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Patterns of partial RNA editing in mitochondrial genes of *Beta vulgaris*

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Abstract RNA editing is a process that modifies the information in transcripts of almost all angiosperm mitochondrial protein-coding genes. In order to determine the frequency and distribution of mitochondrial RNA editing in *Beta vulgaris*, cDNAs were sequenced and compared to the published genome sequence. 357 C to U conversions were identified across the 31 known protein genes and pseudogenes in *Beta*, the fewest so far for a plant mitochondrial genome. Editing patterns in the putative gene *orf518* indicate that it is most likely a functional *ccmC* homolog, indicating that patterns of editing can be a useful determinant of gene functionality. *orf518* also contains a triplicated repeat region whose members are nearly identical yet differentially edited, most likely due to differences in the sequence context of the editing sites. In addition, we show that partial editing in *Beta* is common at silent editing sites but rare at non-silent editing sites, extending previous observations to a complete plant mitochondrial genome. Finally, the degree of partial editing observed for certain genes was dependent on the choice of primers used, demonstrating that care must be taken when designing primers for use in editing studies.

Keywords *Beta vulgaris* · Plant mitochondria · RNA editing · Partial editing

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Introduction

RNA editing, a process that alters the information in a transcript from what is encoded by the genome, occurs in a variety of organisms through insertion, deletion, or modification of nucleotides in RNA (Brennicke et al. 1999; Gott and Emeson 2000). In plants, RNA editing proceeds via an unknown mechanism to convert C to U and U to C in organellar transcripts. Although the mechanism is unknown, the pattern and consequence of editing have been examined in great detail. In some cases, editing can generate new start and/or stop codons (Onodera et al. 1999; Kubo et al. 2000b), increase intron splicing efficiency (Borner et al. 1995), or improve tRNA base pairings in stem regions (Fey et al. 2001). However, most editing sites alter internal amino acids encoded by mRNA (Giege and Brennicke 1999). Editing at these sites usually leads to an increase in protein conservation across species (Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989). This tendency, along with the fact that editing generally increases the hydrophobicity of proteins (Giege and Brennicke 1999), indicates that these sites are important for the proteins to function properly in their

organellar roles. Conversely, silent editing, which has no effect on protein form or function owing to the degeneracy of the genetic code, is less common (Giege and Brennicke 1999) and where present, often occurs in only a subset of transcripts ["partial editing" (Schuster et al. 1990; Kempken et al. 1991)].

The presence of partially edited sites was first detected by direct cDNA sequencing (Gualberto et al. 1989) and soon after by sequencing of cDNA clones (Schuster et al. 1990). In the latter study, 5 out of 16 editing sites were partially edited in the *Oenothera nad3* gene. Interestingly, all three silent editing sites were partially edited and each was observed in only one of eight clones. In contrast, only 2 of 13 nonsilent editing sites were partially edited and they were found in 3 or 7 of 8 clones. This was the first indication that silent editing sites are less efficiently edited than nonsilent edits. Numerous subsequent studies have shown similar patterns, but relatively few have discussed this phenomenon (Kempken et al. 1991; Schuster et al. 1991; Kadowaki et al. 1995; Wilson and Hanson 1996) and none have tested this across a complete genome. Other studies have found a high degree of partial editing at both silent and nonsilent sites. In these cases, the incompletely edited transcripts appear to be intermediates that become more fully edited during maturation (Gualberto et al. 1991; Sutton et al. 1991; Yang and Mulligan 1991).

To date, RNA editing content has been assessed for all known protein genes in the mitochondrial genomes of the angiosperms *Arabidopsis thaliana* (Giege and Brennicke 1999), *Brassica napus* (Handa 2003), and *Oryza sativa* (Notsu et al. 2002), and evidence suggests that the liverwort *Marchantia polymorpha* neither performs nor requires RNA editing (Oda et al. 1992; Ohyama et al. 1993; Steinhauser et al. 1999). To gain a better perspective on the pattern of RNA editing in angiosperms, we have now determined the extent of editing in the mitochondrial genome of the sugar beet *Beta vulgaris*. In particular, we find that partial editing is much more common at silent sites for almost all protein genes. Furthermore, we show that the examination of partial editing patterns can be useful for determining gene functionality, understanding editing site specificity, and evaluating the effect of primer choice on editing site identification.

