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In vivo dissection of *cis*-acting determinants for plastid RNA editing

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Hans Kössel passed away on December 24, 1995. We mourn the loss of a fatherly friend and a great figure in contemporary science. We dedicate this paper to his memory and to his friends all over the world.

Substitutional RNA editing changes single C nucleotides in higher plant chloroplast transcripts into U residues. To determine the *cis*-acting sequence elements involved in plastid RNA editing, we constructed a series of chloroplast transformation vectors harboring selected editing sites of the tobacco *ndhB* transcript in a chimeric context. The constructs were inserted into the tobacco plastid genome by biolistic transformation leading to the production of stable chimeric RNAs. Analysis of RNA editing revealed unexpected differences in the size of the essential *cis* elements or in their distance from the editing site. Flanking sequences of identical size direct virtually complete editing for one pair of editing sites, partial editing for a second and no editing at all for a third pair of sites. Serial 5′ and 3′ deletions allowed us to define the *cis*-acting elements more precisely and to identify a sequence element essential for editing site recognition. In addition, a single nucleotide substitution immediately upstream of an editing position was introduced. This mutation was found drastically and selectively to reduce the editing efficiency of the downstream editing site, demonstrating that position –1 is important for either site recognition or catalysis. Our results indicate that the editing of adjacent sites is likely to be mechanistically coupled. In no case did the presence in the plastome of the additional editing sites have any effect on the editing efficiency of the endogenous *ndhB* sites, indicating that the availability of site-specific *trans*-acting factors is not rate limiting.

Keywords: chloroplast/*ndhB*/plastid transformation/RNA editing/RNA processing

Introduction

Post-transcriptional alterations of single nucleotides within an mRNA have been described for a variety of genetic systems, including higher plant mitochondria (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989) and chloroplasts (Hoch *et al.*, 1991; Kudla *et al.*, 1992; for review, see Kössel *et al.*, 1993). This phenomenon was termed RNA editing and added a novel and unexpected step to the multitude of known transcript maturation processes. RNA editing in different systems involves widely different mechanisms, implying that editing may have evolved several times independently. The editing systems known to date can be roughly classified into insertional and substitutional RNA editing. The insertional type of editing is best known from kinetoplastid mitochondria of trypanosomes where uridine insertions or deletions occur (Benne *et al.*, 1986; for review, see for example, Simpson *et al.*, 1993). RNA editing in plant organelles exemplifies the substitutional type of editing and involves mainly C to U transitions with the exception of few reverse events detected in mitochondrial transcripts (Gualberto *et al.*, 1990; Schuster *et al.*, 1990). The vast majority of chloroplast editing events lead either to the restoration of evolutionarily conserved amino acid residues (Maier *et al.*, 1992a,b) or to the creation of functional initiation codons for translation (Hoch *et al.*, 1991; Kudla *et al.*, 1992). The biological significance of plastid editing was demonstrated directly by incorporation of a heterologous RNA editing site into the tobacco *psbF* gene. The failure to edit this site *in vivo* resulted in a mutant, i.e. photosynthetically deficient, phenotype (Bock *et al.*, 1994). Plastid RNA editing is a very early RNA processing step (Freyer *et al.*, 1993; Ruf *et al.*, 1994) and was shown to be entirely independent of chloroplast translation (Zeltz *et al.*, 1993).

It is currently unknown how a specific cytosine residue within an mRNA is selected for modification. Even though a limited number of editing sites share common sequence motifs (Maier *et al.*, 1992b), no general consensus sequence can be deduced from aligning heterologous sites, thus implying the existence of *trans*-acting specificity factors for individual editing sites. A recent *in vivo* study has substantiated this supposition further: introduction of additional copies of the tobacco *psbL* editing site led to a decrease in the editing efficiency of the endogenous *psbL* mRNA whereas other plastid editing sites remained unaffected (Chaudhuri *et al.*, 1995). It seems reasonable to assume that the sequences immediately surrounding the nucleotide to be edited participate in the recognition of an editing site by such a specificity factor. In this study, we attempted to identify these *cis*-acting elements for selected editing sites of the tobacco *ndhB* transcript and to define a minimum sequence context which is necessary and sufficient to direct editing.

Results

Integration of three pairs of ndhB editing sites into the tobacco plastid genome

The tobacco plastid genome contains in its inverted repeats two identical copies of the *ndhB* gene encoding a subunit of a putative chloroplast NADH dehydrogenase (Matsubayashi *et al.*, 1987). The *ndhB*-encoded transcript

