positions as those determined for *cox2* in turnip

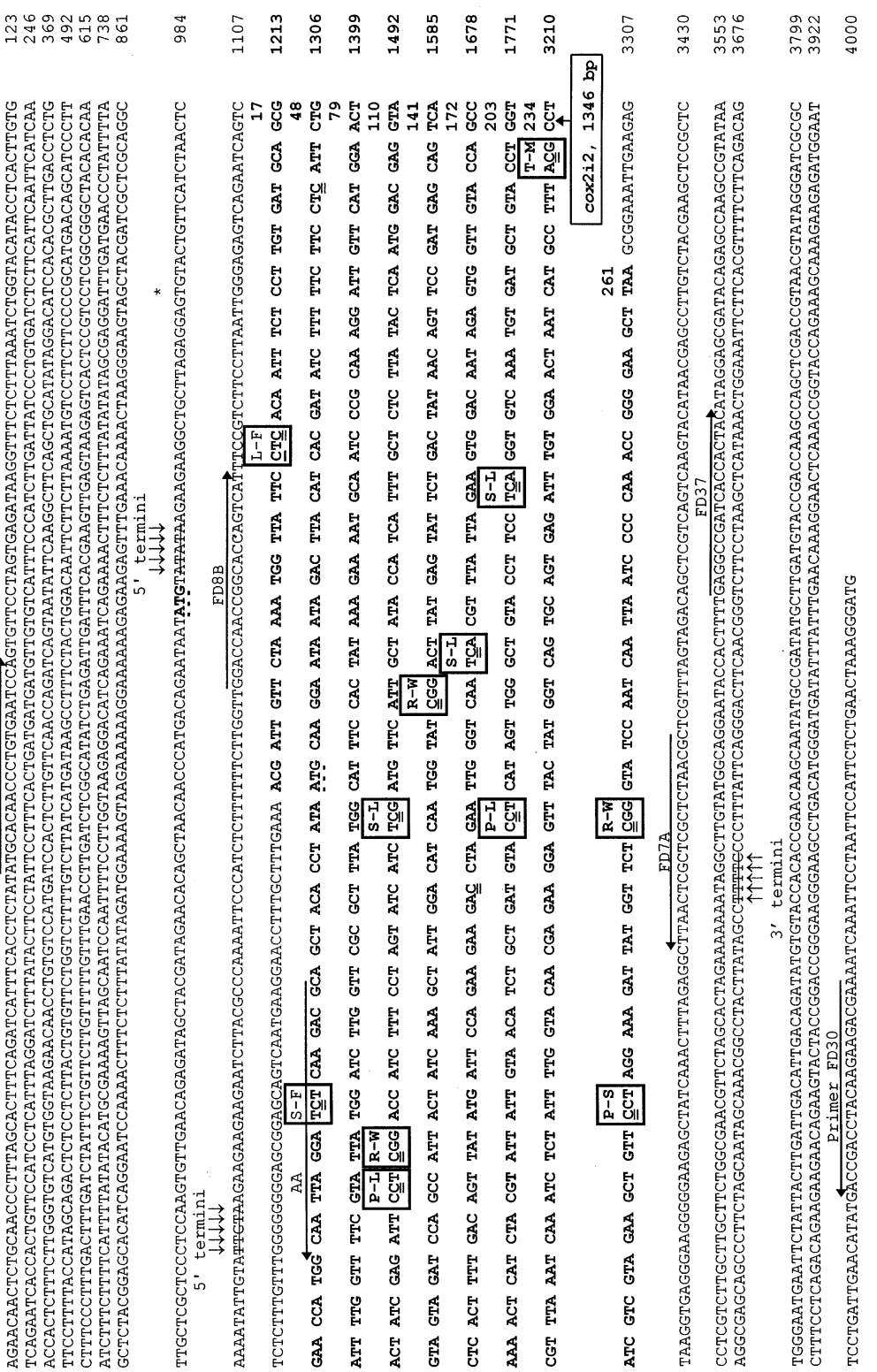

Fig. 1 Nucleotide sequence of the radish *cox2* gene. The *cox2* DNA and cDNA sequences are available under accession numbers AFO36387 and AFO36388, respectively. The *cox2* coding sequence is shown *in bold* in triplets starting at a putative ACG start codon. The position of a 1346-bp intron (*cox*2i2) is indicated by a *vertical arrow*. In-frame ATG codons that flank the putative start codon are underscored by *dots*. A potential GUG start codon is denoted with a (*). Edited positions are either *double* (completely edited) or *single* (incompletely edited) *underlined*. Codons altered by RNA editing are *boxed*, and the resulting changes are indicated. Primer positions are shown as *horizontal arrows* above the sequence. Multiple *vertical arrows* indicate the position of 5′ and 3′ transcript termini determined by S1 nuclease mapping. Codon positions are shown above the last codon of each column. Nucleotide numbering is shown at the end of each column

