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ORIGINAL PAPER

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RNA editing sites in tobacco chloroplast transcripts: editing as a possible regulator of chloroplast RNA polymerase activity

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Abstract Genetic information in chloroplast DNA is sometimes altered at the transcript level by a process known as RNA editing. Sequence analysis of amplified cDNAs for 69 potential editing sites revealed 13 real editing sites in transcripts of 11 tobacco chloroplast genes. Together with those reported previously, these bring the total of edited sites observed in tobacco chloroplast transcripts to 31 (all involve C to U conversion). Alignment of sequences around the 31 editing sites revealed no obvious consensus, apart from an apparent bias for U or C at position -1 and A at position $+2$. Editing in tobacco *rpoA* mRNA restores the conserved leucine residue which is known to be important for transcriptional activation of the α subunit of *E. coli* RNA polymerase. Editing of this site is partial and the extent of editing depends on developmental conditions, suggesting that editing is, at least in part, involved in the regulation of chloroplast-encoded RNA polymerase activity.

Key words Chloroplast · Tobacco · Post-transcriptional regulation · RNA editing · RNA polymerase

Introduction

Many chloroplast genes are transcribed as polycistronic mRNAs which then mature via complex RNA processing pathways that include RNA editing, splicing and cleavage/trimming steps. Editing was first reported in the maize *rpl2* transcript (Hoch et al. 1991) and demonstrated for tobacco *psbL* mRNA (Kudla et al. 1992). In both cases the editing was found to create the initiation codons. Subsequently, RNA editing has been found in internal protein-coding regions and, rarely, in termination codons in various plastid transcripts in all major lineages of land plants (Maier et al. 1992, 1995; Wakasugi et al. 1996; Yoshinaga et al. 1996, 1997; Bock et al. 1997; Freyer et al. 1997). Most editing events in chloroplasts are C to U conversions which lead to the restoration of conserved amino acid residues, suggesting that amino acid residues acquired by editing are important for the function of the encoded protein (e.g. Maier et al. 1995). Indeed, a heterologous (spinach *psbF*) editing site introduced into the tobacco plastid genome remained unmodified, and the lack of RNA editing led to a mutant phenotype (Bock et al. 1994). This finding confirmed that the editing of spinach *psbF* mRNA is essential for protein function.

The RNA editing reaction has been shown to be a rapid post-transcriptional event, which precedes RNA splicing and RNA cutting in *petB* (Freyer et al. 1993; Hirose et al. 1994) and IRF170 (*ycf3*) transcripts (Ruf et al. 1994). Editing in *rpoB* and *rpl2* transcripts was found not to be impaired in ribosome-deficient plastids in the barley mutant *albostrians*, suggesting that it is not dependent on translation in chloroplasts (Zeltz et al. 1993; Hess et al. 1994). On the other hand, Karcher and Bock (1998) reported that elevated temperature and inhibition of chloroplast translation with antibiotics selectively blocked RNA editing at a small number of sites, suggesting a role for the chloroplast translation apparatus in RNA editing. The chloroplast transformation technique (Svab and Maliga 1993) has provided a powerful tool for defining *cis* acting elements required for RNA editing of *psbL* mRNA (Chaudhuri et al. 1995; Chaudhuri and Maliga 1996) and *ndhB* mRNA (Bock et al. 1996, 1997). It has also been reported that editing of tobacco *psbL* and *ndhD* mRNAs, but not *ndhB* mRNA, requires depletable *trans* acting factors. Using

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an interspecific protoplast fusion approach, site recognition factors were suggested to be extraplastidic in origin (Bock and Koop 1997).

As the transplastome approach is available among land plants, only for tobacco, we searched for further editing sites in transcripts from tobacco chloroplasts. Among 13 newly identified sites, one interesting site happens to be in *rpoA* transcripts and it might be involved in the regulation of chloroplast RNA polymerase activity.