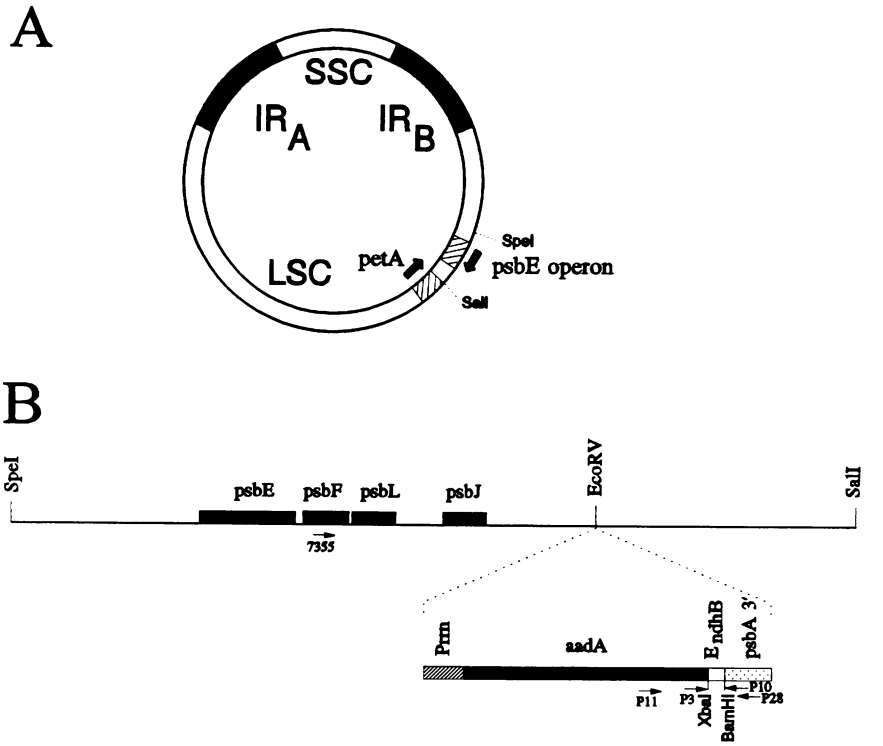

Fig. 1. Insertion of *ndhB* gene segments containing RNA editing sites into the tobacco plastid genome. (**A**) Position of the targeting site for insertion of the chimeric *aadA* gene constructs within the large single copy region (LSC) of the tobacco chloroplast genome. A schematic representation of the circular plastid DNA molecule is shown with its two inverted repeats (IR_A and IR_B) separating the large single copy region from the small single copy region (SSC). The transgenes were inserted into the intergenic spacer between the *psbE* operon and the *petA* gene. The direction of transcription is indicated by arrows. Restriction sites used for construction of the transformation vectors are marked. (**B**) Map of the plastid DNA fragment in the transformation vectors pRB52–pRB66. The chimeric *aadA* gene is ligated into a unique *Eco*RV site downstream of the *psbJ* gene, the last cistron of the *psbE* operon. The *ndhB* segments are inserted as *Xba*I–*Bam*HI fragments in between the *aadA* coding region and the *psbA* 3′ untranslated region which confers transcript stability (Stern and Gruissem, 1987). Prrn, chimeric plastid rRNA operon promoter (Svab and Maliga, 1993); E_{ndhB}, *ndhB* segment carrying two RNA editing sites. The location and orientation of oligonucleotide primers derived from vector sequences are indicated by horizontal arrows.

has been shown to undergo numerous changes by RNA editing. It contains six editing sites in maize (Maier *et al.*, 1992b), nine sites in tobacco (Freyer *et al.*, 1995) and is thus probably the most frequently edited mRNA encoded in the plastid genome (Maier *et al.*, 1995). Interestingly, six of the tobacco editing sites are grouped in three pairs where the two sites of a pair can even share common sequence motifs (Figure 2).This raises the attractive possibility that they are edited by common specificity factors or that the editing of closely adjacent sites is mechanistically coupled. We chose these three pairs of *ndhB* editing sites for a set of experiments aimed at determining the *cis*-acting sequence requirements for plastid RNA editing.

The development of a transformation technology for higher plant chloroplasts (Svab *et al.*, 1990; Svab and Maliga, 1993) has facilitated the study of virtually all aspects of plastid gene expression *in vivo*. Recently, this technique was exploited to realize the interspecific transfer of a plastid RNA editing site (Bock *et al.*, 1994) as well as to make the expression of resistance genes dependent on RNA editing (Chaudhuri *et al.*, 1995). In the absence of efficient *in vitro* editing assays, chloroplast transformation is also the method of choice for the study of the *cis* elements involved in editing site selection.

An intergenic spacer region of the tobacco plastid genome known to be a suitable target site for transgene insertion (Bock *et al.*, 1994) was chosen for the incorporation of small *ndhB* segments containing a pair of editing sites (Figure 1A and B). The segments were linked to a selectable marker gene (*aadA*) conferring resistance to spectinomycin (Svab and Maliga, 1993) as follows: the *aadA* gene was modified by inserting a minilinker in between the coding region and the downstream transcript stabilizing sequence derived from the *psbA* 3′ untranslated region (Figure 1B). The minilinker contains an *Xba*I restriction site, part of which is the *aadA* translation termination codon, and a downstream *Bam*HI site. Small *ndhB* fragments containing a pair of editing sites were prepared by PCR amplification of the respective genomic sequences simultaneously introducing a 5′ *Xba*I site and a 3′ *Bam*HI site. Any such fragment can be inserted into the *Xba*I–*Bam*HI minilinker of the *aadA* gene and yields, in a single cloning step, the final transformation vector. Furthermore, this strategy ensures 100% linkage of the selectable marker with the *ndhB* gene segment since the two transgenes are not separated by plastid genome-derived sequences which could be a target for unwanted homologous recombination. Owing to the incorporation of the *ndhB* fragments downstream of the *aadA* stop codon, the *ndhB* sequences will remain untranslated in this context. They will be co-transcribed with the *aadA* and thus can be viewed as a second RNA gene-like cistron of a dicistronic operon. The lack of translation is not expected to have any influence on transcript editing since RNA editing has been demonstrated to be entirely independent of chloroplast translation (Zeltz *et al.*, 1993).

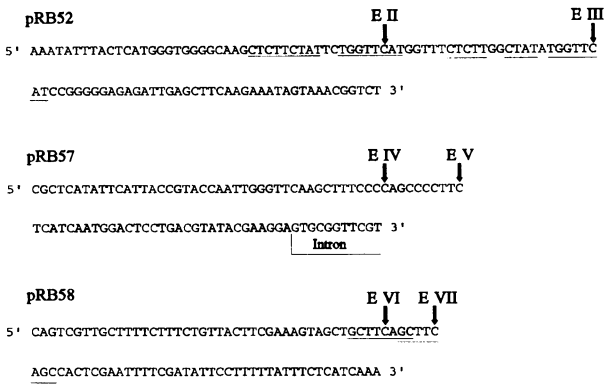

Fig. 2. Sequences adjacent to the three pairs of *ndhB* RNA editing sites chosen for this study. The sequences shown reflect the insertions in the plastid transformation vectors pRB52, pRB57 and pRB58, respectively. Each of the sequences spans an *ndhB* segment from –42 with respect to the 5′ editing site to +42 with respect to the 3′ editing site of the pair. The total size of the three fragments is not identical owing to different spacing between the two sites. Editing sites are marked by vertical arrows. Sites IV and V are located close to the intervening sequence interrupting the *ndhB* reading frame, the intron–exon boundary is marked. Repetitive sequences observed for editing sites II/III and VI/VII (Freyer *et al.*, 1995) are underlined.

The first three constructs (pRB52, pRB57 and pRB58) carried *ndhB* segments with the editing sites II/III, IV/V and VI/VII, respectively. To allow for direct comparison, each of the segments contains a sequence from –42 with respect to the 5′ editing site to +42 with respect to the 3′ editing site of the pair. The total size of the three fragments, however, differs slightly due to small differences in spacing between the two sites (Figure 2).

