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Site-specific factor involved in the editing of the *psbL* mRNA in tobacco plastids

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In tobacco plastids, functional *psbL* mRNA is created by editing an ACG codon to an AUG translation initiation codon. To determine if editing may occur in a chimeric mRNA, the N-terminal part of *psbL* containing the editing site was translationally fused with the *aadA* and *kan* bacterial genes. The chimeric constructs were introduced into the tobacco plastid genome by targeted gene insertion. Editing of the chimeric mRNAs indicated that the 98 nt fragment spanning the *psbL* editing site contains all *cis* information required for editing. Expression of the chimeric gene transcripts led to a significant decrease in the editing efficiency of the endogenous *psbL* mRNA. However, the efficiency of editing in the transplastomic lines was unchanged for four sites in the *rpoB* and *ndhB* mRNAs. Reduced efficiency of *psbL* editing, but not of the other four sites, in the transplastomic lines indicates depletion of *psbL*-specific editing factor(s). This finding implicates the involvement of site-specific factors in editing of plastid mRNAs in higher plants.

Key words: chimeric mRNA/plastid transformation/*psbL* gene/RNA editing/tobacco

Introduction

Certain mRNA sequences can be altered post-transcriptionally by a process known as RNA editing, so that their final nucleotide sequence differs from that encoded by the DNA sequence. RNA editing encompasses widely different molecular mechanisms (Chan, 1993). For example, in the mitochondria of kinetoplastid protozoa, the uridine deletion/addition editing system involves small guide RNAs (Simpson *et al.*, 1993). In the mammalian apolipoprotein B mRNAs a cytidine to uridine change creates a termination codon. The information for this editing event resides in the RNA sequence directly downstream of the edited site (Chan, 1993; Hodges and Scott, 1993). Editing occurs by the deamination of cytidine to uridine by cytidine deaminase (Teng *et al.*, 1993). For another mammalian nuclear gene, GluR2, editing of an adenosine to inosine is directed by a distal downstream intronic sequence and is thought to involve an adenosine deaminase specific for double-stranded RNA (Higuchi *et al.*, 1993).

Extensive RNA editing has been reported in the plastids and mitochondria of higher plants. In plastids, mRNA editing has been found to involve exclusively cytidine to uridine transitions. In plant mitochondria this is also the predominant form of editing, with rare cases of uridine to cytidine transitions. Editing in plants most frequently alters coding sequences, resulting in conservation of functionally important amino acids. In addition, editing creates start codons, stop codons and alters the 5′- and 3′-non-coding as well as intron sequences. The editing site selection process and the biochemical mechanism of editing in plant organelles is largely unknown. Guide-type RNAs that could potentially direct editing in plants have not been identified (Bock and Maliga, 1995). In addition, sequence comparisons of the various editing sites have failed to identify any conserved primary and/or secondary structure motifs that could direct the selection of the editing sites. For reviews on RNA editing in plant organelles see Gray and Covello (1993), Kossel *et al.* (1993) and Schuster and Brennicke (1994).

The availability of plastid transformation in tobacco (Maliga, 1993) made feasible a transgenic approach towards defining the *cis*-acting sequence requirements for plastid RNA editing. To determine if editing may occur in a chimeric mRNA, the N-terminal part of *psbL* containing the editing site was translationally fused with the *aadA* and *kan* bacterial genes. Since the *psbL* translation initiation codon is created by C→U editing (Kudla *et al.*, 1992), the expression of chimeric spectinomycin (E*aadA*) and kanamycin (E*kan*) genes was made dependent on editing. We report that a 98 nt fragment spanning the *psbL* editing site is sufficient to direct editing of the chimeric mRNAs and creates an AUG translational initiation codon from an ACG codon, as reported for the *psbL* mRNA (Kudla *et al.*, 1992). Furthermore, expression of the chimeric mRNAs leads to a decrease in editing efficiency of the *psbL* mRNA, but does not affect the editing efficiency of four editing sites present on other transcripts, implicating the involvement of site-specific editing factors in the plastids of higher plants.

Results

Construction of the chimeric genes

The *psbL* gene encodes a peptide of photosystem II and is part of the *psbE* operon (Carillo *et al.*, 1986; Figure 1). A 98 nt fragment spanning the *psbL* editing site, Δ*psbF*/Δ*psbL*, was cloned upstream of the spectinomycin resistance gene (*aadA*) coding sequence such that the N-terminus of *psbL* was translationally fused with *aadA*. The Δ*psbF*/Δ*psbL* fragment contains 40 nt of the *psbF* C-terminus, 22 nt of the intergenic region between *psbF* and

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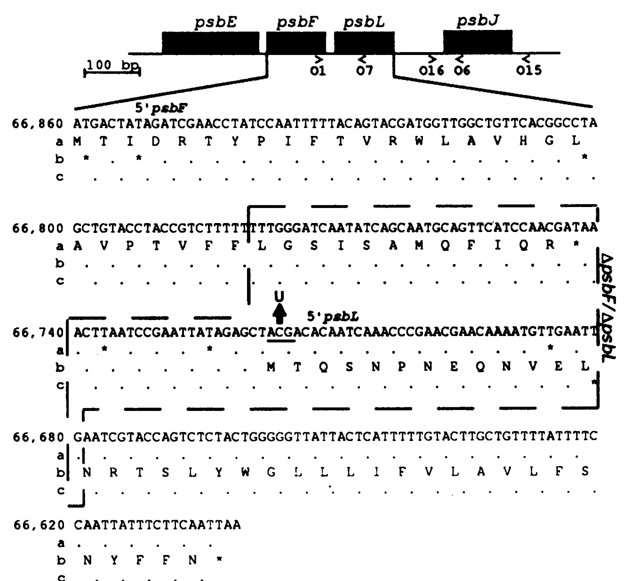