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              -2 1            ↓                 *                 48
Radish           TIVLKWLF LTISPCDAAE PWQLGSQDAA TPIMQGIIDL HHDIFFFLIL
Turnip           MIVLKWLF LTISPCDAAE PWQLGSQDAA TPIMQGIIDL HHDIFFFLIL
Arabidopsis      MIVLKWLF LTISPCDAAE PWQLGSQDAA TPIMQGIIDL HHDIFFFLIL
Pea               MKLEWLF LTIAPCDAAE PWQLGFQDAA TPMMQGIIDL HHDIFFFLIL
Soybean (mt)      MKFEWLF LTIAPCDAAE PWQLGFQDAA TPMMQGIIDL HHDIFFFLIL
Carrot          MSFTGIFHF FTNSPCDAAE PWQLGSQDAA TPMMQGIIDL HHDIFFFLIL
Petunia          MIVLEWLF LTIAPCDAAE PWQLGSQDAA TPIMQGITDL HHDVFFFVIL
Sugar beet       MIVREWLF FTMAPCDAAE PWQLGFQDAA TPMMQGIIDL HHDIFFFLIL
Oenothera        MIVNECLF LTIAPCDAAE PWQLGSQDAA TPMMQGIIDL HHDIFFFLIL
Maize          MILRSLECRF LTIALCDAAE PWQLGSQDAA TPMMQGIIDL HHDIFFFLIL
Rice           MILRSLECRF LTIALCDAAE PWQLGSQDAA TPMMQGIIDL HHDIFFFLIL
Wheat          MILRSLSCRF LTIALCDAAE PWQLGSQDAA TPMMQGIIDL HHDIFFFLIL
Marchantia        MNLMWM FPIAFCDAAE PWQLGFQDPA TPMMQGMIDL HNDIFFFLIV
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Fig. 2 Conserved COX2 amino termini. The predicted amino termini of *cox2* genes from turnip (Dong et al. 1998), *Arabidopsis* (Unseld et al. 1997), pea (Moon et al. 1985), soybean (Grabau 1987), *Oenothera* (Hiesel and Brennicke 1983), sugar beet (Senda et al. 1991), *Petunia* (Pruitt and Hanson 1989), carrot (Lippok et al. 1992), maize (Fox and Leaver 1981), wheat (Bonen et al. 1984), rice (Kao et al. 1984) and *Marchantia* (Oda et al. 1992) are shown below the deduced sequence for radish *cox2*. The position of the mature COX2 protein from sweet potato (Maeshima et al. 1989) is indicated with an *arrow*. The first in-frame methionine in radish *cox2* transcripts is denoted with an (*)

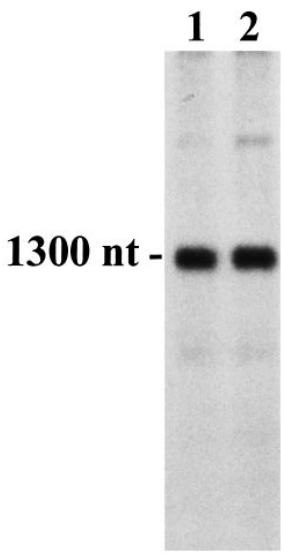

Fig. 3 Northern analysis of *cox2* transcript patterns. Transcript patterns observed when mitochondrial RNAs (5.0 μg) from *R. sativus* (*lane 1*) and *B. campestris* (*lane 2*) are probed with a ^{32}P-labeled radish *cox2* cDNA probe. Transcript size is shown in nucleotides to the left

(Dong et al. 1998). This is as expected given that the two genes are nearly identical.