The chimeric *aadA–ndhB* genes were introduced into the tobacco plastid genome by biolistic bombardment of sterile leaves with plasmid DNA-coated tungsten particles and subsequent selection for spectinomycin resistance. Plastid transformants were identified by a PCR-based assay which simultaneously verified correct integration into the *psbJ–petA* intergenic spacer (Figure 3). Transplastomic lines were purified to homoplasmy by typically two to four additional rounds of regeneration under selective conditions. The presence of a homogeneous population of transformed plastid DNA molecules was confirmed by a very sensitive PCR assay that highly favors the amplification of residual wild-type genome copies due to their smaller size (Figure 3). For each of the constructs, three independently transformed lines were studied further. However, in no case was any difference with respect to RNA editing detected among different transplastomic lines resulting from transformation with one and the same vector plasmid.

RNA accumulation and editing in transplastomic tobacco lines

Editing of the three pairs of *ndhB* editing sites is virtually complete in wild-type tobacco. To test if editing of the *ndhB* sequences occurs also in the chimeric context, cDNAs were amplified with the primer pair P10–P11 (Figure 1B) and sequenced directly. The sequence analysis revealed no detectable editing activity at sites II and III in the Nt-pRB52 transplastomic lines (Figure 4). Sites IV and V in the Nt-pRB57 lines, however, are edited efficiently. As in wild-type *ndhB* transcripts, the C→U transition

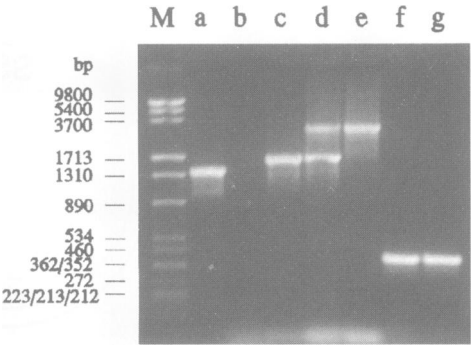

Fig. 3. PCR strategy to identify and analyze chloroplast transformants. The example shown describes the characterization of two spectinomycin-resistant clones obtained from a transformation experiment with vector pRB62. Primary transformants were detected by an amplification reaction using primer pair P11–1020. P11 binds within the *aadA* coding region (Figure 1B). Thus only lines carrying the transgene yield a PCR product of ~1420 bp (lane a). Primer 1020 anneals within the *petA* gene (Figure 1A) which was not part of the transformation vector (Figure 1B). Hence, this amplification reaction also tests for correct integration into the chloroplast genome. Lane b shows the result of the same PCR reaction for a second spectinomycin-resistant clone which is likely to be a spontaneous resistance mutant. Homoplasmic transformed lines were identified by amplification reactions with the primer pair 7355–1020 (lanes c–e). Amplification of wild-type DNA gives rise to a product of ~1.7 kb (lane c), amplification of transformed DNA molecules yields a product of ~3 kb owing to the insertion of the chimeric *aadA* gene. Lane d, PCR products obtained for a heteroplasmic transformant (after the second round of regeneration on spectinomycin-containing medium); lane e, product obtained for a homoplasmic transformant (after the third round of regeneration). Lanes f and g show PCR reactions with the primer pair P10–P11 (Figure 1B) used to generate the substrate for the DNA and cDNA sequencing reactions: lane f, amplified DNA and lane g, amplified cDNA from the homoplasmic plastid transformant. M, molecular weight marker.

is nearly complete (Figure 4). Editing was also detected for pRB58-derived transformants carrying sites VI and VII, although in this case editing is clearly incomplete.

The results of the quantitation for the three pairs of *ndhB* sites are summarized in Table I. Interestingly, the editing efficiency is identical in all three cases for the two sites of a pair. In view of the identical size of both the upstream and downstream sequence environment (–42 to +42; Figure 2), the large differences in editing between the three pairs of sites are rather surprising. This finding may indicate that the *cis*-acting signals for editing vary in either size or distance from the editing site. As an alternative explanation, possible differences in RNA turnover were considered. If one assumes that insertion of the *ndhB* segments downstream of the *aadA* coding region (Figure 1B) affects transcript stability, it seems possible that rapid mRNA degradation in the case of the Nt-pRB52 and Nt-pRB58 lines leads to a lower or even undetectable amount of edited transcript. To exclude this possibility, RNA gel blots were performed in order to compare directly the accumulation of chimeric *aadA* mRNA in the Nt-pRB52, Nt-pRB57 and Nt-pRB58 transplastomic tobacco lines with a control line carrying no *ndhB* segment insertion. The transcripts accumulated to identical high levels in all lines (Figure 5), which is consistent with the high level of spectinomycin resistance (500 μg/ml) conferred by the chimeric *aadA* gene in all the lines. Since the transcripts are identical except for the *ndhB* segment insertion and, more importantly, initiate at the same

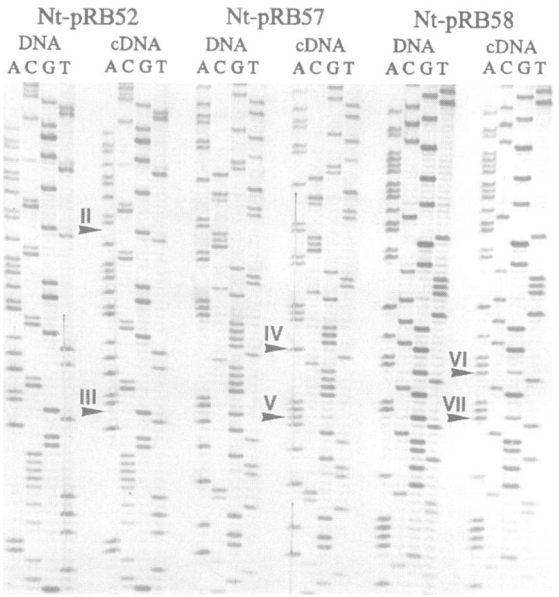

Fig. 4. Sequence analysis to test for transgene mRNA editing in the Nt-pRB52, Nt-pRB57 and Nt-pRB58 transplastomic lines. DNA and cDNA were amplified with primer pair P10–P11 and directly sequenced with primer P10. Owing to the polarity of the sequencing primer, the sequence ladder shown here reflects the strand complementary to the mRNA sequence. Arrowheads point to the editing positions in the cDNA lanes. Roman numerals indicate the editing sites (see Figure 2). Note the lack of editing at sites II and III (G signal only in cDNA lane), nearly complete editing of sites IV and V (strong A signal and very faint G signal) and partial editing of sites VI and VII.