Materials and methods

Searches for potential editing sites were carried out using the SwissProt protein sequence database and a HP9000-845 computer (Hewlett-Packard). Tobacco (*Nicotiana tabacum* BY4) leaves were harvested from 4-week-old plants grown in a growth chamber at 28° C under 16 h light/8 h dark. Chloroplast RNA and DNA were prepared from tobacco leaves as described earlier (Tanaka et al. 1987). cDNA synthesis, PCR and direct DNA sequencing were carried out as described (Hirose et al. 1996).

Results and discussion

RNA editing sites in tobacco chloroplast transcripts

Seventy different protein-coding genes and nine different hypothetical chloroplast reading frames (*ycf*s) have so far been identified in the tobacco chloroplast genome (Shinozaki et al. 1986; Wakasugi et al. 1998). Based on the alignment of polypeptide sequences deduced from these genes and eight *ycf*s from tobacco, rice, maize, black pine and liverwort (*ycf15* was omitted because it is present only in tobacco), 69 RNA editing sites (55 C-to-U and 14 U-to-C sites) were anticipated. Direct sequencing of PCR-amplified cDNA fragments (500–1000 bp) derived from tobacco chloroplast transcripts encompassing all of the 69 sites revealed 13 real editing sites in the transcripts from 11 chloroplast genes. Together with those reported previously (Kudla et al. 1992; Hirose et al. 1994, 1996; Neckermann et al. 1994; Chaudhuri et al. 1995; Freyer et al. 1995), this brings the total of RNA editing sites observed in tobacco chloroplasts to 31 (Table 1).

All 31 of these editing events are C-to-U conversions: 29 cause amino acid substitutions, two create initiation codons and one occurs at the third position of a codon in *atpA* mRNA which results in no amino acid alteration. U-to-C inverse editing, which has been reported in higher plant mitochondria (Gualberto et al. 1990; Schuster et al. 1990) and in the chloroplast of the hornwort *Anthoceros formosae* (Yoshinaga et al. 1996, 1997), was not observed in tobacco chloroplasts, despite cDNA sequencing of the 14 predicted U-to-C editing sites. Given the overall number of codons (23,079) in the tobacco chloroplast genome, the percentage of codons changed by editing is 0.13%. This list of editing sites provided here is by no means complete because our search does not take account of editing sites outside the protein-coding regions (5′-UTRs, 3′-UTRs and introns), the third positions of codons, or tRNA/rRNA transcripts. Moreover, RNA editing that results in non-conserved amino acid substitutions also cannot be ruled out.

RNA editing occurs in various transcripts in tobacco chloroplasts; six editing sites in transcripts of five photosynthetic genes (*petB*, *psbE*, *psbL*, *atpA* and *atpF*), 11 sites in transcripts of seven housekeeping genes (*rpoA*, *rpoB*, *rpoC1*, *rpoC2*, *rps2*, *rps14* and *rpl20*) and 14 sites

Table 1 RNA editing sites identified in chloroplast transcripts from tobacco and maize