Materials and methods

Seeds from cytoplasmic male-fertile *B. vulgaris* (line TK81-O) were obtained from the National Agricultural Research Center for Hokkaido Region (Hokkaido, Japan). Seedlings were grown for 2 weeks at 20°C in 16 h of light and 8 h of darkness.

Total RNA was isolated from whole tissue with the RNeasy Plant Mini Kit (QIAGEN) and then treated with DNase I (Promega) for 30 min at 37°C to remove any contaminating genomic DNA. DNase I was removed by treating with chloroform:isoamyl alcohol (24:1) and RNA was recovered by ethanol precipitation. cDNA was synthesized using M-MulV reverse transcriptase (New England Biolabs) and random hexamers according to manufacturer's instructions. Gene-specific cDNA products were amplified by polymerase chain reaction (PCR) using primers (Table S1) designed in the 5′ and 3′ untranslated regions (UTR) of each gene. Each reaction was performed using 35 cycles of 45 s at 94°C, 45 s at 50°C, and 2.0–2.5 min at 72°C, with an initial step of 3 min at 94°C and a final step of 10 min at 72°C. No amplification was observed when a cDNA synthesis reaction that excluded reverse transcriptase was used as a template, indicating that there was no contaminating genomic DNA in the cDNA preparation. Amplified cDNA products were purified with the QIAquick PCR Purification Kit (QIAGEN) and then directly sequenced on both strands at the Indiana Molecular Biology Institute. In addition, some products were cloned using the TOPO TA Cloning Kit (Invitrogen). Colonies were screened following the colony PCR protocol provided in the TOPO TA Cloning Kit manual, and positive clones were sequenced.

Sites of RNA editing were determined by comparison of cDNA sequences to the published mitochondrial genome for TK81-O (Kubo et al. 2000a). Sites were scored as partially edited when peaks for both T and C were present and clearly above background in both strands of the directly sequenced cDNA. This approach likely underrepresents the true number of partially edited sites, because sites that are mostly edited (in more than 80–90% of transcripts) will be scored as fully edited and sites that are rarely edited (in fewer than 10–20% of transcripts) will be scored as unedited. Nevertheless, the direct sequence approach used here is preferred over sequencing multiple cDNA clones for three reasons. The latter approach requires a large amount of additional sequencing, cannot accurately determine the degree of partiality unless a large number of clones are analyzed, and will introduce nucleotide changes during the cloning process that can be erroneously scored as rare editing events. Sites of RNA editing in the mitochondrial genomes of *Arabidopsis thaliana* (GenBank: Y08501, Y08502), *Brassica napus* (GenBank: AP006444), and *Oryza sativa*

(GenBank: BA000029) were taken from annotations in these GenBank files.

Results and discussion

RNA editing in *B. vulgaris* mitochondrial genes and pseudogenes

The complete mitochondrial genome of male-fertile *B. vulgaris* contains 31 known protein genes, including the putative pseudogenes *ccmC* and *sdh4* (Kubo et al. 2000a; Siqueira et al. 2002). Sites of RNA editing have been determined for eight of these genes (Kubo et al. 1993; Senda et al. 1993; Kubo and Mikami 1996; Estiati et al. 1998; Onodera et al. 1999; Kubo et al. 2000b; Itchoda et al. 2002). In order to obtain a more complete picture of the frequency and distribution of RNA editing in this genome, an analysis of RNA editing was undertaken for the remaining 23 genes and pseudogenes. In total, 357 edited sites were found across the 31 genes and pseudogenes in the *Beta* mitochondrial genome (Table 1), the lowest number of editing sites reported so far for a plant mitochondrial genome. Six additional editing sites were identified in UTR regions. All editing changes are C to U conversions. As described previously, RNA editing generates new start codons for *atp6, nad1, and nad4L* and new stop codons for *atp6* and *atp9* (Onodera et al. 1999; Kubo et al. 2000a; Kubo et al. 2000b). No additional start or stop codons are created from editing.