Fig. 1. The tobacco plastid *psbE* operon and the *psbF* and *psbL* DNA and amino acid sequences. The edited *psbL* initiation codon (ACG→AUG) is underlined. The Δ*psbF*/Δ*psbL* region is in a dashed box. The positions of oligonucleotides O1, O6, O7, O15 and O16 are marked. The DNA sequence is numbered according to Shinozaki *et al.* (1986).

psbL and 36 nt of the *psbL* N-terminus. The construct was cloned in the P*rrn*/T*rps16* plastid expression cassette (Figure 2A). P*rrn* contains the plastid rRNA operon promoter, a ribosome binding site and a translation initiation codon (ATG). In the chimeric construct, the truncated *psbF* coding region forms an open reading frame with the P*rrn* initiation codon (ATG), whereas translation of the E*aadA* reading frame (psbL–aadA fusion peptide) is dependent on the creation of a translation initiation codon (AUG from ACG) by editing the *psbL* site. Note that the two coding regions are in different reading frames in the E*aadA* mRNA (Figure 2A).

The E*kan* gene was obtained by translationally fusing *psbL* with *kan*, a kanamycin resistance gene encoding neomycin phosphotransferase, using the same Δ*psbF*/Δ*psbL* fragment (Figure 2B). E*kan* is similar to the E*aadA* gene, except that it has 39 nt instead of 36 nt of the *psbL* N-terminus.

Transformation and selection of antibiotic-resistant transplastomic lines

The E*aad*A gene was cloned into the plastid transformation vector pPRV100B (Zoubenko *et al.*, 1994) to yield plasmid pHC94, which was introduced into tobacco chloroplasts by the biolistic process. The chimeric gene integrated into the plastid genome by two homologous recombination events in the *trnV–rps7/12* intergenic region. In a sample of 50 bombarded leaves, selection for spectinomycin resistance resulted in the isolation of 43 spectinomycin-resistant clones. Out of these, 34 were confirmed to carry the E*aad*A gene by DNA gel blot analysis (data not shown). Expression of antibiotic resistance indicated editing of chimeric E*aadA*. The efficiency of selection for the E*aadA* gene, ~1 plastid transformant/bombarded leaf sample, was comparable with the efficiency of selection for an *aadA* gene whose expression was independent of editing (Svab and Maliga, 1993). Three independently transformed lines, Nt-pHC94-1, Nt-pHC94-10 and Nt-pHC94-11, were further studied.

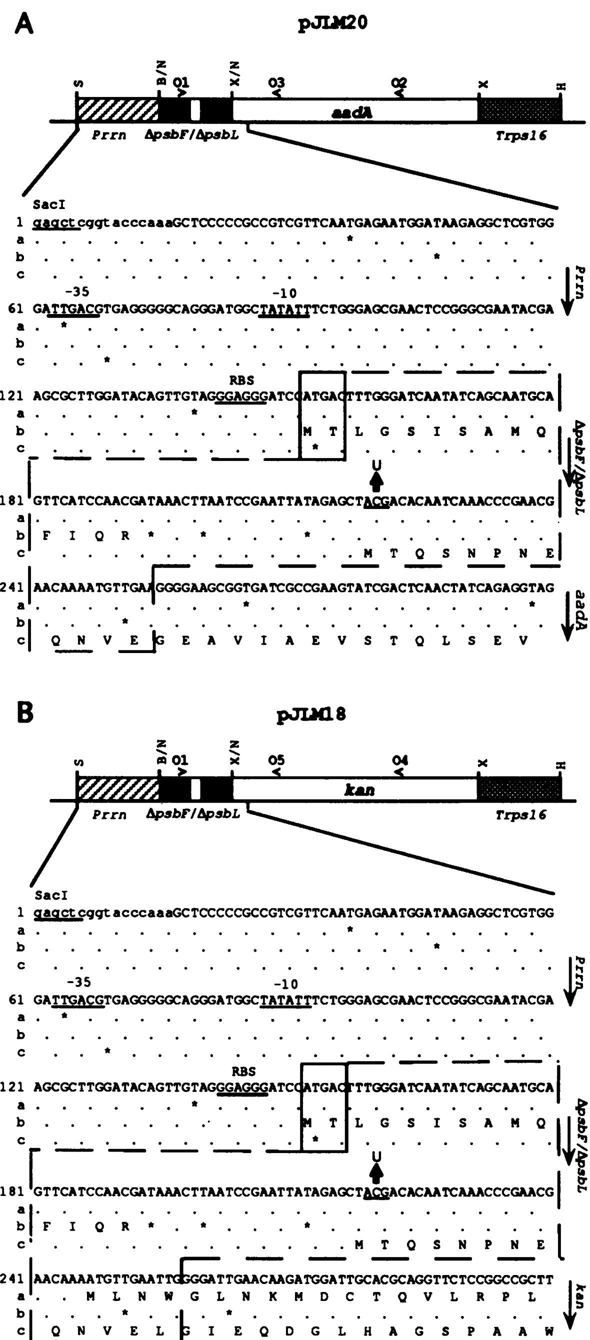

Fig. 2. Physical map and partial DNA sequence of (**A**) the E*aadA* gene in plasmid pJLM20 and (**B**) the E*kan* gene in plasmid pJLM18. The conserved –10/–35 promoter elements and ribosome binding site (RBS) are underlined in the P*rrn* sequence. DNA sequence derived from the Δ*psbF*/Δ*psbL* region is within dashed lines, new sequence introduced during construction is in the solid box. The edited *psbL* initiation codon (ACG→AUG) is underlined. T*rps16* is the 3′-untranslated region of the plastid *rps16* ribosomal protein gene. The positions for oligonucleotides O1, O2 and O3 in E*aadA* and for O1, O4 and O5 in E*kan* are indicated. Abbreviations of restriction sites: B, *Bsp*HI; H, *Hin*dIII; N, *Nco*I; S, *Sac*I; X, *Xba*I.