Gene	Site	Position[a]	Conversion	Tobacco	Maize[b]
rpoA		277	S (uCa) → L (uUa)	+	−
rpoB	1	113	S (uCu) → F (uUu)	+	−
	2	158	S (uCa) → L (uUa)	+	+
	3	184	S (uCa) → L (uUa)	+	+
	4	189	S (uCg) → L (uUg)	−	+
	5	208	P (cCg) → L (cUg)	−	+
	6	667	S (uCu) → F (uUu)	+	−
rpoC1		21	S (uCa) → L (uUa)	+	−
rpoC2	1	925	S (uCg) → L (uUg)	−	+
	2	1248	S (uCa) → L (uUa)	+	−
rps2		83	S (uCa) → L (uUa)	+	−
rps3		61	S (uCa) → L (uUa)	−	+
rps14	1	27	S (uCa) → L (uUa)	+	+
	2	50	P (cCa) → L (cUa)	+	−
rpl2		1	T (aCg) → M (aUg)	−	+
rpl20		103	S (uCa) → L (uUa)	+	+
atpA	1	264	P (cCc) → L (cUc)	+	−
	2	265	S (ucC) → S (ucU)	+	−
	3	383	S (uCa) → L (uUa)	−	+
atpF		31	P (cCa) → L (cUa)	+	−
psbE		72	P (Cca) → S (Uca)	+	−
psbL		1	T (aCg) → M (aUg)	+	−
petB		204	P (cCa) → L (cUa)	+	+
ndhA	1	17	S (uCg) → L (uUg)	−	+
	2	114	S (uCa) → L (uUa)	+	−
	3	159	S (uCa) → L (uUa)	−	+
	4	189	S (uCa) → L (uUa)	−	+
	5	358	S (uCc) → F (uUc)	+	+
ndhB	1	50	S (uCa) → L (uUa)	+	−
	2	156	P (cCa) → L (cUa)	+	+
	3	196	H (Cau) → Y (Uau)	+	+
	4	204	S (uCa) → L (uUa)	+	+
	5	246	P (cCa) → L (cUa)	+	+
	6	249	S (uCu) → F (uUu)	+	−
	7	277	S (uCu) → L (uUa)	+	+
	8	279	S (uCu) → L (uUa)	+	−
	9	494	P (cCa) → L (cUa)	+	+
ndhD	1	1	T (aCg) → M (aUg)	+	−
	2	128	S (uCa) → L (uUa)	+	−
	3	241	S (uCa) → L (uUa)	−	+
ndhF	1	21	S (uCa) → L (uUa)	−	+
	2	97	S (uCa) → L (uUa)	+	−
ycf3	1	15	S (uCc) → F (uUc)	−	+
	2	62	T (aCg) → M (aUg)	−	+
matK		426	H (Cau) → Y (Uau)	−	(+)
5′UTR of *ndhG*		−10	C → U	−	+

[a] Position is given with reference to the first base of the initiation codon as 1

[b] The editing site of maize *matK* gene (+) was proposed by Vogel et al. (1997). Remaining editing sites in maize are from Maier et al. (1995) and Bock et al. (1997) + indicates editing and − indicates no editing (T in the genome)

in four NADH dehydrogenase gene transcripts (*ndhA*, *ndhB*, *ndhD* and *ndhF*) (Table 1). This indicates that RNA editing is a general mRNA maturation process with no apparent correlation with gene function. Among the 31 sites, editing is partial in the *atpA* (site 2) (Hirose et al. 1996), *ndhD* (site 1; Neckermann et al. 1994; Hirose and Sugiura 1997), *rps14* (site 2; Hirose et al. 1998) and *rpoA* (this work) transcripts, resulting in the existence of two forms of these mRNAs. In vitro translation assays have shown that translation efficiency does not differ between edited and unedited mRNAs from tobacco *rps14* (Hirose et al. 1998).

Features of the surrounding sequence of editing sites and codon transitions

To search for possible structural motif(s), sequences surrounding all the 31 editing sites (−50 to +51; +1 is the editing site) were compared (Fig. 1), because the *cis*-acting elements for editing have been reported to reside within 50 nucleotides of editing sites (Bock et al. 1996, 1997; Chaudhuri and Maliga 1996). No obvious consensus sequence nor any secondary structure could be found in these regions. It has been postulated that a depletable *trans* acting factor is involved in the editing of tobacco transcripts from *psbL*, *ndhD* (Chaudhuri and Maliga 1996) and *rpoB* (Reed and Hanson 1997) genes. We favor the idea that site-specific *trans* acting factors are involved in recognition of editing sites.