Even though *cox2* appears to be expressed normally at the RNA level in radish mitochondria, it was possible that the true active form of the gene is located in the nucleus. To investigate this possibility Southern hybridization and PCR analyses were conducted. When Southern blots of purified radish nuclear DNA were probed with a ^{32}P-labeled *cox2* cDNA probe the only hybridization observed was a faint signal corresponding to mitochondrial DNA contamination (data not shown). Likewise, PCR-amplification of purified radish nuclear DNA with primers capable of amplifying cowpea and mungbean nuclear *cox2*

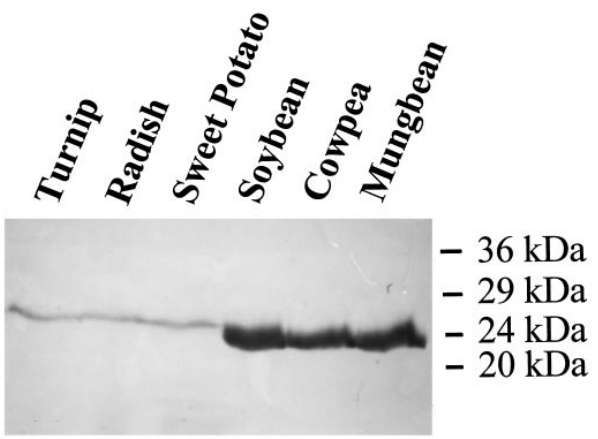

Fig. 4 Western-blot analysis of cytochrome c oxidase subunit II proteins. Mitochondrial proteins (approximately 200 μg) purified from turnip, radish, sweet potato, soybean, cowpea and mungbean were separated on an 12% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with COXII monoclonal antibody (Hybridoma cell line CCO-6) and alkaline phosphatase-conjugated secondary antibody. The sizes of the protein markers are shown to the right

only produced mitochondrial-specific fragments (data not shown). These results indicate that the *cox2* gene has not been transferred to the radish nucleus.

Western-blot analysis

In order to determine if an internal ATG codon had been recruited for translation initiation, a Western blot analysis was conducted. Translation initiation from the first internal AUG codon would produce a mature protein 17 amino acids shorter than that expected for turnip COX2, if turnip COX2 is processed at the same position as the sweet potato protein, and 32 amino-acids shorter than an unprocessed turnip COX2 protein. Mitochondrial proteins isolated from radish, turnip, sweet potato, soybean, cowpea and mungbean were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose and probed with a COX2 monoclonal antibody (Fig. 4). One unique band was detected in each of the mitochondrial protein preparations that showed a strict correlation with whether the *cox2* gene is nuclear or mitochondrial localized. COX2 from radish, turnip and sweet potato are the same size and of the same intensity; while those from soybean, cowpea and mungbean are of similar size and intensity. The COX2 bands in plants with nuclear-localized *cox2* genes are slightly

smaller, but at least ten-times more intense than those with mitochondrially localized genes. While the difference in signal intensity appears to be a result of the presence of more COX2 protein in the cowpea, soybean and mungbean samples, the difference in relative signal intensity is not due to unequal sample loading. When a duplicate blot was probed with antibody to mitochondrial ATPA, all six samples produced the same-intensity bands (data not shown). These results suggest that differences in the expression/accumulation of COX2 protein may exist between plants that contain nuclear- and mitochondrially encoded genes. However, it is also possible that the observed signal differences are due to different affinities of the yeast COX2-specific antibody for the proteins. Additional experiments are required to investigate in more detail the nature of the observed differences.

The most important observation, with respect to our current work, is the finding that radish mitochondrial proteins produce a COX2 signal of the same size and intensity as turnip and sweet potato. This result, taken together with the observed differences in the cowpea, soybean and mungbean samples, suggests that the active radish *cox2* gene is mitochondrially localized and that the mature amino-terminus of the protein is located at approximately if not the same position as that found in turnip and sweet potato. This result indicates that translation most-likely does not initiate from an internal methionine and that radish *cox2* may in fact utilize the ACG codon for translation initiation.