As direct selection for kanamycin resistance is inefficient (Carrer *et al.*, 1993), the E*kan* gene was linked to a

spectinomycin resistance gene in transformation vector pPRV111B to yield plasmid pJLM23. Direct selection of plastid transformants was attempted after bombardment with pJLM23 plasmid-coated tungsten particles. No kanamycin-resistant clones were obtained in a sample of 200 bombarded leaves (100 each selected on 50 and 100 μg/ml kanamycin sulfate). However, transgenic plants containing the E*kan* gene were obtained by selection for the linked spectinomycin resistance gene. Three independently transformed lines, Nt-pJLM23-2, Nt-pJLM23-14 and Nt-pJLM23-18, were further studied. Leaf segments from each of the clones proliferated on kanamycin medium (50 μg/ml), indicating phenotypic expression of the E*kan* gene.

Editing of EaadA, Ekan and psbL transcripts

The phenotypic expression of antibiotic resistance by E*aadA* and E*kan* plants indicated that the chimeric genes were edited, since their translation was made dependent on editing of an ACG to an AUG initiation codon. To directly test for editing of E*aadA* and E*kan* mRNAs, cDNAs were PCR amplified with primer O1 within the *psbF* coding region and primers O2 and O4 within the E*aadA* and E*kan* coding sequences respectively. The positions of primers are shown in Figure 2, the PCR amplification products are shown in Figure 3A. Direct sequencing of the PCR products from three independently transformed E*aadA* lines and phosphorimager analysis indicated that ~70% of the E*aadA* transcripts are edited (Figure 3B and Table I). A similar extent of editing was found for the E*kan* mRNAs (Figure 3C and Table I). The partial editing was not due to the presence of contaminating DNA in the RNA samples, since no PCR-amplified products were obtained from non-reverse transcribed, DNase I-treated RNA samples (Figure 3A, lanes 3 and 7).

The *psbL* site in the chimeric transcripts was only partially (~70%) edited, while in leaves of wild-type plants the *psbL* mRNA is >99% edited (Kudla *et al.*, 1992; Bock *et al.*, 1993). Therefore, it was of interest to determine whether or not editing of the *psbL* mRNA is affected in the transgenic plants. The *psbL* cDNAs were PCR amplified with primers O1 and O6 within the *psbF* and *psbJ* coding regions (Figure 1A) from wild-type and transgenic plants. Direct sequencing of the PCR products revealed that the transgenic plants contained ~10% unedited *psbL* mRNA. This indicates a >10-fold increase in the level of unedited *psbL* mRNA in the transgenic plants (Figure 4 and Table I). Artifacts due to DNA contamination of RNA samples were excluded by the lack of PCR products from non-reverse transcribed, DNase I-treated RNA samples (Figure 4A, lanes 3, 7 and 11).

Relative abundance of psbL, EaadA and Ekan mRNAs

Accumulation of partially edited *psbL* mRNA in the transgenic lines could be due to its competition with the chimeric E*aadA* or E*kan* mRNAs for a limiting common factor(s) that is required for editing. Therefore, the relative abundances of the *psbL* and chimeric transcripts were determined. It should be noted that both the polycistronic *psbE* (Carillo *et al.*, 1986) and the E*aadA* and E*kan*