Table I. RNA editing efficiencies for three pairs of tobacco *ndhB* editing sites in identical transgenic contexts (see text for details)

Editing sites	Transformation vector	Editing efficiency (5′ site)	Editing efficiency (3′ site)
II/III	pRB52	0%	0%
IV/V	pRB57	95%	95%
VI/VII	pRB58	70%	70%

promoter, it seems reasonable to conclude that they are equally stable.

Editing of the endogenous ndhB sites is not affected in the transplastomic tobacco plants

Incomplete editing of a chimeric *ndhB* sequence could also be due to depletion of site-specific factors involved in *ndhB* editing. In all transformation experiments, the chimeric *ndhB* sequences were introduced as additional copies, leaving the two endogenous *ndhB* genes in the inverted repeats of the ptDNA unchanged. One could envisage that the presence of supernumerary editing sites could lead to competition for rate-limiting amounts of *trans*-acting factor(s) between the endogenous *ndhB* transcripts and the chimeric mRNA molecules. Depending on the titer of the limiting site-specific factor, the competition effect may be more or less severe. This could, for example, explain efficient editing of sites IV and V compared with partial editing at sites VI and VII. If such a competition were the case, one would expect an equivalent decrease in the editing efficiency of the endogenous sites. Indeed, when additional copies of the tobacco *psbL* editing site

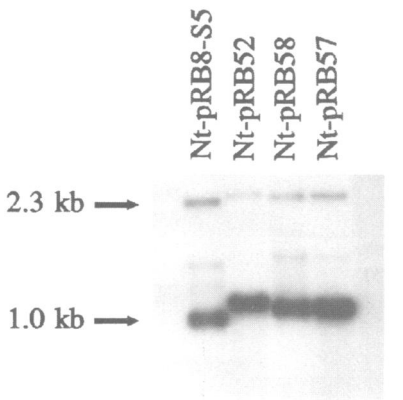

Fig. 5. Northern blot analysis to compare the stabilities of chimeric transcripts carrying *ndhB* editing sites. Two μg of total plant RNA from each of the homoplasmic transplastomic lines Nt-pRB8-S5 (control line without an *ndhB* insertion; Bock *et al.*, 1994), Nt-pRB52, Nt-pRB57 and Nt-pRB58 were hybridized with a radiolabeled *aadA* probe. Subsequently, the blot was stripped and reprobed with a 16S rRNA-specific probe (not shown), thus allowing normalization for RNA loading and comparative quantitation using a PhosphorImager. No significant difference in RNA accumulation could be measured for any of the transformed lines, indicating that the 3′-terminal insertion of *ndhB* segments does not affect transcript stability. Hybridization with the *aadA*-specific probe leads to the detection of two prominent RNA species: the major band of ~1.1 kb corresponds to the monocistronic *aadA* mRNA. The appearance of a second RNA species of ~2.3 kb is the result of readthrough transcription initiating at the *psbE* operon promoter (Bock *et al.*, 1994). Note that the presence of the *ndhB* insertions leads to small differences in transcript size which are clearly visible.

were introduced into the chloroplast genome, editing of the endogenous *psbL* mRNA was shown to be significantly less efficient (Chaudhuri *et al.*, 1995).

To test whether depletion of site-specific editing factor(s) is the cause for the observed differences in editing efficiencies of the three pairs of *ndhB* sites, editing of the endogenous *ndhB* sites was measured for the Nt-pRB52, Nt-pRB57 and Nt-pRB58 transplastomic lines. However, none of the endogenous *ndhB* sites exhibited any significant reduction in editing efficiency (data not shown). Thus inefficient editing of transgene editing sites cannot be explained with a factor competition model.

Besides the observed absence of editing factor competition, the inserted *ndhB* sequences also seem to lack any other deleterious effect on chloroplast gene expression since none of the generated transplastomic plants exhibited a detectable mutant phenotype under greenhouse conditions.

Deletion mapping of cis-acting sequence elements for ndhB editing sites IV and V

In order to define the *cis* elements essential for editing more precisely, the *ndhB* editing sites IV and V were studied further. When compared with the other two pairs of sites, they offer the obvious advantage of harboring all the elements required for complete editing in the –42/+42 sequence context. We first deleted 20 nucleotides from the 5′ end (pRB60) and 20 nucleotides from the 3′ end (pRB59). The 5′ deletion resulted in a small decrease in editing efficiency affecting both sites about equally (Table II). The analogous 3′ deletion turned out to have a different effect: it does not affect the 5′ editing site (site IV) but leads to a detectable reduction in editing of the

Table II. RNA editing efficiencies for chimeric transcripts harboring the *ndhB* editing sites IV/V

Transformation vector	*ndhB* segment	Editing efficiency (site IV/siteV)
pRB57	-42 EIV EV +42	95 % / 95 %
pRB59	-42 EIV EV +22	95 % / 75 %
pRB60	-22 EIV EV +42	80 % / 75 %
pRB61	-22 EIV EV +22	75 % / 60 %
pRB62	-42 EIV EV +12	75 % / 55 %
pRB63	-42 EIV EV +2	60 % / 45 %
pRB65	-12 EIV EV +42	20 % / 25 %
pRB66	-2 EIV EV +42	0 % / 0 %

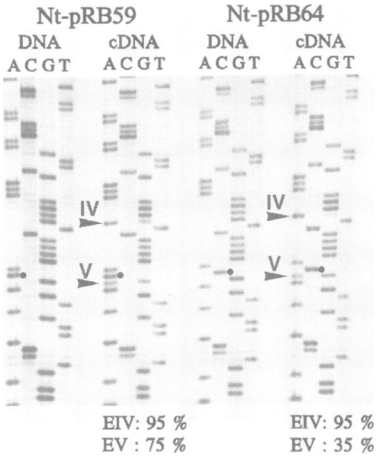

Fig. 6. Sequence analysis to test the effect of a single point mutation on *ndhB* editing sites IV and V. DNA and cDNA samples were amplified with primer pair P11–P28 and directly sequenced using primer P28. Due to the polarity of this primer, the autoradiograph shows sequences complementary to the mRNA. Arrowheads mark the editing positions in the cDNA lanes, Roman numerals indicate editing site numbers. Black dots denote the mutated nucleotide position (A in pRB59 and C in pRB64 reflecting a T→G change in the mRNA-like strand). The editing efficiencies for the two sites are given below the cDNA ladders.

3′ editing site (site V). As expected, the double deletion mutant (–22 to +22: pRB61) showed an additive effect (Table II), i.e. a slightly greater reduction in editing efficiency.