An ACG codon is present at the translation initiation site of *Raphanus cox2* genes

In order to determine if the putative ACG initiation codon is limited to *R. sativus* or if it is found in other species, *cox2* mtDNA fragments corresponding to this region were amplified from several *Brassica* and *Raphanus* cultivars using primers FD8B and FD7A (Fig. 1). The *Brassica* plants analyzed were *B. nigra, B. juncea* and *B. oleacea*, while the *Raphanus* plants included *R. raphanistrum*, two wild California radish isolates (*R. sativus* No. 5260 and 5262) and CMS Ogura radish (*R. sativus*). Sequence analysis of the PCR fragments showed that the ACG codon was present in every *Raphanus cox2* gene examined, but in none of the *Brassica* genes (data not shown). This result indicates that this mutation probably occurred after the emergence of the genus *Raphanus*.

Discussion

In this report we present data indicating that the *cox2* gene in *Raphanus* species utilizes a non-conventional initiation codon for translation. The *cox2* gene in *R. sativus* and related *Raphanus* cultivars contains an ACG codon in place of a highly conserved AUG codon, predicted to be the translation initiation site in other plants. There have been several other reports of chloroplast and mitochondrial genes that contain an ACG codon at positions that are normally AUG start codons. Most of these genes, including *rpl*12 in maize (Hoch et al. 1991), *psb*L in tobacco (Kudla et al. 1992) and spinach (Bock et al. 1993), *ndh*D in tobacco, spinach and snapdragon (Neckermann et al. 1994) and *orf33* in black pine (Wakasugi et al. 1996) are chloroplast genes. Two mitochondrial genes, wheat *nad1* (Chapdelaine and Bonen 1991), and tomato *cox1* (Kadowaki et al. 1995) and potato *cox*1 (Quinones et al. 1995) have also been shown to contain an ACG at the translation initiation site. In every example described above, RNA editing generates a normal AUG codon in the mRNAs, thereby restoring the conventional translation start codon. In contrast, we find that while *cox2* transcripts are extensively edited in radish mitochondria, no editing of the ACG codon in question was observed. Editing at this position was not observed in approximately 40 individual cDNA clones and total reverse-transcribed *cox2* cDNA. While we can not rule out the possibility that a very small number of transcripts are edited to produce a conventional AUG start codon, it is clear that essentially all radish *cox2* transcripts contain an ACG at this position. This finding raises two important questions: (1) why is the ACG codon not edited to generate a conventional initiation codon in radish?, and (2) is ACG utilized as the translation initiation site in radish *cox2*?

Almost all plant mitochondrial protein-encoding RNAs examined to-date undergo RNA editing involving the post-transcriptional conversion of specific cytidine residues to uridines (reviewed in Maier et al. 1996). While some silent editing events are observed, most C-to-U changes result in amino-acid substitutions. These changes in messenger RNA sequence result in improved amino-acid conservation of the encoded proteins. In addition to restoring highly-conserved amino acids, editing also creates functional start and stop codons in serveral proteins. Therefore, RNA editing plays a significant role in conserving protein structure and function in plant mitochondria. Why then do radish mitochondria not edit the ACG codon to restore the highly conserved predicted start codon?

There are several examples of “unusual” RNA editing patterns in radish mitochondrial transcripts. The only example of an unedited mRNA corresponding to a normally occurring plant mitochondrial gene is Scarlet Knight radish *atp6* (Krishnasamy et al. 1994). The radish *atp6* gene contains every base found to be edited in other species (Makaroff et al. 1989). Analysis of potential coding regions and editing patterns in radish *cox1* (Makaroff et al. 1991) and *orfB* genes (Krishnasamy and Makaroff 1994) indicates that they also require little to no editing. However, except for the lack of editing at the ACG codon corresponding to the putative translation initiation site, radish *cox2* exhibits relatively normal editing patterns. Fifteen positions are edited in radish *cox2* transcripts, including an ACG codon (#233) later in the gene (Fig. 1). The editing pattern is essentially the same as that observed in other plant mitochondrial *cox2* transcripts. Therefore, the editing machinery is present and the transcript is accessible for

editing; however, ACG codon (#1) is not recognized as an editing site. Currently, the factors and sequences necessary for editing in plant mitochondria are unknown, and a direct correlation between sequence context and editing has yet to be identified (Gray 1996; Wilson and Hanson 1996). It is interesting that wheat and potato/tomato have evolved the ability to convert an ACG into an AUG start codon for the *nad1* and *cox1* genes respectively, but that radish has not yet developed this ability.

