Nucleic Acids Research, Vol. 20, No. 21 5699–5703

A single nuclear gene specifies the abundance and extent of RNA editing of a plant mitochondrial transcript

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Received July 20, 1992; Revised and Accepted September 17, 1992

ABSTRACT

A number of cytosines are altered to be recognized as uridines in transcripts of the NADH-dehydrogenase subunit 3 (*nad3*) gene in the mitochondria of the higher plant *Petunia hybrida*. Here we show that the extent of editing for three of the edit sites, all of which change the encoded amino acid, varies between different *Petunia* lines. Genetic analysis indicates that a single nuclear gene is responsible for this variation. Interestingly, according to RNA blot hybridization analysis, RNA editing extent and transcript abundance are correlated. This observation is consistent with the hypothesis that RNA editing is a post-transcriptional event.

INTRODUCTION

RNA editing is a recently discovered phenomenon (1,2) that alters the actual sequence of an RNA molecule after it has been transcribed, thereby making it impossible to deduce the protein sequence directly from its gene sequence. Most of the RNA editing events described so far are observed in mitochondrially-encoded genes (3–7), except for three cases in mammalian nuclear genes (8,9,10) and a few cases in plant chloroplast genes (11,12,13). In higher plant mitochondria, all known protein-encoding genes except T-*urf13* (14) undergo a C-to-U type of editing event, which is very similar to the well-studied editing event in mammalian apolipoprotein B mRNA (8). However, the mechanism by which this C-to-U change occurs is unknown. The vast majority of known editing sites are found at the first or second positions of codons and thus lead to changes in the amino acids specified (15,16). In most cases proteins translated from edited mRNAs are more similar to their nonplant homologs than the protein sequence deduced from the corresponding genomic sequence (5–7,17).

Another interesting feature of plant mitochondrial RNA editing is that partially edited transcripts also exist (18–25); i.e, in some transcripts, editing has not occurred at all potential editing sites. Although some partially edited sites are at silent positions (19,20,24), some do change the encoded amino acids (18,21,25). Though it has not been proven, it is likely that partially edited transcripts are editing intermediates subject to further editing (21,22,23).

We were interested in determining whether nuclear genes could affect extent of editing of particular mitochondrial genes. In this paper a reverse transcription/polymerase chain reaction (RT/PCR) technique was used to show that extent of RNA editing at three partial edit sites, which all change the encoded amino acid, varies between different *Petunia* lines that have the same mitochondrial genome but different nuclear backgrounds. Genetic analysis indicates that a single nuclear gene is responsible for this variation. Transcript analysis shows a tight correlation between the extent of RNA editing and transcript abundance.

MATERIALS AND METHODS

Genotypes

Lines 3688, 11127, 2423 contain different nuclear genomes but the same male-sterility-encoding mitochondrial genome. Fertile lines 3699 and 3704 contain the same nuclear genomes as 3688 and 11127, in the presence of the *P.parodii* and *P.hybrida* cytoplasmic genomes, respectively. Lines 3688 and 3699 carry the *P.parodii* nuclear genome; 3704, 11127, and 2423 are *P.hybrida* lines.

Nucleic acid isolation

Total RNA was extracted from young *Petunia* leaves following the procedure as detailed (26). After LiCl precipitation, the supernatant was saved and total DNA was precipitated by adding 2 volumes of EtOH.

Northern analysis

Approximately 50 μg of total leaf RNA was loaded on each lane and size fractionated on formaldehyde-agarose gel and blotted onto nitrocellulose filters. Ethidium bromide was added to RNA samples before electrophoresis for visualization. Prehybridization and hybridization were done as described (27). A PCR-amplified 680 bp fragment of the *nad3* gene and a 0.5 kb *Pst*I-*Eco*RI fragment from *Petunia atp6* cDNA were used as probes. Probes were labelled to high specific activity using Sequenase (USB). After hybridization, the filter was washed at room temperature in 2×SSC, 0.5% SDS and then at 65°C in 0.2×SSC,0.5% SDS. For rehybridization, the filter was washed at 68°C in 0.2×SSC, 0.5% SDS for 3 hrs and hybridized as above.