In *Beta*, the frequency of editing is not consistent across genes, ranging from 0% (0 sites in 1,575 bp) for *cox1* to 4.8% (30 sites in only 621 bp) for *ccmB* (Table 1). Similar inconsistencies in editing frequency as a function of gene length are also observed in *Arabidopsis, Brassica, and Oryza* (Table S2). However, the frequency of editing does seem to be fairly consistent for each gene across species; genes that are frequently edited in *Beta* are also frequently edited in *Arabidopsis, Brassica, and Oryza* (e.g. *ccmB* and *ccmC*), while rarely edited *Beta* genes are also rarely edited in the other species (e.g. *atp1* and *cox1*). There are some obvious differences as well, evidenced by the large standard deviations relative to the mean for some genes, which may be indicative of a history of retro-processing (reverse transcription of edited mRNA and reinsertion into the genome). Consistent with its low level of RNA editing overall, *Beta* has the lowest frequency of editing for 16 of 30 genes, while the frequency is highest for only five genes. The similar patterns of editing in homologous genes from *Beta, Arabidopsis, Brassica*, and *Oryza* indicate that editing

frequencies have remained relatively stable throughout angiosperm history. It further suggests that, barring retroprocessing, these patterns are likely to hold for most, if not all angiosperms and possibly for other plant groups as well.

RNA editing as a determinant of gene functionality

The *ccmC* gene was initially reported to be a pseudogene in the mitochondrial genome of *Beta* (Kubo et al. 2000a), and this assessment has been perpetuated in comparisons of mitochondrial gene content across species (Notsu et al. 2002; Handa 2003; Turmel et al. 2003; Clifton et al. 2004; Sugiyama et al. 2005). It seems that the pseudogene status was originally assigned based on the absence of a start codon at the same position as in other plants (Satoh et al. 2004). However, because the *ccmC* pseudogene does not contain any stop codons, Satoh et al. (2004) renamed it as *orf518* and *orf496* in male-fertile and male-sterile *Beta* mitochondria, respectively. The 3′ halves of *orf518* and *orf496* are homologous to *ccmC* from other species, and the 5′ ends contain a fragment of *atp9* (Fig. 1a; Satoh et al. 2004).

We designed primers flanking the entire *orf518* gene and verified that it is edited at 28 sites in the *ccmC*-homologous region (Table 1, Fig. 1a). Editing patterns indicate that the pseudogene designation is not warranted. Only 6 of the 28 editing sites are silent, consistent with the low levels of silent editing observed across angiosperm mitochondrial genes. Furthermore, all 22 nonsilent edits increase the similarity of the predicted protein to CCMC proteins from other angiosperms (data not shown); such conservation is a hallmark of RNA editing in functional genes but not necessarily pseudogenes (see below). Finally, all 22 nonsilent editing sites are fully edited, whereas 4 of 6 silent editing sites are partially edited. The dichotomous frequency of partial editing at nonsilent vs. silent editing sites found in the *ccmC*-homologous region mirrors the pattern observed across the other functional genes in the *Beta* mitochondrial genome. Taken together, these results indicate that *orf518* is an intact and likely functional *ccmC* homolog in *Beta*, and they demonstrate that patterns of editing can provide important clues regarding the functionality of plant mitochondrial genes.

If *ccmC* in *Beta* is indeed functional, it is not clear where translation starts since it does not share the position of the start codon found in other plants. However, five other ATG codons could serve as the translational start site (Fig. 1a), all of which are found in both male-fertile and male-sterile *Beta* (an in-frame

Table 1 RNA editing in *Beta vulgaris* line TK81-O

Gene	Coding length (bp)	Editing sites	Nonsilent edits		Silent edits		Source
			Total	Partial[a]	Total	Partial[a]	
Complex I							
nad1	978	20	18	0	2	0	This study
nad2	1,467	24	20	0	4	1	This study
nad3	357	12	10	0	2	1	This study
nad4	1,488	19	17	NR	2	NR	Itchoda et al. (2002)
nad4L	303	10	10	1	0	0	Kubo et al. (2000b)
nad5	2,013	17	17	0	0	0	This study
nad6	618	11	9	0	2	2	This study
nad7	1,176	20	20	0	0	0	This study
nad9	579	5	5	NR	0	0	Kubo et al. (1993)
Complex II							
sdh4-ψ[b]	305	4	2	2	2	2	This study
Complex III							
cob	1,182	13	10	0	3	3	This study
Complex IV							
cox1	1,575	0	0	0	0	0	This study
cox2	783	9	8	0	1	0	This study
cox3	798	4	2	0	2	2	This study
Complex V							
atp1	1,521	3	3	NR	0	0	Senda et al. (1993)
atp4	597	12	10	0	2	2	Kubo et al. (2000b)
atp6	774	12	12	0	0	0	Onodera et al. (1999)
atp8	489	2	1	0	1	1	Kubo and Mikami (1996)
atp9	225	5	4	0	1	0	This study
Cytochrome c biogenesis							
ccmB	621	30	24	0	6	2	This study
ccmC[c]	687	28	22	0	6	4	This study
ccmFc	1,317	13	11	0	2	2	This study
ccmFn	1,734	23	22	2	1	0	This study
Ribosomal proteins							
rpl5	561	5	5	0	0	0	This study
rps3	1,656	6	6	0	0	0	This study
rps4	948	11	10	0	1	1	This study
rps7	447	3	2	0	1	0	Estiati et al. (1998)
rps12	378	6	6	0	0	0	This study
rps13	351	2	1	0	1	1	This study
Other							
MatR	1,968	9	8	3	1	0	This study
MttB	831	19	19	15	0	0	This study
Total	28,727	357	314	23	43	24	