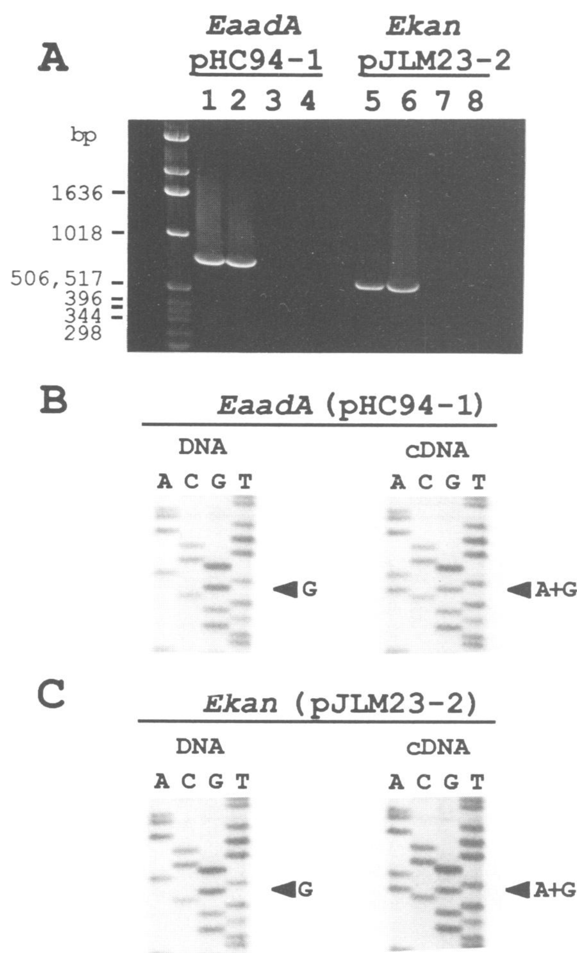

Fig. 3. Editing of the E*aadA* mRNA in the Nt-pHC94-1 plant and of the E*kan* mRNA in the Nt-pJLM23-2 plant. (**A**) PCR amplification products from DNA (lanes 1 and 5) and cDNA (lanes 2 and 6) with primers O1 and O2 for E*aadA* and primers O1 and O4 for E*kan* templates. The location of primers is shown in Figure 2. Controls were amplification reactions carried out with DNase I-treated RNA (lanes 3 and 7) and buffer only (lanes 4 and 8) using the same primers. The DNA and cDNA sequence of (**B**) E*aadA* in the Nt-pHC94-1 plant and of (**C**) E*kan* in the Nt-pJLM23 2 plant. The amplified products were directly sequenced with primers O3 (E*aadA*) and O5 (E*kan*). Due to the polarity of primers, the sequence shown is complementary to the mRNA. The editing site in the sequence is marked by an arrowhead. Note a mixture of A and G nucleotides at the editing site in the cDNA samples, indicating partial editing.

Table I. Unedited mRNAs (%) in the wild-type and transgenic leaves

Plant line	Sample	*psbL*	E*aadA*	E*kan*
Nt-wt	1	<0.1		
	2	0.7		
	3	0.3		
Nt-pJLM23-2	1	8.8		30.3
Nt-pJLM23-14	1	9.2		28.2
Nt-pJLM23-18	1	10.2		28.4
Nt-pHC94-1	1	9.5	28.7	
Nt-pHC94-10	1	9.0	30.4	
Nt-pHC94-11	1	10.4	29.9	

Radioactivity in bands corresponding to nucleotides was determined by phosphorimager analysis. The values were normalized for DNA loading and labeling efficiency against six other bands in the same lanes. Percent unedited mRNA = [corrected unedited signal/(corrected edited + corrected unedited signal)]×100.

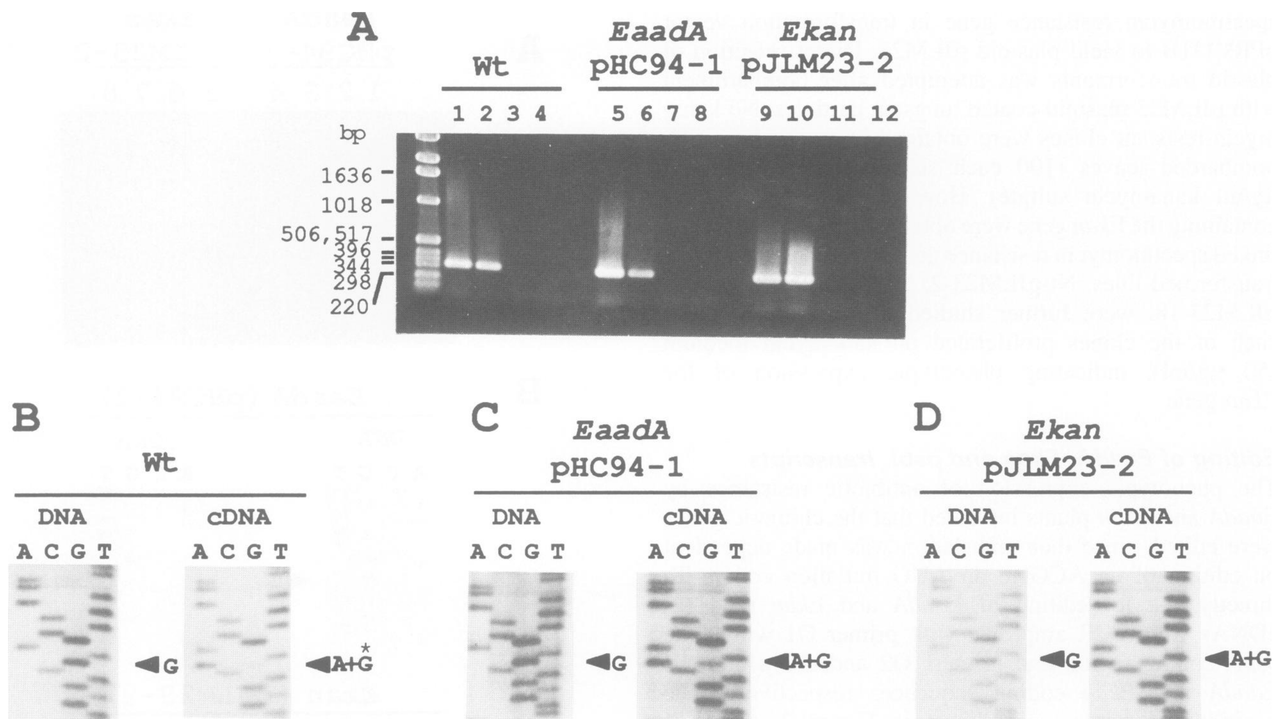

Fig. 4. Editing of the *psbL* mRNA in the transgenic plants. (**A**) PCR amplification products from DNA (lanes 1, 5 and 9) and cDNA (lanes 2, 6 and 10) from wild-type, Nt-pHC94-1 (E*aadA*) and Nt-pJLM23-2 (E*kan*) plants with primers O1 and O6 (see Figure 1). Controls were amplification reactions carried out with DNase I-treated RNA (lanes 3, 7 and 11) and buffer only (lanes 4, 8 and 12). The DNA and cDNA sequence of *psbL* in (**B**) wild-type, (**C**) Nt-pHC94-1 (E*aadA*) and (**D**) Nt-pJLM23-2 (E*kan*) plants. The amplified products were directly sequenced with primer O7. The sequence shown is complementary to the mRNA sequence due to the polarity of the O7 primer. The editing site is indicated by an arrowhead. Note nearly complete editing in the wild-type (G* is very faint) and partial editing in the transgenic plants.

