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Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype

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The *psbF* mRNA is edited in spinach plastids by a C to U conversion, changing a serine to a conserved phenylalanine codon. In tobacco at this position a phenylalanine codon is present at the DNA level, and the *psbF* mRNA here is not edited. To test if the *psbF* editing capacity is evolutionarily conserved, the tobacco *psbF* gene was modified to match the corresponding spinach sequence. The endogenous tobacco gene was replaced with the modified copy using biolistic transformation. We report here that the heterologous editing site remains unmodified in transplastomic tobacco plants. The lack of editing is associated with slower growth, lowered chlorophyll content and high chlorophyll fluorescence, a phenotype characteristic of photosynthetic mutants. This finding confirms that the editing of the *psbF* mRNA is an essential processing step for protein function and thus provides direct proof for the biological significance of plant organellar RNA editing. Given that a mutant phenotype is associated with the lack of editing, it seems likely that the evolutionary loss of the site-specific capacity for *psbF* editing was preceded by the mutation that eliminated the editing requirement.

Key words: cytochrome b559/plastid mutant/plastid transformation/*psbF* gene/RNA editing

Introduction

Higher plant organellar mRNAs can be subject to post-transcriptional pyrimidine to pyrimidine conversions, a phenomenon referred to as RNA editing. RNA editing in most instances results in an alteration of the amino acid sequence which is deduced from the DNA sequence. Cytosine to uridine transitions predominate in mitochondrial transcripts (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989; reviewed in Gray and Covello, 1993) and are the only type of modification found in chloroplasts (Hoch *et al.*, 1991; Kudla *et al.*, 1992, for a review see Kössel *et al.*, 1993). While the frequency of RNA editing events is considerably higher in mitochondria than in plastids, the general features of the editing process appear to be similar (Maier *et al.*, 1992). So far, very little is known about the underlying mechanisms and the site-specific sequence requirements.

The phylogenetic data strongly suggest that RNA editing in plant organelles evolved in vascular plants and occurs in both gymnosperms and angiosperms (Gray and Covello, 1993). The fact that many editing sites are not conserved implies that point mutation(s) led to the elimination of specific editing sites in some lineages of higher plants during evolution. A good example for the loss of an editing site is the plastid *psbF* gene which is part of a tetracistronic transcription unit and encodes the β subunit of cytochrome b559, a highly conserved integral membrane protein of photosystem II. The tobacco and the spinach proteins are 100% identical at the amino acid level. We have demonstrated previously in spinach that codon 26 for a phenylalanine residue is created by RNA editing: a C to U conversion at the second codon position changes a UCU triplet (serine) to a UUU triplet (phenylalanine) (Bock *et al.*, 1993). In tobacco, the codon for phenylalanine is already present at the DNA level (Shinozaki *et al.*, 1986). The evolutionary loss of the *psbF* RNA editing site may or may not be accompanied by the loss of the editing capacity for this particular site. To test if the *psbF* editing capacity is evolutionarily conserved, the spinach editing site was introduced into the tobacco *psbF* gene. We report here that tobacco plastids lack the capacity to edit the spinach site resulting in a mutant phenotype. This experiment provides the first direct proof for the biological significance of mRNA editing in higher plant organelles.

Results

Introduction of a spinach RNA editing site into the tobacco plastid genome

The *psbF* gene is part of the plastid *psbE* operon and is located upstream of the *psbL* coding region as shown in Figure 1A. The initiation codon of the *psbL* mRNA is created by RNA editing in both tobacco and spinach (Kudla *et al.*, 1992; Bock *et al.*, 1993). In contrast, codon 26 of the *psbF* reading frame is edited in spinach, but not in tobacco plastids (Figure 1B). The spinach *psbF* editing site and the surrounding sequence motif from −16 to +18 were introduced into the tobacco *psbF* gene in a cloned fragment by oligonucleotide-directed mutagenesis. This involved mutations at three different nucleotide positions. One of the point mutations led to the elimination of a *Sau*3AI site normally present in the wild-type tobacco sequence (Figure 1B), generating a screenable RFLP marker. To facilitate direct selection, a spectinomycin resistance (*aadA*) gene was cloned downstream of the *psbE* operon. As a result, the *aadA* gene in plasmid pRB8 is flanked by the *psbE* operon and ptDNA sequences downstream of the *petA* gene (Figure 1A). The mutant *psbF* gene was introduced into the tobacco plastid genome by bombardment of tobacco leaves with plasmid pRB8-coated tungsten particles and selection for the linked spectinomycin resistance gene. Spectinomycin-resistant