NR, partial editing information was not reported in cited reference

[a] For consistency between methodologies, editing sites from cited references were scored as partial if found to be edited in less than 80% of clones (see methods)

[b] Silent and nonsilent editing sites in *sdh4* were scored after correcting for the frameshift

[c] Only includes the *ccmC*-homologous region from *orf518* (see Fig. 1 and text)

stop codon upstream of the first putative start codon precludes any additional possibilities). Of these possibilities, the most likely candidates seem to be the second and the fifth start site. The second putative start codon is in the duplicated *atp9* region, and is in fact homologous to the start codon for *atp9*. Thus, *ccmC* in *Beta* may have co-opted the promoter and ribosomal binding site of *atp9*. The fifth putative start codon is found only 11-13 codons downstream from the reported start codons for *Arabidopsis*, *Brassica*, *Pisum*, *Oenothera*, *Oryza*, *Triticum*, *Zea*, and *Marchantia* (data not shown). Interestingly, the above species, as
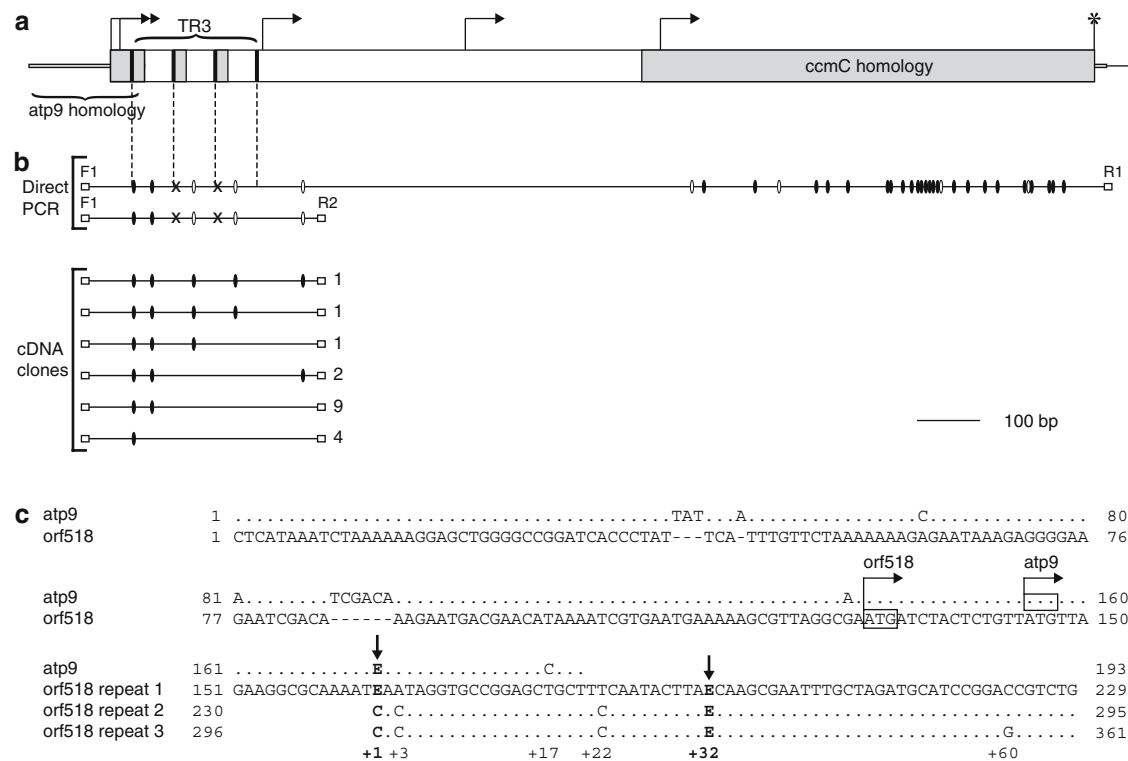
Fig. 1 The *orf518* gene in *Beta vulgaris*. **a** Structure of the *orf518* gene. *Shaded areas* indicate regions homologous to known mitochondrial genes. Possible translational start codons are indicated by *arrows*; the second start codon is homologous to the start site of *atp9*. Stop codon is indicated by a *star*. The three tandem repeats that comprise the TR3 region are bracketed. **b** RNA editing in *orf518*. RNA editing in the entire *orf518* gene and in the TR3 region was analyzed by direct sequencing of amplified cDNA. *Thin horizontal lines* indicate the sizes of the analyzed cDNA products. Primers positions are indicated by *open boxes*. *Open* and *closed ovals* represent partially and fully edited sites, respectively. Unedited sites in the TR3 region are marked by the letter X. RNA editing in the TR3 region was also analyzed by sequencing 18 clones. The number of times each clone type was found is indicated to the right of each type. Nucleotide changes observed in only a single clone are not shown due to the possibility that they were introduced during PCR and/or cloning. **c** Nucleotide sequence of TR3 and the *atp9*-homologous region. Nucleotide states for all sequences are shown in editing columns (marked by *arrows*); for all other columns, nucleotides identical with *orf518* or the *orf518* repeat 1 sequence are plotted as *periods*. Columns of interest within the repeat region are numbered according to their position in the repeat. Edited cytidines are represented by the letter *E*; frequency of editing at each site is shown in (**b**). Putative start codons are *boxed*. *orf518* begins at the first start codon, while *atp9* begins at the second start codon