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cDNA synthesis and PCR amplification

Approximately 50 μg of total leaf RNAs were treated with 100 U/ml of RQ1-DNase I (RNase free) (Promega) for 30 minutes at 37°C in the presence of RNasin (Promega). After phenol/chloroform, chloroform extractions and ethanol precipitation, the pellet was dissolved in diethylpyrocarbonate-treated water and heated at 95°C for 3 minutes and quickly chilled on ice. First strand cDNAs were made by adding 100 ng of 3′ PCR primer, 40 units of RNasin, 0.5 mM of each deoxynucleotide and 1×PCR buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$), in the presence of 200 units of M-MLV-reverse transcriptase (BRL) for 1 hr at 37°C. RNAs were then degraded by adding EDTA (pH 8.0) and NaOH to final concentrations of 25 mM and 0.5 N respectively, and incubated at 65°C for 1 hr. cDNAs were precipitated by adding 1/10 volume of 3 M NaOAC (pH 4.8), equal volume of isopropanol and 20 μg of glycogen (Boehringer). PCR amplification of cDNAs was done by adding 200 ng of each primer, 10 μl of 10×PCR buffer, 2 μl of dNTPs (10 mM), 2.5 U of Taq DNA polymerase in a final volume of 100 μl. Taq DNA polymerase and dNTPs were added at step 1 after 5 minutes at 92°C. The PCR was performed on a Hybaid cycler (National Labnet) under the following conditions: Step 1: 10 minutes at 92°C, 1 minute at 60°C, 1 minute at 72°C, 1 cycle; Step 2: 30 seconds at 92°C, 1 minute at 60°C, 1 minute at 72°C, 35 cycles; Step 3: 10 minutes at 72°C, 1 cycle. Control PCRs were done on total leaf DNAs that were prepared as described above. About 200 ng of total leaf DNA was used in PCR under the same amplification conditions as were used to amplify cDNAs.

10 μl of PCR products were exhaustively digested with 10 units of *Rsa*I or *Msp*I overnight at 37°C. Restriction fragments were size-fractionated in a 2.5% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV-light.

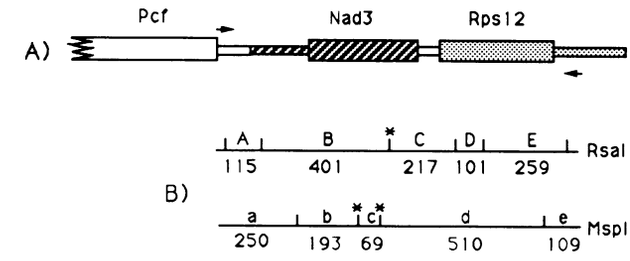

Figure 1. (A) Schematic of a genomic region in *Petunia* CMS lines' mitochondrial genome containing cotranscribed *pcf*, *nad3* and *rps12* genes. Arrows indicate position of upstream primer (5′-GCCTTGACAAGTTAGTACGGGTACTG-3′) and downstream primer (5′-GTGACTTCGTACCTATCCTTACC-3′) used in PCR amplification. **(B)** Restriction map of the amplified PCR product containing *nad3-rps12* region. a, b, c, d, and e represent restriction fragments after *Msp*I digestion. A, B, C, D, and E represent restriction fragments after *Rsa*I digestion. The number below each fragment is the size (bp) of that fragment. Asterisks indicate sites that will be disrupted on PCR products amplified from edited RNA.