As noted previously (Maier et al. 1995, 1996; Hanson et al. 1996; Bock et al. 1997), there is an obvious bias toward pyrimidine (C or U; in 29 of the 31 sites) at position −1 and adenosine (22 among the 31 sites) at position +2 (Fig. 2). Exceptions to this bias are the adenosines at −1 in *psbL* and *ndhD* (site 1) mRNAs in which editing creates initiation codons. Mutation of this A to C in tobacco *psbL* mRNAs impairs editing, suggesting that this A is a critical determinant for ACG to AUG editing (Chaudhuri and Maliga 1996). On the other hand, alteration of U to G at −1 in tobacco *ndhB* (site 5) mRNA also inhibits editing, suggesting that a pyrimidine at −1 is important for editing of internal codons (Bock et al. 1996).

Despite the lack of a consensus sequence for the 31 editing sites, we found highly similar sequences (21 nt) surrounding the editing sites in *rpoC2* and *ndhD* (site 2) mRNAs (Fig. 2), suggesting that a common factor might recognize these sites. Computer analysis revealed

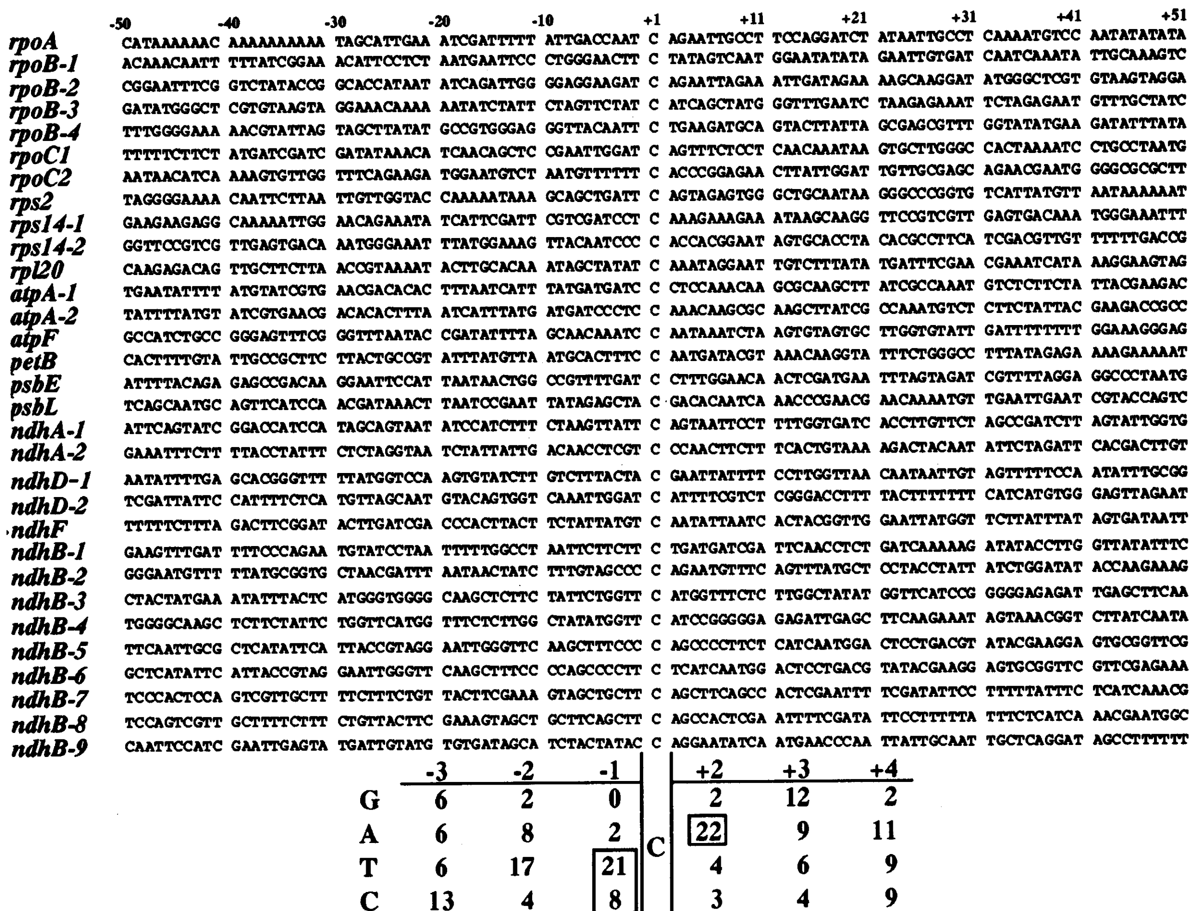

Fig. 1 Alignment of sequences spanning the 31 editing sites identified in tobacco chloroplast transcripts (+1 is editing site). The frequencies of the four nucleotides in positions −3 to +4 are shown *below* the alignment

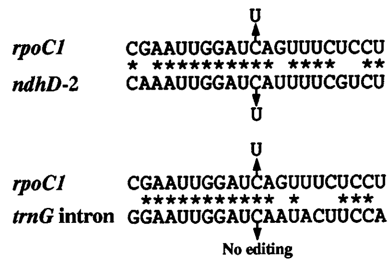

Fig. 2 Sequences around editing sites in *rpoC1* and *ndhD* (site 2) mRNAs and a sequence within the *trnG*(UCC) intron. Editing sites are indicated by *arrows*

a similar sequence within the group II intron of *trnG*(UCC) gene. Sequencing of cDNA fragments derived from the unspliced pre-tRNA^{Gly} showed no editing at this site, indicating that sequence similarity around editing sites is not sufficient for editing. An additional sequence element(s) or secondary structure may be necessary for recognition of editing sites.