well as the green alga *Chara* and the jakobid *Reclinomonas*, also code for a methionine at this downstream position, raising the possibility that it may actually serve as the translational start site for all of these species.

The *sdh4* gene was originally described as absent from the mitochondrial genome of *Beta* (Kubo et al. 2000a). However, it was later shown to be present as a pseudogene, containing a 5 bp insertion that creates multiple premature stop codons (Siqueira et al. 2002). We found *sdh4* to be transcribed and edited at four positions (Table 1). All four editing sites are downstream of the first introduced stop codon and are partially edited. After correcting for the frameshift caused by the 5 bp insertion, two editing sites are silent while the other two are nonsilent and increase protein simi-

larity to homologous proteins from other species (data not shown). The two nonsilent editing sites are also found in *Lycopersicon esculentum* and *Solanum tuberosum* (Adams et al. 2001; Siqueira et al. 2002).

The relaxation of constraints on editing sites appears to be a common phenomenon for mitochondrial pseudogenes. Similar to *Beta sdh4*, the *rps14* pseudogene from *Triticum* contains one conserved nonsilent editing site that is only partially edited (Sandoval et al. 2004). In some pseudogenes, such as *rps12* from *Oenothera* and *rps14* from *Solanum*, an evolutionarily conserved editing site has been completely lost (Grohmann et al. 1992; Quinones et al. 1996). The most spectacular example of relaxed editing constraints in pseudogenes comes from *Oenothera rps19*, which shows not only two losses of editing sites that

increase protein conservation, but also five gains of editing sites that decrease protein conservation (Schuster and Brennicke 1991). For a number of genes, unusual editing patterns are the only indication that they are not functional (Mundel and Schuster 1996; Handa 2003; Mower 2005). Although these genes are intact, in-frame, and transcribed, they have lost editing at evolutionarily conserved positions, suggesting pseudogenization of their mitochondrial copies and the presence of functional copies in the nucleus.