Table II. List of tested editing sites in wild-type and transgenic plants

Editing site	Codon no.		Codon (amino acid)		Reference
	Maize	Tobacco	Unedited	Edited	
rpoB site I	156	158	TCG (Ser)→	TTG (Leu)[a]	Zeltz *et al.*, 1993
rpoB site II	182	184	TCA (Ser)→	TTA (Leu)	Zeltz *et al.*, 1993
ndhB site I	156	156	CCA (Pro)→	CTA (Leu)	Maier *et al.*, 1992
ndhB site II	196	196	CAT (His)→	TAT (Tyr)	Maier *et al.*, 1992

[a]In tobacco, a TCA codon is edited to a TTA codon.

mRNAs are ~1.1 kb in size. To quantify the accumulation of these transcripts, we employed differential DNA probes on Northern blots (Figure 5). Probing with the *psbJ* coding sequence fragment indicated that the 1.1 kb *psbE* operon mRNA, which contains the *psbJ* and *psbL* reading frames, accumulates to a similar extent in the wild-type and transformed plants (Figure 5B, lower). The O14 oligonucleotide probe hybridizes to the mRNA containing the Δ*psbF*/Δ*psbL* region present in both the *psbE* operon and the chimeric E*aadA* and E*kan* transcripts. The O14 probe detected approximately four times more RNA in the transgenic plants, indicating a 1:3 ratio of the polycistronic *psbE* to chimeric mRNAs (Figure 5B, upper).

Editing of other mRNAs are not affected in the transgenic plants

Increased demand for *psbL* editing in the transgenic plants led to a reduction in its editing efficiency. We tested if editing of other mRNAs is also affected in the transgenic plants. Two sites were tested in the *rpoB* and two in the *ndhB* transcripts (Table II). The *rpoB* and *ndhB* editing sites were originally reported for maize and confirmed for tobacco in this study.

Editing sites I and II of *rpoB* are almost fully edited in wild-type tobacco (Figure 6), as has been observed for maize and barley (Zeltz *et al.*, 1993). Similarly, sites I and II of the *ndhB* transcript are fully edited in wild-type tobacco (Figure 6), as reported for maize (Maier *et al.*, 1992). The editing efficiency for the same sites was tested in three lines each of the E*aadA*- and E*kan*-expressing plants. No significant difference in the editing efficiency between wild-type and transformed plants was found for any of the four sites. Data in Figure 6 are shown for a Nt-pHC94-1 plant, one of the E*aadA*-expressing lines. Lack of change in the editing efficiency at any of the sites other than *psbL* indicates that expression of the chimeric genes specifically compromises the editing efficiency of the *psbL* site.

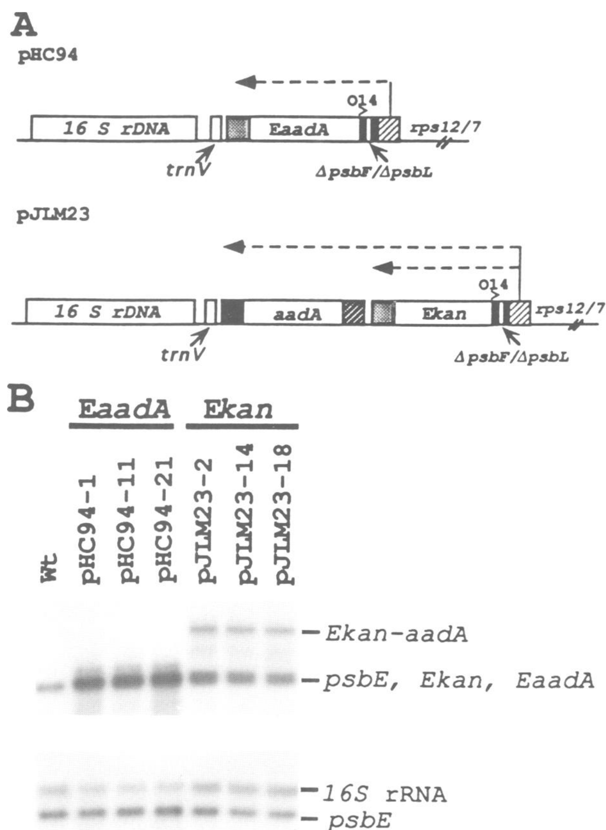

Fig. 5. Steady-state levels of *psbL* and chimeric mRNAs. (**A**) Partial map of the plastid genome with the E*aadA* and E*kan* genes obtained by transformation with the pHC94 or pJLM23 plasmids. The *16SrDNA* and *trnV* genes and the *rps12/7* operon are marked. Horizontal arrows indicate mRNAs detected by the O14 oligonucleotide probe.
(**B**) (Upper) The O14 oligonucleotide detects the similar size (1.1 kb) *psbE*, E*aadA* and E*kan* and the 2.2 kb E*kan–aadA* transcripts. Additional, minor uncharacterized RNA species are also visible, which were not included in the quantitation. Total cellular RNA (2 µg/lane) was loaded from a wild-type plant (Wt), plasmid pHC94-transformed plants (Nt-pHC94-1, Nt-pHC94-11 and Nt-pHC94-21) and plasmid pJLM23-transformed plants (Nt-pJLM23-2, Nt-pJLM23-14 and Nt-pJLM23-18). (Lower) Accumulation of *psbE* mRNA detected by the *psbJ* probe and of 16S rRNA as the loading control. The filter was stripped of the labeled O14 oligonucleotide and probed with a mixture of the *psbJ* and 16SrDNA probes. The *psbE* probe was obtained by PCR amplification of the *psbJ* region with primers O15 and O16 (Figure 1). The 16SrDNA probe was a 2.4 kb *Eco*RI–*Eco*RV ptDNA fragment defined by the restriction sites at nucleotides 138448 and 141847 of the plastid genome (Shinozaki *et al.*, 1986).