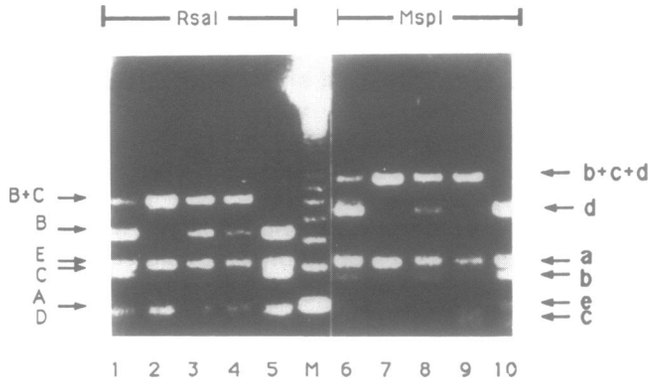

Figure 2. Restriction analysis of RT/PCR products amplified from total RNAs of 3 different petunia CMS lines and a progeny of 3688×3704. PCR products amplified from cDNAs reverse transcribed from total RNAs of 3688 (lanes 1, 6), 11127 (lanes 2, 7), 2423 (lanes 3, 8) and a progeny plant of 3688×3704 (lanes 4, 9) were digested with either *Rsa*I (lanes 1, 2, 3, 4) or *Msp*I (lanes 6, 7, 8, 9). Amplification products from total DNA (lanes 5, 10) were cut with *Rsa*I (lane 5) or *Msp*I (lane 10) and served as controls. 123 bp DNA ladder was used as molecular weight marker (M). Arrows on the left indicate position of restriction fragments from *Rsa*I digest, those on the right indicate *Msp*I restriction fragments. B+C and b+c+d represent intermediates that can no longer be digested by *Rsa*I or *Msp*I as a result of RNA editing. See Fig. 1 for symbols.

RESULTS

Influence of nuclear background on the extent of RNA editing in *nad3* mRNAs

Comparison of genomic and cDNA sequences of *Petunia* mitochondrial *nad3* gene revealed 19 editing sites (R.Wilson, personal communication). Three of the edit sites are within the recognition sites of endonuclease *Msp*I or *Rsa*I on genomic DNA. Therefore on cDNAs reverse-transcribed from edited *nad3* mRNAs, these restriction sites will be disrupted (Fig. 1). This provides us with a convenient assay to look for the extent of RNA editing at these sites by comparing the percentage of fragments unrestrictable with *Msp*I or *Rsa*I in the total PCR-amplified cDNA population. PCR products containing *Msp*I and *Rsa*I sites not altered by RNA editing serve as an internal control.

When total leaf RNAs from three isocytoplasmic *Petunia* lines (3688, 11127, 2423) were RT/PCR amplified and digested with either *Rsa*I or *Msp*I, we could consistently see a significant difference in the percentage of fragments unrestrictable at these sites. In 3688 only a small portion of fragments are unrestrictable (lanes 1, 6, Fig. 2), while in 11127 (lanes 2, 7, Fig. 2) and 2423 (lanes 3, 8, Fig. 2) lines a major portion of the fragments are not cleaved, with the 11127 line having the greatest percentage of digestion-resistant fragments. In the control lanes (lanes 5, 10, Fig. 2), PCR products of genomic DNA were completely digested. Based on these data, we conclude that of the total *nad3* mRNA population in 3688, a much lower percentage of molecules are edited at the three sites than that in 11127 and 2423. Since all three of these lines share the same mitochondrial genome but different nuclear backgrounds, this result indicates that a nuclear factor(s) is affecting the extent of RNA editing for at least these three edit sites in *nad3* mRNA.

Segregation of editing extent variation in a genetic cross

To further test the influence of the nuclear genome on the editing extent in *nad3* mRNA, we analyzed RNA editing in F1 progeny of a cross between line 3688 and line 3704, which contains the same nuclear genome as the line 11127 (Fig. 3). Line 3704 was used as the male parent instead of line 11127 because 11127 is male sterile. The F1 progeny from this cross inherit the cytoplasm solely from 3688 but nuclear genomes from both 3688 and line 3704. We found that in F1 plants, as in 11127, a major portion