Differential editing in a triplicated repeat region

Within the *Beta* mitochondrial genome are a number of VNTR (variable number of tandem repeats) regions (Nishizawa et al. 2000). One such VNTR, TR3, is 66 bp in length and is repeated, nearly perfectly, three times near the 5′ end of *orf518* in male-fertile *Beta* (Fig. 1a; Satoh et al. 2004). The first 20 bp of each repeat is the 3′ end of the partial *atp9* duplication found at the beginning of *orf518*. During our investigation of the extent of RNA editing in the *orf518* gene, we were surprised to find differential editing among the three copies of this tandem repeat region. To explore this result in more detail, we generated cDNA covering the entire TR3 region and analyzed editing in 18 clones (Fig. 1b). The first nucleotide in each repeat is a cytidine that is homologous to a fully edited cytidine in the native *atp9* gene. This site is also found to be fully edited (18/18 clones) in the duplicated *atp9* region of the first repeat, but is completely unedited (0/18) in the second and third repeats. A second editing site is located at position 32 in each repeat and is usually edited (14/18) in the first repeat but rarely edited in the second and third repeats (3/18 and 2/18, respectively).

The differential editing of the first editing site is almost certainly the result of the different flanking sequences in repeat 1 versus repeats 2 and 3 (Fig. 1c). The upstream sequence of the first editing site in repeat 1 is identical for 35 bp to the upstream sequence of the homologous *atp9* editing site, and both sites are fully edited. In contrast, the upstream region in repeats 2 and 3 is the end of the previous repeat. Since these editing sites share none of the homology with the upstream *atp9* sequence and are completely unedited, it seems likely that site specificity is contained in the upstream *atp9* region. It is also possible that the downstream sequence has an effect on conferring specificity. Repeats 2 and 3 have a C only 2 bp downstream of the editing site, while repeat 1 and *atp9* have an A at this same position. These findings are consistent with in vitro and *in organello* RNA editing assays that have shown that editing site specificity lies in the sequences immediately flanking editing sites (Choury et al. 2004; Neuwirt et al. 2005).

For the second editing site, the difference in editing frequency is most likely due to the single nucleotide difference found 10 bp upstream of the editing site (Fig. 1c). Repeat 1 has a T at this position, whereas repeats 2 and 3 have a C. A second difference between repeat 1 and repeats 2 and 3 lies 29 bp upstream and may also contribute to the observed frequency differential, although this difference may be too far from the editing site to have an effect. A downstream difference, at position 60, is unique to repeat 3 and thus cannot account for the differential editing in repeat 1 versus repeats 2 and 3. The fact that only one or two changes in the sequence surrounding this editing site can have such a large effect on the frequency of editing indicates that the editing machinery is highly sensitive to specific sequence context.

Abundance of partial editing at silent editing sites

In *Beta*, the proportion of partial editing at silent and nonsilent editing sites is highly biased (Table 1). In total, 47 out of 330 editing sites are partially edited (editing sites for *atp1, nad4, and nad9* were not counted since partial editing information was not reported for these genes). Of the 41 silent editing sites for which partial editing data are available, 24 (58.5%) are partially edited. In contrast, only 23 of the 289 nonsilent editing sites (8.0%) are partially edited. Most of these partially edited nonsilent sites occur in three genes: *mttB, matR, and sdh4*. For all other genes, partial editing at nonsilent editing sites is extremely rare. If partially edited sites were distributed without regard to their effect on the amino acids produced, then we would expect 5.8 silent (= $41 \times 47/330$) and 41.2 nonsilent (= $289 \times 47/330$) editing sites to be partially edited. The observed numbers of partially edited silent and nonsilent sites differ significantly from random expectations ($P = 9.7e^{-16}$; $X^2 = 64.5$; d.f. = 1).