Discussion

Experiments reported here are the first demonstration of the editing of chimeric mRNAs in plastids. Editing of both E*aadA* and E*kan* transcripts indicates that 98 and 101 nt respectively of the Δ*psbF*/Δ*psbL* fragment are sufficient to direct editing at the *psbL* site. Accumulation of E*aadA* or E*kan* mRNA at levels ~3-fold above that of the *psbE* polycistronic message containing the *psbL* reading frame led to a significant (>10-fold) increase in the level of unedited *psbL* transcript. An increase in the level of unedited *psbL* mRNA from <1% to ~10% did not have any deleterious consequence that could have been detected at the phenotypic level.

The chimeric mRNAs were also partially edited in the transgenic plants. The somewhat lower editing efficiency of the chimeric transcripts (~70%) compared with the *psbE* transcripts (~90%) suggests that sequences outside the 98 nt Δ*psbF*/Δ*psbL* fragment may be required for maximum editing efficiency.

Partial editing of both *psbL* and chimeric mRNAs suggests depletion of a limiting *trans*-acting factor(s) that is required for editing of the shared site. However, the editing efficiency of four other sites was unaffected, suggesting that the depleted factor is specifically required for editing of the *psbL* transcript and is not a component of the general editing machinery. It is therefore conceivable that each of the editing sites in the chloroplast genome requires some factors for editing that are unique to them. This conclusion is reinforced by the lack of any obvious sequence motif common to the 98 nt Δ*psbF*/Δ*psbL* fragment and sequences surrounding the other four tested editing sites. Therefore, it appears likely that the editing of these sites is directed by sequences and factors that are unique to each.

As an alternative to depletion of a site-specific factor, existence of 'strong' and 'weak' editing sites was also considered. Accordingly, the *psbL* site would be weak and its editing frequency would be lowered by the presence of excess chimeric RNA competing for a limiting but common editing factor, whereas the others would be strong sites that remain unaffected. We consider this explanation unlikely, based on other data in the literature, which are consistent with the existence of site-specific editing factors in plastids. The *psbF* mRNA is edited in spinach plastids by a C→U conversion, changing a serine to a conserved phenylalanine codon. In tobacco at this position a phenylalanine codon is already present at the DNA level. When the tobacco *psbF* gene was modified to match the spinach sequence, the heterologous editing site was unedited, although the adjacent *psbL* site is edited in both species (Bock *et al.*, 1994). It appears, therefore, that tobacco lacks the capacity to edit the spinach *psbF* mRNA, while maintaining the capacity to edit the *psbL* site which is common to both species. Another case consistent with site-specific editing is site IV of *rpoB* mRNA, which is edited in maize but not in barley, although the sequences surrounding the site are highly conserved. Interestingly, the editing of three other sites in the same transcript is conserved between the two species (Zeltz *et al.*, 1993). These observations suggest that the editing capacity of an individual site may be lacking without affecting the editing capacity of other sites, supporting site-specific editing in plastids. Further experiments will have to identify the molecular nature of the site-specific editing factors.

Materials and methods

Construction of chimeric genes

The E*kan* gene (Figure 2B) in plasmid pJLM18 was constructed in a pBluescript KS+ plasmid (Stratagene). The E*kan* coding region in pJLM18 is expressed in the P*rrn*/T*rps16* cassette. The P*rrn* 5′ regulatory region consists of the plastid rRNA operon promoter and a ribosome binding site and is on an *Eco*RI–*Nco*I fragment. P*rrn* derives from plasmid pZS195, the progenitor of plasmid pZS197 (Svab and Maliga, 1993) in which the translational initiation codon (ATG) is included in the *Nco*I site. The *Nco*I site of P*rrn* was ligated to the *Bsp*HI site of a *Bsp*HI–*Xba*I fragment; the *Nco*I–*Bsp*HI fusion eliminated both restriction sites. The *Bsp*HI–*Xba*I oligonucleotide was obtained by annealing the overlapping 5′-CATTCATGACTTTGGGATCAATATCAGCATATG-CAGTTCATCCAACGATAAACTTAATCCGAATTATAGAGC-3′ and