In the Nt-pRB59, Nt-pRB60 and Nt-pRB61 transplastomic lines, the majority of transgene mRNA molecules are still edited, suggesting that only elements required for maximum editing efficiency were deleted. The essential elements, however, seem to reside within –22/+22. In an attempt to narrow down these elements further, we proceeded with the 5′ and 3′ deletions in steps of 10 nucleotides. All of the 3′ deletions had only little effect on RNA editing efficiency (Table II). Even removal of all but two 3′ nucleotides (pRB63) does not abolish editing. The analogous 5′ deletions, however, exhibited much more severe effects: –12 (pRB65) directs editing of both sites only very poorly and –2 (pRB66) completely abolishes editing of both sites (Table II).

We have also tried to identify a sequence context for sites II and III that would permit editing of the chimeric transcripts. We tested both smaller (–22 to +22: pRB53) and larger segments (–62 to +62: pRB55; –82 to +82: pRB56). However, in none of the obtained homoplasmic transformed lines could any editing of sites II and III be detected. We did not increase the size of the *ndhB* segment further since this is likely to increase the risk of unwanted homologous recombination between the endogenous *ndhB* genes and the transgene. In fact, such recombination-induced genome rearrangements were observed for some of the Nt-pRB56 plants but were not characterized further.

A single nucleotide substitution drastically reduces RNA editing efficiency

Plastid RNA editing was shown previously to exhibit a strong bias towards pyrimidines as the 5′ neighboring position of editing sites (Maier *et al.*, 1992b). Of the 25 editing positions identified in the maize chloroplast genome (Maier *et al.*, 1995), only one is preceded by an A nucleotide and none by a G. These data may imply that an upstream purine nucleotide renders a C less susceptible to RNA editing.

To test whether or not editing activity is maintained in the presence of a 5′ neighboring G, we changed the T upstream of *ndhB* editing site V into a G. For technical reasons, the mutation was introduced in a –42/+22 segment which yielded 95% editing of site IV and 75% of site V for the wild-type sequence (pRB59; Table II). Transplastomic lines obtained from transformation with the vector carrying the T→G substitution (pRB64) showed no significant change in editing of site IV with respect to which the point mutation is eight nucleotides downstream (Figure 6). Site V, however, seems to be severely affected: its editing efficiency dropped to 35% as compared with 75% in the Nt-pRB59-transformed lines. Thus the preceding G nucleotide does not completely exclude site V from C→U transition but clearly renders it a less efficient substrate for the plastid editing machinery.

Discussion

Seven years after its initial discovery, higher plant organellar RNA editing still holds many mysteries. Whereas the general features of the editing process are well characterized (for review, see for example, Bonnard *et al.*, 1992; Kössel. *et al.*, 1993; Schuster and Brennicke, 1994), none of the determinants involved in site selection and catalysis has as yet been identified. In order to gain a better understanding of the mechanism of how an individual C residue is selected for modification, we have analyzed three pairs of plastid *ndhB* editing sites in a transgenic *in vivo* study. We report here that flanking sequences of identical size (–42 to +42) direct nearly complete editing for one pair of editing sites, partial editing for a second

and no detectable editing for a third pair of sites. We have shown that this rather unexpected observation can be attributed neither to differences in transcript stability nor to depletion of site-specific editing factors. We propose that the different behavior of the three pairs of editing sites is caused by differences in either the size of essential *cis* elements or in their distance from the editing site. As an alternative explanation, aberrant RNA secondary or ternary structure formation has to be considered. Interactions between the *ndhB* segment and other parts of the chimeric transcript could theoretically inhibit editing, for example by masking the editing site(s) in a double-stranded RNA structure. At present we cannot completely exclude this possibility. However, we consider it less likely since computer analyses did not reveal good candidate sequences for interaction either with editing sites II and III or with sites VI and VII. Moreover, the fact that the two sites of a pair behave identically with respect to editing may argue against this possibility since it would involve the additional assumption that both sites interact equally strongly with other transcript sequences.

Deletions in the 3′-flanking region of sites IV and V exhibited only minor effects on editing efficiencies (Table II). They can be explained by the presence of 3′ sequence elements enhancing editing or, alternatively, by negative effects of the 3′ deletions on RNA conformation and thus on the accessibility of the sites. The corresponding 5′ deletions revealed much stronger effects and eventually led to the loss of any detectable editing activity at both sites. Therefore, we conclude that an essential element for editing of sites IV and V is located in the –12/–2 region upstream of editing site IV. The presence of an essential recognition element 5′ upstream of an editing site could be also demonstrated for the tobacco *psbL* site (S.Chaudhuri and P.Maliga, personal communication).

C→U editing of the mammalian apolipoprotein B mRNA was shown to involve an RNA sequence element close to the editing position which was termed the 'mooring sequence' (for review, see Chan, 1993). It is thought to mediate substrate recognition as well as editosome assembly. By analogy to ApoB editing, the identified upstream sequence element essential for editing of the *ndhB* sites IV and V could serve as a mooring sequence that allows for binding of the editing machinery to its RNA substrate and thus is absolutely necessary for editing of the downstream C nucleotides. Additional specificity factors would be required to distinguish the two editing positions from the many other C nucleotides in this region (see Figure 2).

The essential sequence element located in the –12/–2 region could be either a general *cis*-guiding factor for plastid RNA editing or a site-specific element for editing of *ndhB* sites IV and V only. To distinguish between these two possibilities, we performed a computer screen on the entire tobacco chloroplast genome sequence. This analysis failed to detect homologous sequences upstream of other chloroplast RNA editing sites. Therefore, we conclude that this sequence motif is likely to be a binding site for the site-specific *trans*-acting factor rather than a general recognition device for chloroplast editing sites. Evidence for the existence of such a *trans* factor has been provided recently for the tobacco *psbL* mRNA editing site (Chaudhuri *et al.*, 1995).

None of the deletions described here selectively abolished the editing of only one of the two editing sites IV and V, implying that the editing of closely adjacent sites may be somehow mechanistically coupled. The identical behavior of sites II/III and VI/VII (Table I) may lend further support to this hypothesis. The coupling could be caused, for example, by sharing a common recognition element for binding of the editing machinery. However, editing of the two sites of a pair is clearly not absolutely linked: for some of our constructs we observe significant differences in the editing efficiencies of the two sites (Table II, Figure 6), implying the existence of RNA molecules that are edited at one site of the pair but not at the other.