Thus, the nonrandom distribution of partial editing extends to most, if not all (see next section) genes in the mitochondrial genome of *Beta* (Table 1). The predominance of partial editing at many silent sites may simply represent an inefficiency of editing at these sites, with translation proceeding irrespective of their editing status. Alternatively, partially edited silent sites, despite having no effect on protein sequence, may nevertheless serve a functional role. For example, editing of these sites may be one of the final steps in mRNA maturation, signaling that the transcript is ready for translation.
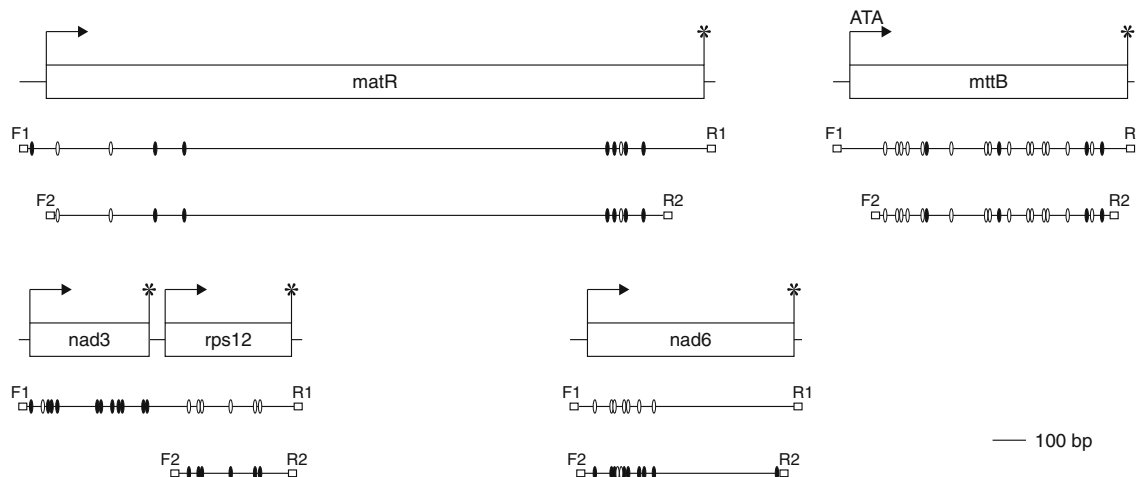
Fig. 2 Effect of primer choice on editing site detection. Symbol usage follows that of Fig.1. RNA editing in all four transcripts was analyzed by direct sequencing of amplified cDNA. The upper cDNA sequence for each gene was the result of the initial analysis. The lower cDNA sequence was the result of the reanalysis using internal primers that were designed in regions that lack editing. The *mttB* gene is shown starting at an ATA codon based on genomic annotations (GenBank: BA000009)

Effect of primer choice on RNA editing site detection

Initial assessment of partial editing in *Beta* mitochondrial genes revealed four genes (*matR, nad6, mttB, rps12*) for which many editing sites appeared to be partially edited (Fig. 2). Because the cDNA primers were designed based on the genomic sequence, it is possible that we preferentially selected for editing intermediates during PCR. This preferential amplification could occur if editing sites are located in the primer binding sites, which would create mismatches between the primer and edited transcripts but not unedited or incompletely edited transcripts. It is also possible that processing of these transcripts during maturation may proceed into or beyond the primer binding sites (which are in the UTR regions); thus, only immature transcripts would be amplified.

To eliminate these possibilities, we reevaluated the editing patterns of these four genes (Fig. 2) using an internal set of primers designed in regions that are free of editing. In our initial analysis of editing in *nad6*, seven editing sites were identified and all were partially edited. Upon reanalysis, however, all previously identified sites are fully edited. In addition, four new editing sites were found, two of which are silent and only partially edited. For *rps12*, we initially evaluated the entire *nad3/rps12* cotranscription product and observed six partially edited sites in *rps12* (*nad3*, on the other hand, was partially edited at only one silent editing site). Using new primers, all six editing sites in *rps12* are fully edited. This suggests that the primers initially used for *nad6* and *rps12* were selecting for incompletely edited transcripts, and implies that primer choice is critical in studies on RNA editing.

In contrast, the frequency and completeness of editing did not change upon reevaluation of *matR* and *mttB* (Fig. 2). It is not clear why *matR* and *mttB* continue to have such a high degree of partial editing at nonsilent sites. Partial editing seems to be the rule for these two genes, as this is observed at many sites in *matR* from *Glycine, Solanum, Triticum,* and *Zea* (Thomson et al. 1994; Begu et al. 1998) and in *Arabidopsis mttB* (Sunkel et al. 1994). Although little is known about the actual mitochondrial function of *matR* and *mttB*, they are most likely essential because they have not been lost from the mitochondrial genome in any of the 280 angiosperms examined (Adams et al. 2002) and because they are intact and in-frame in all angiosperms from which they've been isolated. Interestingly, both of these genes have uncertain translational start sites for many angiosperms (Sunkel et al. 1994; Thomson et al. 1994; Begu et al. 1998). Although non-canonical start sites may be used, it is possible that further maturation steps (e.g. processing or trans-splicing) are required. Thus, the high degree of partial editing for *matR* and *mttB* may also be due to amplification of immature transcripts. However, it remains a possibility that *matR* and *mttB* are in fact pseudogenes that have simply not acquired the usual identifying characteristics, such as in-frame stop codons or frameshifting indels.

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