Based on the available data, we can not conclude with certainty that radish *cox2* utilizes the ACG codon (#1) for translation initiation. However, it is clear that translation must initiate from a non-conventional start codon and the most likely candidate is the ACG codon. There are two in-frame AUG codons surrounding the ACG codon, neither of which appears to be utilized. The first, which is located 76 codons 5′ to the ACG codon, is not contained in the mature *cox2* transcript, and therefore, can not be used as a start codon. The second in-frame AUG codon is located 32 amino acids downstream from the predicted start codon. This codon, which is conserved in all plant *cox2* genes examined to-date, is located 17 codons internal to the mature N-terminus of COX2 in sweet potato (Maeshima et al. 1989). If this codon were used to initiate the translation of radish *cox2*, the protein would not only be missing the N-terminal leader peptide, but also 17 highly conserved amino acids from the mature protein. This truncated protein would be approximately 2 kDa smaller than the size expected if the protein was processed at the same site as sweet potato COX2.

When compared with animal *cox2* genes, the *cox2* gene from plants and fungi encodes approximately 15 extra amino acids at its N-terminal end (Maeshima et al. 1989). These extra amino-acids form a leader peptide that is removed in both fungi (Coruzzi and Tzagoloff 1979, Van den Boogaart et al. 1982) and plants (Maeshima et al. 1989). In *Saccharomyces* the leader peptide is cleaved by an inner-membrane-bound protease (IMP) after it has been transported to the intermembrane (Pratje et al. 1983; Schneider et al. 1991; Nunnari et al. 1993). The leader peptide in *Saccharomyces* has been demonstrated to be crucial to the stability of COX2 and was proposed to be a signal peptide for membrane insertion (Torello et al. 1997). If the same is true in plant mitochondria, then a shortened peptide translated from the downstream AUG codon would not be expected to be correctly localized and should have reduced stability. Western-blot analysis of radish mitochondrial proteins (Fig. 4) clearly shows that radish COX2 is the same size, and is present in the same relative abundance, as those from sweet potato and turnip. This result suggests that radish COX2 is translated and processed normally.

These results are also consistent with expression of COX2 from a mitochondrial gene. The COX2 signal is significantly stronger in mitochondrial protein preparations from soybean, cowpea and mungbean than those from radish, turnip and sweet potato (Fig. 4). In soybean, cowpea and mungbean, COX2 is encoded by a nuclear gene and imported into the mitochondrial inner membrane (Nugent and Palmer 1991; Covello and Gray 1992), while in turnip, sweet potato and radish *cox2* is a mitochondrial gene. The reason for this difference in signal between proteins where *cox2* is nuclear-encoded and mitochondrially encoded is not clear; however, it is consistent with Southern and PCR data indicating that *cox2* sequences are not present in the nucleus of radish. Therefore, the mitochondrial gene is the only *cox2* gene present in radish and it produces an apparently normal COX2 protein.

Taken together, these data suggest that radish *cox2* must be translated from a non-conventional start codon. In addition to the ACG codon, an in-frame GUG codon is located 66 codons further upstream (Fig. 1). The GUG codon is located within the largest mature transcript, but not the shorter *cox2* transcript. A GUG codon has been proposed to be the translation initiation codon for the *rpl*16 gene in *Oenothera*, maize, *Petunia* and *Marchantia* mitochondria (Bock et al. 1994). However, this has not been experimentally confirmed. Both GUG and ACG can be utilized as translation initiation in plant cells at the same relative efficiency, but at levels considerably lower that AUG codons (Gordon et al. 1992). While our data does not allow us to distinguish between the two potential start codons, we favor the theory that the ACG codon is utilized for translation initiation. Translation from the ACG codon would yield a protein with a conventional leader peptide that would be processed normally. However, further experiments are clearly required to further investigate the translation of radish *cox2*.

Acknowledgements This work was supported in part by Academic Challenge Awards from the Miami University Botany Department and a Dissertation Research Award from the Miami University Graduate School to D. F. G. We thank Dr. Jeffrey D. Palmer for the COX2 antibody and mungbean genomic DNA, Drs. Lisa Earle and Maureen Stanton for some of the plant material used in this study, Dawn Paxson-Sowders for critical reading of the manuscript and Lucinda Swatzell for help with the photography.

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