The absence of a 5′ neighboring G from all editing sites in the maize chloroplast genome (Maier *et al.*, 1995) raises the interesting possibility that a 5′ G would render a C non-editable. We have, therefore, tested the effect of a single point mutation changing the 5′ neighboring nucleotide of editing site V from T to G. The point mutation was shown to be silent with respect to the upstream editing site (site IV) but had a strong effect on the downstream site (V). This finding provides further edivence for the greater importance of the 5′ upstream region in editing site selection, and demonstrates that a 5′ G can be accepted by the chloroplast editing machinery albeit, at least in the case of *ndhB* site V, with lower efficiency.

This study also aimed to define suitable substrates for *in vitro* editing assays. To date, no system is available to study plastid RNA editing *in vitro*. Plant mitochondrial extracts that appear to contain some editing activity have been described (Araya *et al.*, 1991; Yu and Schuster, 1995). However, this activity acted only poorly on the added *in vitro* transcripts. Our results indicate that the choice of the substrate for *in vitro* editing reactions can be crucial: a 190 nucleotide *in vitro* transcript containing the *ndhB* editing sites II and III (as in pRB56) will most probably not be a substrate that can be utilized by the plastid editing machinery and thus would never allow the detection of any editing activity *in vitro*. A much smaller transcript covering sites IV and V, however, should provide a suitable test substrate. Future *in vitro* experiments that make use of such substrates are hoped to provide insights into the biochemistry of the RNA editing reaction as well as into the molecular nature of the *trans*-acting specificity factors involved.

Materials and methods

Plant material

Tobacco plants (*Nicotiana tabacum* cv Petit Havana) were grown under sterile conditions on agar-solidified MS medium (Murashige and Skoog, 1962) containing 30 g/l sucrose. Homoplasmic transformed lines were rooted and propagated on the same medium.

Oligonucleotides

The following synthetic oligonucleotides were employed in this study: P2, 5′-TTTTTCTAGATTTTTGGATCCGATCCTGGCCTAGTC-3′; P3, 5′-CAGTTGGAAGAATTTGTCC-3′; P4, 5′-TTTTTCTAGAAAATATTTACTCATGGGTGG-3′; P5, 5′-TTTTGGATCCAGACCGTTTACTATTTCTTG-3′; P6, 5′-TTTTTCTAGAGGCAAGCTCTTCTATTCTG-3′; P7, 5′-TTTTGGATCCAAGCTCAATCTCTCCCCC-3′; P10, 5′-AACCTCCTATAGACTAGGC-3′; P11, 5′-AGCGAAATGTAGTGCTTACG-3′; P12, 5′-TTTTTCTAGAGGTCTAATGAGGCTACTATG-3′;

Table III. Transformation vectors

Vector	Primer pair
pRB52	P4/P5
pRB53	P6/P7
pRB55	P12/P13
pRB56	P14/P15
pRB57	P16/P17
pRB58	P18/P19
pRB59	P16/P21
pRB60	P20/P17
pRB61	P20/P21
pRB62	P16/P22
pRB63	P16/P23
pRB64	P16/P24
pRB65	P26/P17
pRB66	P27/P17

P13, 5′-TTTTGGATCCTATACATTTGTGTATTGATA-3′; P14, 5′-TTTTTCTAGAATATACCAAGAAAGATGTAC-3′; P15, 5′-TTTTGGATCCAATTGAAATTCCTGGGGAGT-3′; P16, 5′-TTTTTCTAGACGCTCATATTCATTACCGTA-3′; P17, 5′-TTTTGGATCCACGAACCGCACTCCTTCGTA-3′; P18, 5′-TTTTTCTAGACAGTCGTTGCTTTTCTTTCT-3′; P19, 5′-TTTTGGATCCTTTGATGAGAAATAAAAAGG-3′; P20, 5′-TTTTTCTAGAGGAATTGGGTTCAAGCTTT-3′; P21, 5′-TTTTGGATCCTACGTCAGGAGTCCATTGA-3′; P22, 5′-TTTTGGATCCGTCCATTGATGAGA-3′; P23, 5′-TTTTGGATCCGAGAAGGGGCTGGG-3′; P24, 5′-TTTTGGATCCTACGTCAGGAGTCCATTGATGAGCAGG-3′; P26, 5′-TTTTTCTAGATCAAGCTTTCCCCAGCC-3′; P27, 5′-TTTTTCTAGACCCAGCCCCTTCTCATC-3′; P28, 5′-TAGCACCCTCTTGATAGAAC-3′; 7355, 5′-GACTATAGATCGAACCTATCC-3′; 1020, 5′-CAAGATCCATTACGTGTCCAAGG-3′; 16R1, 5′-CGGGTGAGTAACGCGTAAG-3′; 16R2, 5′-TTGCATCGAATTAAACCACATGCTCCACC-3′; nb5, 5′-CAAGGAGATTCCCCAATATC-3′; nb11, 5′-TTCATGCTTGTTTGAGTAATAGC-3′.

Construction of plastid transformation vectors

The chimeric genes containing plastid editing sites were constructed in a 2384 bp *Sal*I–*Spe*I tobacco plastid DNA (ptDNA) fragment cloned in a Bluescript KS+ vector (Shinozaki *et al.*, 1986; Bock *et al.*, 1994). The *Xba*I site was removed from the polylinker by digestion with *Not*I and *Spe*I followed by a fill-in reaction with Klenow enzyme. After religation, the plasmid was linearized with *Eco*RV and a chimeric *aadA* gene was inserted into the intergenic spacer between the *psbJ* and *petA* genes as described by Bock *et al.* (1994) (Figure 1). The plasmid clone obtained (pRB50) carries the *aadA* gene in the same orientation as the *psbE* operon. Subsequently, a *Bam*HI restriction site was introduced downstream of the *aadA* termination codon (which is part of an *Xba*I site) by PCR-based mutagenesis as follows: the region containing the *Xba*I–*Sal*I fragment (Figure 1B) was amplified with the primer P2 and the Reverse Primer (Stratagene). P2 contains an *Xba*I site, the first 15 nucleotides of the *psbA* 3′ region separated by five T nucleotides (to facilitate *Xba*I–*Bam*HI double digestion) and a *Bam*HI site (see above). The *Xba*I–*Sal*I fragment of pRB50 was now replaced with the similarly cut PCR product generating plasmid pRB51. The vectors used for plastid transformation are derivatives of pRB51 and carry segments with *ndhB* editing sites as *Xba*I–*Bam*HI fragments. These fragments were prepared by PCR amplification of the respective *ndhB* sequences and simultaneous introduction of a 5′ *Xba*I restriction site and a 3′ *Bam*HI site. After double digestion with *Xba*I and *Bam*HI, the fragments were inserted into the similarly cut pRB51, and the correctness of both amplification and cloning was verified by DNA sequencing with primer P3 (derived form the 3′ portion of the *aadA* coding region) or P28 (complementary to the *psbA* 3′ untranslated region).