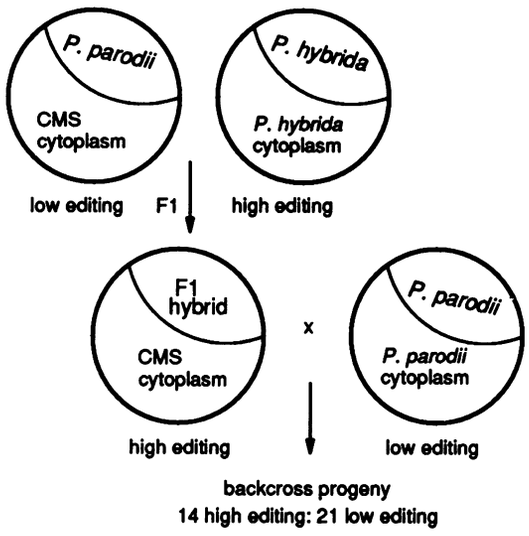

Figure 3. A diagram showing crosses used for genetic analysis. Male sterile line 3688 (female parent) was pollinated by a male fertile line 3704, which is the isogenic line of 11127. The F1 plants have the same CMS cytoplasm as 3688 and thus are male sterile. A F1 plant was then used as female parent and was pollinated by a fertile line 3699, which is the isogenic line of 3688. Seeds from this cross were collected and planted. Young leaves from individual plant were used to isolate total RNA.

of the transcripts are edited at the three sites tested (lanes 4, 9, Fig. 2). This result suggests that 3704 and 11127 nuclear genomes contain a dominant allele(s) of the nuclear gene(s) that confer extensive editing while 3688 contains the recessive allele(s).

We then scored for editing extent in individual progeny from a backcross of the F1 hybrid (3688×3704) to 3699, a male fertile isonuclear line of 3688 (Fig. 3). Because the F1 hybrid is male sterile, it could only serve as female parent. In this cross we found that the progeny segregate for the editing extent variation (Fig. 4B, 4C). In a total of 35 progeny plants analyzed, 21 have the less extensive editing characteristic of line 3688, while 14 of them have the extensive editing as in 11127. Each RNA sample was amplified with and without reverse transcription to make sure there was no genomic DNA contamination (Fig. 4A). Repeat experiments gave the same result. The ratio we observed suggests that a single nuclear gene is segregating in this cross. Consistent with the conclusion that the 11127 and 3704 nuclear background contains a dominant allele of the nuclear gene affecting editing extent, in a population derived from backcrossing the F1 hybrid (3688×3704) to 3704, all progeny show extensive editing at the 3 edit sites (data not shown).

The variation in editing extent is *nad3* transcript-specific

In order to obtain a general picture of the effect of this nuclear gene on RNA editing in *Petunia* mitochondria, we tried to find other transcripts whose editing extents are affected by nuclear background as are *nad3* transcripts. We have examined *coxII, nad1, atp6* and *atp9* transcripts. To date, no transcript has been found whose editing is affected by nuclear background in the same way as *nad3* transcripts (data not shown). Although transcripts of additional genes need to be analyzed, our preliminary results suggest that the nuclear effect on RNA editing is *nad3* transcript-specific.