Of the 31 editing sites, 28 are located in the second position in the codon (Tables 1 and 2), two at the first position (*psbE* and *ndhB* site 3) and one at the third position (*atpA* site 2). This strong bias leads to preferential amino acid substitution, especially leucine for proline and serine. A similar bias has previously been observed in maize chloroplast transcripts (Maier et al. 1995). However, it was not so obvious in the case of chloroplast transcripts from black pine (Wakasugi et al. 1996).

Comparison of editing sites between tobacco and maize

Twenty-seven editing sites have so far been identified in maize chloroplast transcripts (Maier et al. 1995; Bock

Table 2 Codon transitions caused by RNA editing of tobacco chloroplast transcripts

First base	Second base				Third base
	U	C	A	G	
	Pho ← 3	Ser ←	Tyr ←	Cys	U
U	Pho ← 1, 15	Ser 1	Tyr	Cys	C
	Lau ←	Ser	Stop	Stop	A
	Lau	Ser	Stop	Trp	G
C	Lau	Pro 1	His 1	Arg	U
	Lau ← 1, 6	Pro	His	Arg	C
	Lau ←	Pro	Gln	Arg	A
	Lau	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Mct ← 2	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

The arrows show the direction of the codon transitions with the numbers above the arrows indicating the observed frequencies of the respective transitions

et al. 1997). Comparison of the editing sites between tobacco (dicot) and maize (monocot) shows that 11 sites are common, while the remaining 21 sites in tobacco and 15 sites in maize are not conserved (Table 1). Thus, editing sites are highly variable between these species. Furthermore, editing sites within homologous gene transcripts are different. For example, the *rpoB* transcript of tobacco and maize has four editing sites each, but only two are shared (sites 2 and 3 in Table 1). Reed and Hanson (1997) investigated whether two editing sites (sites 2 and 4 in Table 1) present in maize *rpoB* mRNA could be recognized by the editing machinery of transformed tobacco chloroplasts. The heterologous maize editing site 2 common to maize and tobacco was found to be recognized by the tobacco machinery, but site 4 (unique to maize) was not, indicating that editing factors necessary for recognition of site 4 are not present in tobacco. This result suggests co-evolution of editing sites and cognate editing factors.