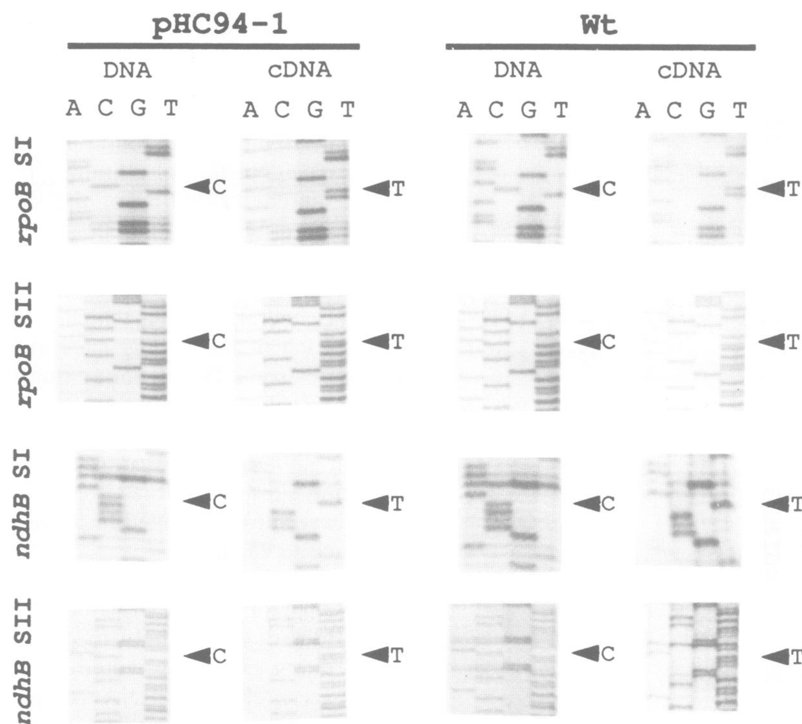

Fig. 6. Editing of the *rpoB* and the *ndhB* transcripts in the wild-type and transgenic Nt-pHC94-1 plants. The DNA and cDNA sequences corresponding to each gene were PCR amplified using the following primers: O8 and O9 for *rpoB*; O10 and O11 for *ndhB*. The sequencing primer for *rpoB* was O8, for *ndhB* O1. The editing site in the sequence is marked by an arrowhead. The arrowhead points at the C in DNA which is edited to T at sites I and II of the *rpoB* and *ndhB* transcripts.

5′-CGGTCTGAATTCAATTCAACATTTTGTTCGTTCGGGTTTGA-TTGTGTCGTAGCTCTATAATTCGGATTAAG-3′ single-stranded oligonucleotides and extension with the Klenow fragment of DNA polymerase I. The *Bsp*HI–*Xba*I fragment contains the sequence framed in Figure 2B, including the Δ*psbF*/Δ*psbL* sequence encoding the C-terminal end of *psbF*, the intergenic region and the N-terminal portion of *psbL*. As the result of the *Nco*I–*Bsp*HI fusion, the C-terminal end of *psbF* is translated from the P*rrn* translation initiation codon (ATG). To translationally fuse the 14 N-terminal codons of *psbL* with the *kan* coding region, the *Xba*I single-stranded overhang of the *Bsp*HI–*Xba*I fragment and the single-stranded overhang of the *Nco*I site of *kan* (including the translation initiation codon) was removed by mung bean nuclease treatment and subsequently ligated. The *kan* coding region derives from plasmid pTNH4 as an *Nco*I–*Xba*I-fragment (Carrer *et al.*, 1993). The T*rps16* fragment is contained within an *Xba*I–*Hin*dIII fragment and was linked to the E*kan* coding region via the *Xba*I site. The T*rps16* fragment contains the *rps16* gene 3′ regulatory region between nucleotides 5087 and 4939 in the ptDNA (Shinozaki *et al.*, 1986). The *Xba*I site at the 5′-end of the fragment was created by oligonucleotide-directed mutagenesis; the 3′-end of the fragment was excised from the plastid genome at an *Eco*RI site at nucleotide position 4938. (Staub and Maliga, 1994). The *Eco*RI site was subsequently converted to a *Hin*dIII site by linker ligation. For introduction into the plastid genome, the E*kan* construct was cloned as an *Eco*RI–*Hin*dIII fragment in the multiple cloning site of plastid vector pPRV111B (Zoubenko *et al.*, 1994), which is adjacent to a selectable *aadA* gene.

The E*aad*A gene (Figure 2A) in plasmid pJLM20 was constructed in a pBluescript KS+ plasmid as described for the E*kan* gene. The *Nco*I–*Xba*I fragment containing the *aadA* coding region is derived from plasmid pHC1 (Carrer *et al.*, 1991) and the *aadA* coding region is translationally fused with the 12 N-terminal codons of the tobacco *psbL* gene. For introduction into the plastid genome, the E*aad*A gene was cloned in plastid insertion vector pPRV100B (Zoubenko *et al.*, 1994). The pPRV100B vector carries a multiple cloning site flanked by ptDNA sequences, but no selectable plastid marker gene.

Plastid transformation and plant regeneration

Tobacco (*Nicotiana tabacum* cv. Petit Havana) plants were grown aseptically on agar-solidified medium containing MS salts (Murashige and Skoog, 1962) and sucrose (30 g/l). Leaves were placed abaxial side up on RMOP media for bombardment. The RMOP medium consists of MS salts, N^6-benzyladenine (1 mg/l), 1-naphthaleneacetic acid (0.1 mg/l), thiamine (1 mg/l), inositol (100 mg/l), agar (6 g/l), pH 5.8, and sucrose (30 g/l). The DNA was introduced into chloroplasts on the surface of 1 μm tungsten particles using the DuPont PDS1000He Biolistic gun (Maliga, 1995). Spectinomycin-resistant clones were selected on RMOP medium containing 500 μg/ml spectinomycin dihydrochloride. Resistant shoots were regenerated on the same selective medium and rooted on MS agar medium (Svab and Maliga, 1993). Kanamycin-resistant clones were selected on RMOP medium containing 50 or 100 μg/ml kanamycin sulfate (Carrer *et al.*, 1993).