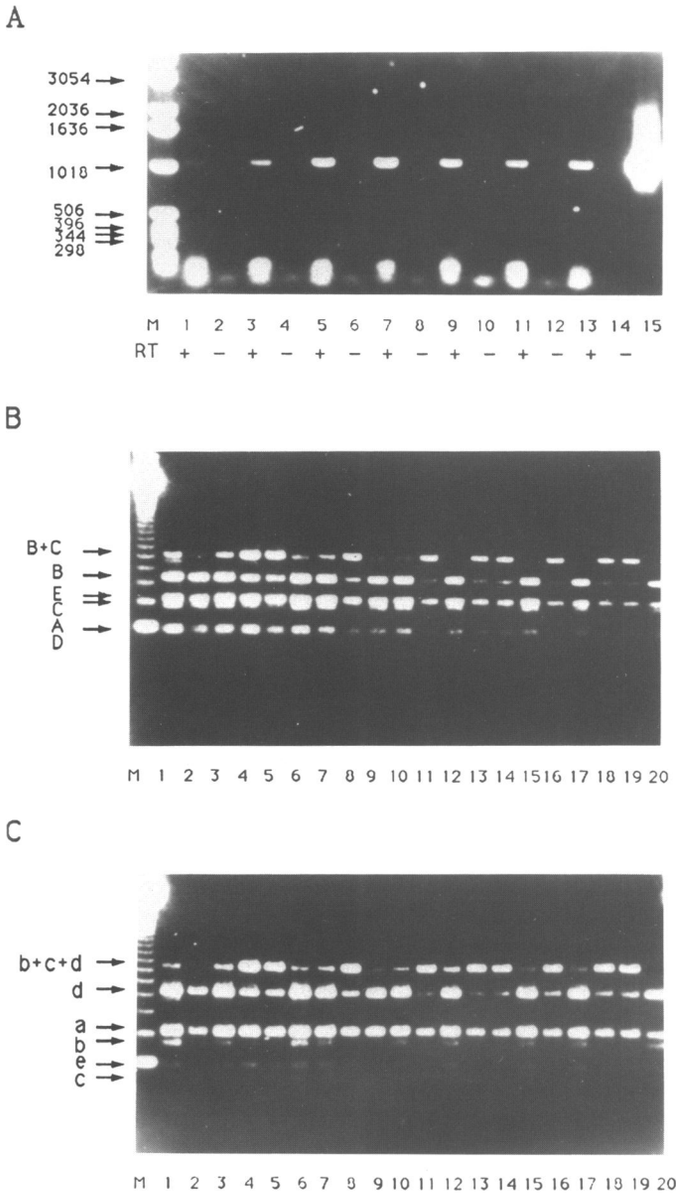

Figure 4. **(A)** RT/PCR of total RNAs isolated from individual plants of the backcross. About 50 μg of total RNAs from 7 individual plants were DNase I treated and reverse transcribed in the presence (lanes 1, 3, 5, 7, 9, 11, 13) or absence (lanes 2, 4, 6, 8, 10, 12, 14) of RT, and then PCR amplified. Total DNA was used in PCR as a control (lane 15). One-tenth of the reaction was loaded on a 1.5% agarose gel and size-fractionated. Sizes of the molecular weight marker fragments are indicated by arrows on the left. Products of expected size (1130 bp) were detected in RT (+) lanes. The absence of amplification in RT (−) lanes shows there is no genomic DNA contamination in cDNAs. All PCR products used in (B) and (C) were amplified and controlled as in (A). **(B)** *Rsa*I digest of PCR products amplified from total RNAs of 19 individual progeny of the backcross. About 2 μg of each PCR product was digested with *Rsa*I overnight and size-fractionated on a 2.5% agarose gel. Equal amount of PCR product amplified from total DNA was cut with *Rsa*I and run as a control (lane 20). 123 bp DNA ladder was used as molecular weight marker (M). Arrows on the left indicate position of *Rsa*I restriction fragments of the PCR products. See Fig. 1 for symbols. Out of 19 plants, ten (lanes 1, 2, 3, 6, 7, 9, 10, 12, 15, 17) show less extensive editing and nine (lanes 4, 5, 8, 11, 13, 14, 16, 18, 19) show more extensive editing at the *Rsa*I site. **(C)** The same PCR products as in (B) were cut with *Msp*I. As in (B) the same ten plants (lanes 1, 2, 3, 6, 7, 9, 10, 12, 15, 17) show less extensive editing, and the same nine plants (lanes 4, 5, 8, 11, 13, 14, 16, 18, 19) show more extensive editing at the two *Msp*I sites tested.