Possible significance of RNA editing in the regulatory function of chloroplast RNA polymerase

RNA editing alters the 277th codon from serine to leucine in the mRNA (*rpoA*) for the α subunit of tobacco chloroplast RNA polymerase. The α subunit of *E. coli* RNA polymerase is known to consist of the subunit assembly (N-terminal) and transcription factor contact (C-terminal) domains (Murakami et al. 1996). This leucine residue is located inside the latter domain and is highly conserved among chloroplasts as well as in eubacteria (Fig. 3A). Biochemical investigations of the role of individual amino acid residues in the *E. coli* α subunit revealed that this leucine residue is important for function in transcriptional activation mediated by the cAMP receptor protein (CRP) transcription factors and DNA enhancer (UP) elements, and that the leucine residue is important for protein folding to expose the DNA contact site for direct interaction with UP elements (Murakami et al. 1996). This suggests the importance of editing for regulatory function of chloroplast RNA polymerase. Though no prokaryotic-type transcription factor has been isolated from higher plant chloroplasts, five ORFs encoding putative proteins similar to bacterial transcription regulatory factors were found in the chloroplast genome from the red alga *Porphyra purpuria* (Reith and Munholland 1995). In addition, several upstream DNA enhancer elements have been reported in chloroplast promoters (Christopher et al. 1992; Allison and Maliga 1995; Kim and Mullet 1996).

Editing of this site was, however, found to be partial, with editing occurring in 70% of transcripts in green tobacco leaves and in 50% of cases in cultured non-green BY-2 cells (Fig. 3B). This finding raises the interesting possibility that two forms of chloroplast-encoded RNA polymerases are present in tobacco chloroplasts and suggests that the ratio of the two polymerases is regulated developmentally through RNA

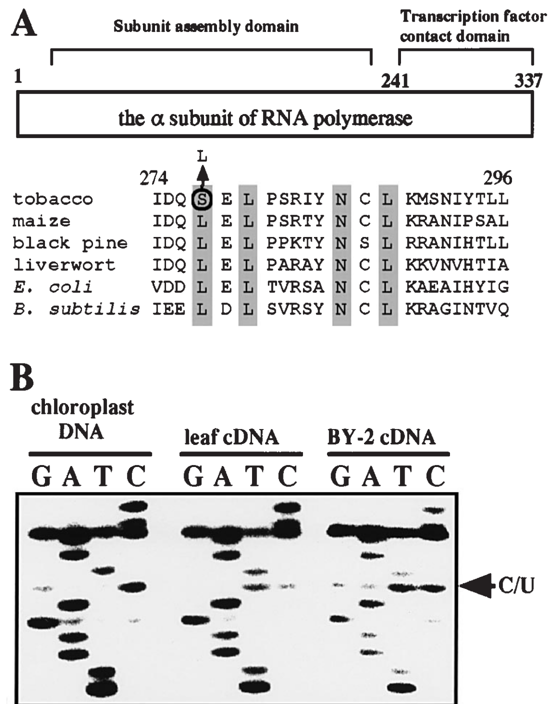

Fig. 3 **A** Schematic representation of the functional domains of the RNA polymerase α subunit based on that in *E. coli* (Murakami et al. 1996). Amino acid sequences around the editing site are shown *below*. Conserved residues are *shaded*. **B** Sequencing ladders showing partial editing of tobacco *rpoA* transcripts in green leaves and nonphotosynthetic BY-2 cells. The editing site is indicated by an *arrow*

editing. The α subunit possibly synthesized from the unedited mRNA may lack the ability to interact with putative transcription factors and UP-like elements. This is reminiscent of the *Chlorella* chloroplast *rpoA* gene, which is short and lacks a region corresponding to the C-terminal domain (Wakasugi et al. 1997). The overall transcription rate from consensus-type promoters may be regulated in part by the ratio of two forms of chloroplast-encoded RNA polymerases in tobacco chloroplasts.

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