RNA gel blot analysis

Total RNA was extracted using TRIzol (Gibco BRL). RNA was electrophoresed in formamide-containing 1% agarose gels and transferred to nylon membrane (Amersham). Hybridization to ^{32}P-end-labeled oligonucleotide probe O14 was carried out in 6× SSPE, 0.5% SDS, 10× Denhardt's solution, 100 mg/ml tRNA, 0.1% sodium pyrophosphate at 45°C. Hybridization to random primed (Boehringer Mannheim) ^{32}P-labeled DNA fragment probes was carried out at 65°C in rapid hybridization buffer (Amersham). RNA levels in samples that hybridized to the probes were quantitated by PhosphorImager analysis (Molecular Dynamics).

cDNA synthesis and PCR amplification

Total cellular DNA was isolated according to Mettler (1987). Total cellular RNA was extracted using TRIzol (Gibco BRL). Reverse transcription of proteinase K- and DNase I-treated RNA samples were carried out as described by Kudla *et al.*(1992). DNA and cDNA were amplified by PCR according to standard protocols: 1 min at 92°C, 2 min at 55°C, 1.5 min at 72°C, 30 cycles.

DNA sequencing

The PCR amplification products were separated in 1.5% agarose gels and purified using the Geneclean II kit (BIO 101 Inc.). Direct sequencing of DNA was performed as described (Bachmann *et al.*, 1990) using the Sequenase kit (US Biochemicals) and the detergent Nonidet P-40.

List of primers

O1 5′-CAATATCAGCAATGCAGTTCATCC-3′
O2 5′-CCAAGCGATCTTCTTCTTGTCCAA-3′

O3 5′-GCGCTCGATGACGCCAAC-3′
O4 5′-CACGACGAGATCCTCGCCG-3′
O5 5′-GAATAGCCTCTCCACCCA-3′
O6 5′-GGAATCCTTCCAGTAGTATCGGCC-3′
O7 5′-GGAAAATAAAACAGCAAGTAC-3′
O8 5′-CAAATATTGCAAAGTCCCGG-3′
O9 5′-CCGGATCGCCACCTACAC-3′
O10 5′-TGGCTATAACAGAGTTTCTC-3′
O11 5′-GGATTTCCAGAAGAAGATGCC-3′
O14 5′-GTTCGTTCGGGTTTGATTGTG-3′
O15 5′-GAACTCAACGGGCCCTTCCCC-3′
O16 5′-GGAGGGAAGTGGAGTAAATGGCCG-3′

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References

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

Bock,R. and Maliga,P. (1995) *In vivo* testing of a tobacco plastid DNA segment for guide RNA function in *psbL* editing. *Mol. Gen. Genet.*, in press.

Bock,R,, Hagemann,R., Kossel,H and Kudla,J (1993) Tissue- and stage-specific modulation of RNA editing of the *psbF* and *psbL* transcript from spinach plastids—a new regulatory mechanism? *Mol. Gen. Genet.*, **240**, 238–244.

Bock,R., Kossel,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.

Carillo,N., Seyer,P., Tyagi,A. and Herrmann,R.G. (1986) Cytochrome b-559 genes from *Oenothera hookeri* and *Nicotiana tabacum* show a remarkably high degree of conservation as compared to spinach. The enigma of cytochrome b-559: highly conserved genes and proteins but no known function. *Curr. Genet.*, **10**, 619–624.

Carrer,H., Staub,J.M. and Maliga,P. (1991) Gentamycin resistance in *Nicotiana* conferred by AAC(3)-I, a narrow substrate specificity acetyltransferase. *Plant Mol. Biol.*, **17**, 301–303.

Carrer,H., Hockenberry,T.N., Svab,Z. and Maliga,P. (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. *Mol. Gen. Genet.*, **241**, 49–56.

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

Gray,M.W. and Covello,P.S. (1993) RNA editing in plant mitochondria and chloroplasts. *FASEB J.*, **7**, 64–71.

Higuchi,M., Single,F.N., Kohler,M., Sommer,B., Sprengel,R. and Seeburg,P.H. (1993) RNA editing of AMPA receptor subunit GluR-B: a base-paired intron–exon structure determines position and efficiency. *Cell*, **75**, 1361–1370.

Hodges,P. and Scott,J. (1993) Editing of mammalian apolipoprotein B mRNA by site-specific RNA deamination. In Benne,R. (ed.), *RNA Editing. The Alteration of Protein Coding Sequences of RNA.* Ellis Horwood, New York, NY, pp. 125–151.

Kossel,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 Kueck,U. and Brennicke,A. (eds), *Plant Mitochondria.* VCH Publishers, Weinheim, Germany, pp. 93–102.

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

Maier,R.M., Neckermann,K., Hoch,B., Akhmedov,N.B. and Kossel,H (1992) 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

Maliga,P. (1993) Towards plastid transformation in flowering plants. *Trends Biotechnol.*, **11**, 101–106.

Maliga,P (1995) Biolistic transformation of tobacco cells with nuclear drug resistance genes. In Maliga,P., Klessig,D., Cashmore,A., Gruissem,W. and Varner,J. (eds), *Methods in Plant Molecular Biology—A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 37–54.

Mettler,I.J. (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. *Plant Mol. Biol. Rep.*, **5**, 346–349.

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

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.

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, NY, pp. 53–85.

Staub,J. and Maliga,P. (1994) Translation of *psbA* mRNA is regulated by light *via* the 5′-untranslated region in tobacco plastids. *Plant J.*, **6**, 547–553.

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.

Teng,B.B., Burant,C.F. and Davidson,N.O. (1993) Molecular cloning of the apolipoprotein B messenger RNA editing protein. *Science*, **260**, 1816–1819.

Zeltz,P., Hess,W.R., Neckermann,K., Boerner,T. and Kossel,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.

Zoubenko,O.V., Allison,L.A., Svab,Z. and Maliga,P. (1994) Efficient targeting of foreign genes into the tobacco plastid genome. *Nucleic Acids Res.*, **22**, 3819–3824.

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