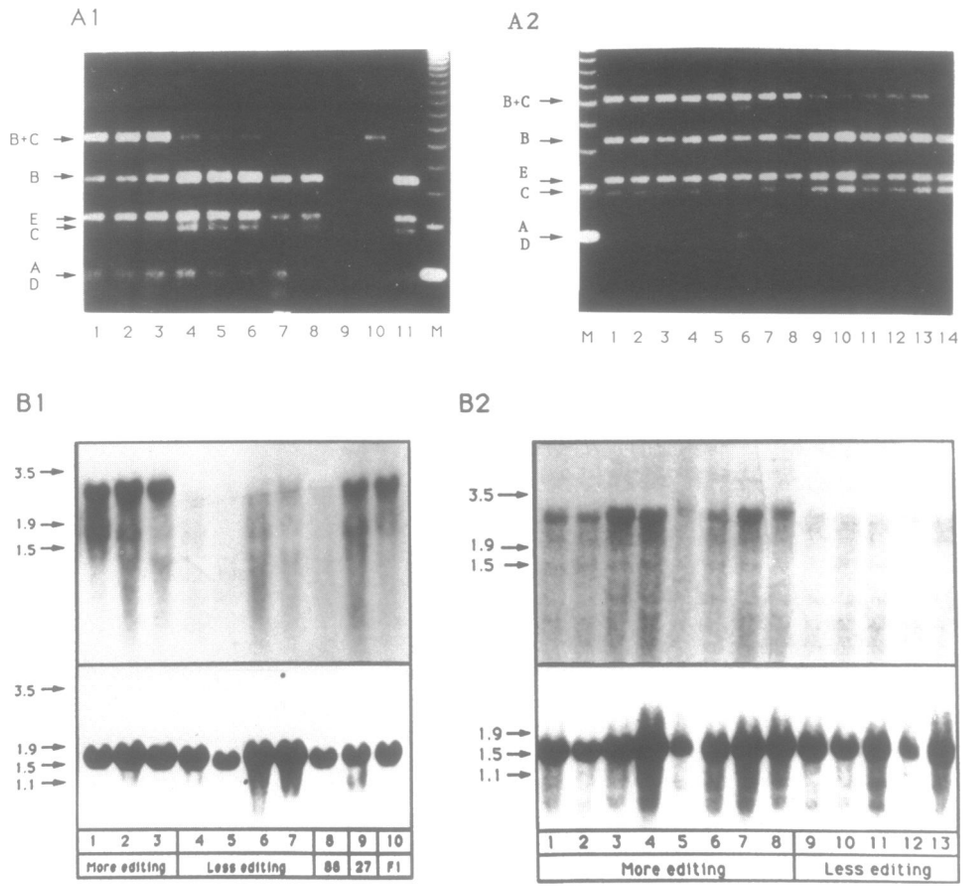

Figure 5. Parallel restriction analysis of RT/PCR amplification products and blot hybridization analysis of total RNAs from the backcross progeny and their parental lines. **(A1), (A2)** PCR products amplified from total RNAs of 20 individual plants of the backcross (A1, lanes 1−7; A2, lanes 1−13) and from total RNAs of 3688 (A1, lane 8), 11127 (A1, lane 9) and 3688×3704 (A1, lane 10) were digested with *Rsa*I. Amplification products of total genomic DNA were cut with *Rsa*I and run as controls (A1, lane 11; A2, lane 14). 123 bp DNA ladder was used as molecular weight marker. Arrows on the left indicate positions of *Rsa*I restriction fragments of the PCR products. See Fig. 1 for symbols. **(B1), (B2)** Hybridization analysis of total RNAs used in (A1), (A2). The same preparations of RNAs used in (A1), (A2) were used in (B1), (B2), respectively. RNAs were loaded in the same order as in (A1), (A2), size-fractionated on formaldehyde-agarose gels and transferred to nitrocellulose filters. Top: Filters hybridized with *nad3* gene probe. Bottom: After the *nad3* probe was stripped off, the same filters were rehybridized with an *atp6* cDNA probe. Arrows on the left indicate sizes (kb) of ribosomal RNA bands.

Correlation of editing extent and transcript abundance

One interpretation for the observed nuclear effect on RNA editing extent is that a nuclear gene is affecting *nad3* transcription rate or transcript turnover and therefore affecting editing indirectly. To test this possibility we did RNA blot analysis of *nad3* transcripts on individual progeny from the segregating backcross as well as from 3688, 11127 and F1 plants. In the *Petunia* CMS-encoding mitochondrial genome, the *nad3* gene and *rps12* (ribosomal protein S12) gene are cotranscribed with a *Petunia* CMS-associated fused (*pcf*) gene (28) (Fig. 1). The size of the cotranscript is about 2.8kb. When *nad3* gene was used as a probe, a major 2.8kb transcript was detected in 11127 leaves (Fig. 5B1, lane 9), while in 3688 leaves this transcript could barely be detected (Fig. 5B1, lane 8). In an F1 plant (Fig. 5B1, lane 10), as in 11127, the 2.8 Kb transcript was easily detected. Thus high transcript level is dominant to low transcript level. Though levels are lower, transcripts encompassing *nad3* are present in line 3688; cDNAs representing partially edited transcripts have been isolated and sequenced by R. Wilson (personal communication).