In this way, the transformation vectors shown in Table III were generated (see Results for details).

Plastid transformation and selection of transplastomic tobacco lines

Young leaves were harvested form sterile tobacco plants and bombarded with plasmid DNA-coated tungsten particles using the DuPont PDS1000He biolistic gun (Svab and Maliga, 1993; Kanevski and Maliga, 1994). Spectinomycin-resistant shoots were selected on RMOP regeneration medium containing 500 mg/l spectinomycin dihydrochloride. Plastid transformants were identified by a PCR strategy that allowed the simultaneous testing for correct integration into the chloroplast genome (see Figure 3). Transplastomic lines were subjected to at least two additional rounds of regeneration on RMOP–spectinomycin to obtain homoplasmic tissue. Homoplasmy was verified by a highly sensitive PCR assay (see Figure 3). Only plants with a uniformly transformed ptDNA population were used for the molecular biological analyses.

Isolation of nucleic acids

Total plant nucleic acids were isolated according to a rapid miniprep procedure described by Doyle and Doyle (1990). For cDNA synthesis, an aliquot of the preparation was treated with DNase I (Promega) as described by Kudla *et al.* (1992). RNA for gel blot analyses was extracted using the TRIzol reagent (Gibco/BRL).

Northern blot analysis

RNA was electrophoresed on formaldehyde-containing 1.5% agarose gels and transferred to nylon membranes (Boehringer Mannheim) using standard protocols (Sambrook *et al.*, 1989). Hybridization was carried out at 65°C in Rapid Hybridization Buffer (Amersham) using [^{32}P]dATP-labeled probes generated by random priming (Boehringer Mannheim). A *Nco*I–*Xba*I restriction fragment covering the entire *aadA* coding region was prepared from a progenitor of pZS197 (Svab and Maliga, 1993) and served as *aadA*-specific probe. A 824 bp PCR product (corresponding to nucleotide positions 10 2851–10 3674 in the tobacco ptDNA; Shinozaki *et al.*, 1986) obtained by cDNA amplification with the primer pair 16R1–16R2 was used to generate a 16S rRNA-specific probe.

cDNA synthesis and PCR

Reverse transcription of DNase I-treated total plant RNA was primed with random hexanucleotide primers. The elongation reaction was performed with Moloney murine leukemia virus RNase H-free reverse transcriptase (Gibco/BRL) following the manufacturer's instructions.

DNA and cDNA templates were amplified according to standard protocols (45 s at 94°C, 1.5 min at 55–60°C, 1.5 min at 72°C; 30 cycles).

DNA sequencing

Plasmid DNA was sequenced by cycle sequencing reactions using the Sequitherm kit (Biozyme). Primer pairs P11–P10 or P11–P28 (Figure 1B) were used to generate the substrate for direct sequencing of transgene-derived PCR products. Primer pair nb11–P14 amplified the endogenous *ndhB* sequences. DNA and cDNA amplification products were purified for sequencing by electrophoresis on 2% agarose gels and subsequent extraction from gel slices using the QIAEX II kit (QIAGEN). Sequence determination was performed by a modified chain termination method described by Bachmann *et al.* (1990). Oligonucleotides P3, P10 or P28 (Figure 1B) served as sequencing primers for the transgenes. Primers P14, P19 and nb5 were used for sequencing of the endogenous *ndhB* sites.

Quantitation of RNA editing efficiency

In order to quantify editing efficiencies, several calculation procedures were tested. In our hands, the direct sequencing method for PCR products (Bachmann *et al.*, 1990) led to significant differences in labeling efficiencies for the four lanes of a sequencing reaction. Thus it is not practicable to quantitate band intensities in the C lane against bands in the T lane directly. On the other hand, the relative peak intensities within one and the same lane turned out to be fairly constant. Editing efficiency can, therefore, be measured as the decrease in relative C signal intensity. Calculation according to the following formula proved to give reliable and reproducible results:

$$E_{ed} = 100\% - \frac{\dfrac{C_{e,c} - C_{o,c}}{C_{r,c} - C_{o,c}} \cdot 100\%}{\dfrac{C_{e,d} - C_{o,d}}{C_{r,d} - C_{o,d}}}$$

E_{ed}: editing efficiency, $C_{e,c}$: C signal intensity at the editing position in the cDNA sequence, $C_{e,d}$: C signal intensity at the editing position in the DNA sequence, $C_{o,c}$: background intensity in the C lane of the cDNA sequence, $C_{o,d}$: background intensity in the C lane of the DNA sequence, $C_{r,c}$: reference peak in the C lane of the cDNA sequence, $C_{r,d}$: reference peak in the C lane of the DNA sequence. (The calculation was carried out for three sets of DNA and cDNA sequencing reactions

and for two different reference peaks each. The average editing efficiency value was rounded to full 5 or 10%. For sequencing reactions with antisense primers, the G lane was used for quantitation.)

Crosses and testing of maternal transgene inheritance

Wild-type and transformed plants were transferred to soil and grown to maturity under greenhouse conditions. Seed pods were collected from selfed plants and from reciprocal crosses of the transplastomic lines with wild-type plants. Surface-sterilized seeds were germinated on spectinomycin-containing (500 mg/l) RM medium and analyzed for uniparental inheritance of the resistance trait. Selfed transformants and crosses with a plastid transformant as the maternal parent give rise to green (i.e. spectinomycin-resistant) progeny, whereas seeds collected from wild-type plants yield white (i.e. drug-sensitive) seedlings.

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References

Araya,A., Domec,C., Begu,D. and Litvak,S. (1991) An *in vitro* system for the editing of ATP synthase subunit 9 mRNA using wheat mitochondrial extracts. *Proc. Natl Acad. Sci. USA*, **89**, 1040–1044.

Bachmann,B., Lüke,W. and Hunsmann,G. (1990) Improvement of PCR amplified DNA sequencing with the aid of detergents. *Nucleic Acids Res.*, **18**, 1309.