In the backcross, the transcript abundance is segregating. When the segregation patterns of transcript abundance and RNA editing extent are compared, there is an excellent correlation (Fig. 5, A1 vs. B1, A2 vs. B2). In all of the backcross progeny tested, this correlation holds true. The correlation was also evident when a portion of the *rps12* coding region was used as a probe (data not shown).

DISCUSSION

In higher plants, only a few nuclear genes that regulate mitochondrial gene expression have been reported (29,30). In this paper we describe a single nuclear locus that controls the transcript abundance and RNA editing extent of the *nad3* transcript in *Petunia* mitochondria. If a single nuclear gene is involved, then a 1:1 ratio of segregation is expected in the backcross, whereas if two nuclear genes are involved, a 3:1 ratio is expected. The ratio we found, 21:14 is statistically most consistent with a single locus hypothesis. At a 0.050 level of significance, the possibility of involvement of two or more nuclear genes is excluded. The excellent correlation of transcript abundance and RNA editing extent suggests that a single nuclear

gene is responsible for both events, although we can not exclude the less likely possibility that there are two tightly linked nuclear genes.

How could this single nuclear gene control both events? First, this nuclear gene might encode a factor that is directly involved in controlling *nad3* transcript abundance by regulating RNA stability. For example, line 11127 may contain such a nuclear-encoded factor so that *nad3* transcripts are stabilized and therefore have a greater chance to be fully edited by the editing activity. Perhaps 3688 contains little of this factor so that *nad3* transcripts are rapidly turned over before they are fully edited. Most of the transcripts that are amplified in 3688 may be editing intermediates that have not yet been degraded. This model is consistent with the concept that plant mitochondrial RNA editing is post-transcriptional (21).

Another interpretation of our observation is that RNA transcripts that are fully edited are more stable than unedited or partially edited transcripts, possibly due to their different secondary or tertiary structures. If so, then the nuclear gene we found might be directly involved in controlling editing efficiency of *nad3* transcripts by modulation of the mitochondrial RNA editing activity and thereby affecting transcript abundance indirectly. Because we observed that the nuclear gene affects *nad3* transcripts but not those of other genes that we analyzed, this possibility is less likely.

In mitochondria of another higher plant, *Oenothera*, *nad3* transcripts are also differentially edited (18). The three edit sites that are tested in this paper are also edited in *Oenothera*. These three editing events all change the encoded amino acid. At codon positions 49, 72 and 83, a UCC ser codon, a CCG pro codon and a CCU pro codon are edited to a UUC phe codon, CUG leu codon and UCU ser codon respectively, by C to U transitions. Therefore RNA editing at these three sites restores codons for conserved amino acids and possibly preserves functionality of the encoded peptide. However, the three sites are more completely edited in *Oenothera* than in *Petunia*. In *Oenothera*, two of the three edit sites were found to be completely edited (codon positions 49, 72), whereas the other site (codon position 83) was edited in 7 out of 8 cDNA clones sequenced (18). In *Petunia* CMS line 3688, less than 10% of the transcripts are edited at these three sites. It remains to be determined whether both unedited and partially edited *nad3* transcripts are translated *in vivo*. If that is the case, a population of isoforms of NAD3 peptides which might be functionally different would be generated. In this case, control of RNA editing extent could be a mechanism of regulating gene expression in plant mitochondria.

ACKNOWLEDGEMENTS

We thank Jay Calfee and Claudia Sutton for helpful discussions and Su Guo for help in manuscript preparation. This work was supported by the USDA NRI Genetic Mechanisms Program. Bingwei Lu was the recepient of a Cornell Graduate School A.D.White Fellowship.

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