Benne,R., Van den Burg,J., Brakenhoff,J.P.J., Sloof,P., Van Boom,J.H. and Tromp,M.C. (1986) Major transcript of the frameshifted *coxII* gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell*, **46**, 819–826.

Bock,R., Kössel,H. and Maliga,P. (1994) Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype. *EMBO J.*, **13**, 4623–4628.

Bonnard,G., Gualberto,J.M., Lamattina,L. and Grienenberger,J.M. (1992) RNA editing in plant mitochondria. *Crit. Rev. Plant Sci.*, **10**, 503–524.

Chan,L. (1993) RNA editing: exploring one mode with apolipoprotein B mRNA. *BioEssays*, **15**, 33–41.

Chaudhuri,S., Carrer,H. and Maliga,P. (1995) Site-specific factor involved in the editing of the *psbL* mRNA in tobacco plastids. *EMBO J.*, **14**, 2951–2957.

Covello,P.S. and Gray,M.W. (1989) RNA editing in plant mitochondria. *Nature*, **341**, 662–666.

Doyle,J.J. and Doyle,J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.

Freyer,R., Hoch,B., Neckermann,K., Maier,R.M. and Kössel,H. (1993) RNA editing in maize chloroplasts is a processing step independent of splicing and cleavage to monocistronic mRNAs. *Plant J.*, **4**, 621–629.

Freyer,R., López,C., Maier,R.M., Martin,M., Sabater,B. and Kössel,H. (1995) Editing of the chloroplast *ndhB* encoded transcript shows divergence between closely related members of the grass family (Poaceae). *Plant Mol. Biol.*, **29**, 679–684.

Gualberto,J.M., Lamattina,L., Bonnard,G., Weil,J.-H. and Grienenberger,J.M. (1989) RNA editing in wheat mitochondria results in the conservation of protein sequences. *Nature*, **341**, 660–662.

Gualberto,J.M., Weil,J.H. and Grienenberger,J.M. (1990) Editing of the wheat *coxIII* transcript: evidence for twelve C to U and one U to C conversions and for sequence similarities around editing sites. *Nucleic Acids Res.*, **18**, 3771–3776.

Hiesel,R., Wissinger,B., Schuster,W. and Brennicke,A. (1989) RNA editing in plant mitochondria. *Science*, **246**, 1632–1634.

Hoch,B., Maier,R.M., Appel,K., Igloi,G.L. and Kössel,H. (1991) Editing of a chloroplast mRNA by creation of an initiation codon. *Nature*, **353**, 178–180.

Kanevski,I. and Maliga,P. (1994) Relocation of the plastid *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. *Proc. Natl Acad. Sci. USA*, **91**, 1969–1973.

Kössel,H., Hoch,B., Maier,R.M., Igloi,G.L., Kudla,J., Zeltz,P., Freyer,R., Neckermann,K. and Ruf,S. (1993) RNA editing in chloroplasts. In Brennicke,A. and Kück,U. (eds), *Plant Mitochondria*. VCH Publishers, Weinheim, Germany, pp. 93–102.

Kudla,J., Igloi,G.L., Metzlaff,M., Hagemann,R. and Kössel,H. (1992) RNA editing in tobacco chloroplasts leads to the formation of a translatable *psbL* mRNA by a C to U substitution within the initiation codon. *EMBO J.*, **11**, 1099–1103.

Maier,R.M., Hoch,B., Zeltz,P. and Kössel,H. (1992a) Internal editing of the maize chloroplast *ndhA* transcript restores codons for conserved amino acids. *Plant Cell*, **4**, 609–616.

Maier,R.M., Neckermann,K., Hoch,B., Akhmedov,N.B. and Kössel,H. (1992b) Identification of editing positions in the *ndhB* transcript from maize chloroplasts reveals sequence similarities between editing sites of chloroplasts and plant mitochondria. *Nucleic Acids Res.*, **20**, 6189–6194.

Maier,R.M., Neckermann,K., Igloi,G.L. and Kössel,H. (1995) Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. *J. Mol. Biol.*, **251**, 614–628.

Matsubayashi,T. *et al.* (1987) Six chloroplast genes (*ndhA–F*) homologous to human mitochondrial genes encoding components of the respiratory chain NADH dehydrogenase are actively expressed: determination of the splice sites in *ndhA* and *ndhB* pre-mRNAs. *Mol. Gen. Genet.*, **210**, 385–393.

Murashige,T. and Skoog,F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, **15**, 473–497.

Ruf,S., Zeltz,P. and Kössel,H. (1994) Complete RNA editing of unspliced and dicistronic transcripts of the intron-containing reading frame IRF170 from maize chloroplasts. *Proc. Natl Acad. Sci. USA*, **91**, 2295–2299.

Sambrock,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schuster,W. and Brennicke,A. (1994) The plant mitochondrial genome: physical structure, information content, RNA editing, and gene migration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 61–78.

Schuster,W., Hiesel,R., Wissinger,B. and Brennicke,A. (1990) RNA editing in the cytochrome b locus of the higher plant *Oenothera berteriana* includes a U to C transition. *Mol. Cell. Biol.*, **10**, 2428–2431.

Shinozaki,K. *et al.* (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and gene expression. *EMBO J.*, **5**, 2043–2049.

Simpson,L., Maslov,D.A. and Blum,B. (1993) RNA editing in *Leishmania* mitochondria. In Benne,R. (ed.), *RNA Editing. The Alteration of Protein Coding Sequences of RNA*. Ellis Horwood, New York, pp. 53–85.

Stern,D.B. and Gruissem,W. (1987) Control of plastid gene expression: 3′ inverted repeats act as mRNA processing and stabilizing elements but do not terminate transcription. *Cell*, **51**, 1145–1157.

Svab,Z. and Maliga,P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Natl Acad. Sci. USA*, **90**, 913–917.

Svab,Z., Hajdukiewicz,P. and Malga,P. (1990) Stable transformation of plastids in higher plants. *Proc. Natl Acad. Sci. USA*, **87**, 8526–8530.

Yu,W. and Schuster,W. (1995) Evidence for a site-specific cytidine deamination reaction involved in C to U RNA editing in plant mitochondria. *J. Biol. Chem.*, **31**, 18227–18233.

Zeltz,P., Hess,W.R., Neckermann,K., Börner,T. and Kössel,H. (1993) Editing of the chloroplast *rpoB* transcript is independent of chloroplast translation and shows different patterns in barley and maize. *EMBO J.*, **12**, 4291–4296.

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