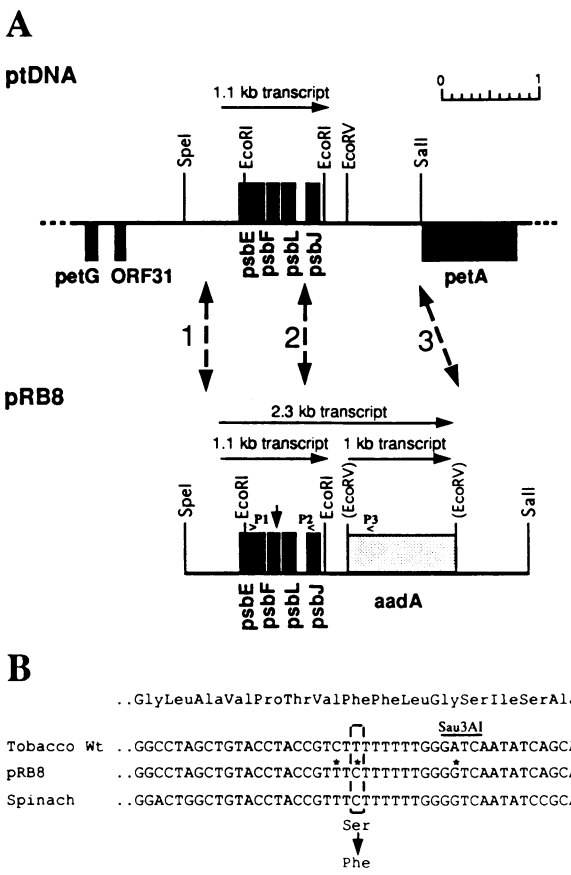

Fig. 1. Experimental design to test the conservation of the *psbF* editing capacity in tobacco plastids. (**A**) Introduction of the mutant *psbF* gene into the tobacco plastid genome. Plastid DNA fragment in plasmid pRB8 and the region of the plastid genome containing the cognate sequences (ptDNA) are shown. The *psbE/F/L/J* gene cluster is depicted with adjacent genes *petG*, ORF31 and *petA* (based on Shinozaki *et al.*, 1986). The *aadA* gene confers resistance to spectinomycin. Genes above the line are transcribed from the left to the right; genes below the line are transcribed in the opposite direction. Transcripts are indicated by horizontal arrows. The vertical arrow marks the mutant *psbF* region shown in (B). P1, P2 and P3 (arrowheads) indicate the position and orientation of the PCR primers. Potential recombination sites (1, 2 and 3) that yield different transplastomes are marked by dashed lines (see text). (**B**) Partial alignment of *psbF* genes. The region containing the editing site in spinach is shown for tobacco (Wt), plasmid pRB8 with the engineered *psbF* gene and spinach. In spinach, the UCU to UUU change results in the incorporation of phenylalanine instead of serine, while in tobacco a UUU codon is encoded at the DNA level. The nucleotides which were changed by mutagenesis are marked by asterisks. Note the *Sau*3AI site in wild-type tobacco which is removed by mutagenesis in plasmid pRB8.

plants were obtained as a result of the incorporation of the *aadA* gene by homologous recombination via the flanking ptDNA sequences. Of the 19 spectinomycin-resistant clones obtained, 16 were verified as plastid transformants by resistance to both spectinomycin and streptomycin (Svab and Maliga, 1993) and by PCR analysis using *aadA*-specific primers (data not shown). Incorporation of the mutant *psbF* region by two homologous recombination events, such as sites 1 and 3 in Figure 1A, was confirmed in three out of 12 clones. The Nt-pRB8-S6, Nt-pRB8-S7 and Nt-pRB8-S11 clones (subsequently referred to as S6, S7 and S11) were initially identified by the absence of the *Sau*3AI site in PCR-amplified DNA fragments. Data for the S6 and S7 clones

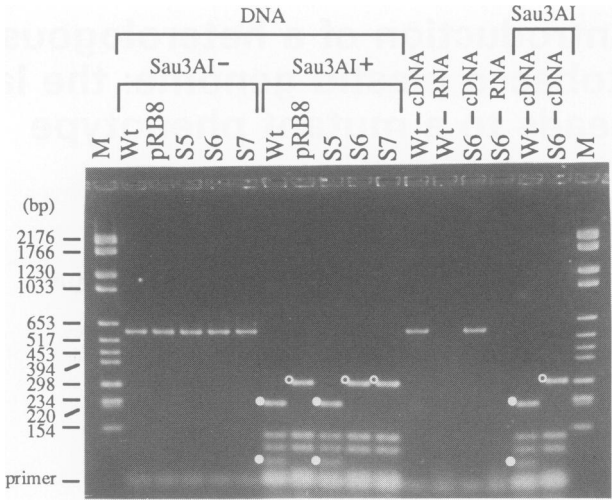

Fig. 2. Identification of transplastomic lines by PCR analysis. Primer pair P1 and P2 (Figure 1A) were used to amplify a 589 bp fragment including the *psbF* and *psbL* genes. DNAs from wild type tobacco plants (Wt), vector pRB8 and plants transformed with vector pRB8 (S5, S6 and S7) yield identical fragments. Digestion of the amplification products with *Sau*3AI facilitated the classification of plastid transformants: the clones with one less *Sau*3AI site (295 bp fragment, open circle) were likely to carry each of the closely-linked *psbF* mutations (S6 and S7), while maintenance of the tobacco-specific *Sau*3AI site (219 and 76 bp fragments; filled circles) indicated a wild-type *psbF* gene (clone S5). The absence of the diagnostic *Sau*3AI site in the plastid transformant S6 was also confirmed at the cDNA level (Wt-cDNA, S6-cDNA, with and without *Sau*3AI digestion). Control reactions were carried out with RNA instead of cDNA as template to prove the absence of contaminating DNA (Wt-RNA and S6-RNA lanes).

are shown in Figure 2. A uniformly transformed plastid genome population is indicated by the complete absence of the diagnostic *Sau*3AI site in the PCR-amplified DNA samples. Incorporation of each of the three point mutations was subsequently confirmed by DNA sequencing (Figure 4). The editing of *psbF* mRNA was studied in homoplasmic S6 and S7 plants. In the remaining nine clones the lack of the diagnostic *Sau*3AI site indicated that the incorporation of the *aadA* gene was not accompanied by the incorporation of the mutant *psbF* gene. Such transplastomes may have formed by two homologous recombination events at sites such as 2 and 3 in Figure 1A. One such transplastomic line, Nt-pRB8-S5 (referred to as S5), was used as a control in the editing study.

The three spectinomycin-resistant, streptomycin-sensitive clones are spontaneous mutants (Svab and Maliga, 1993).

Transcript pattern and RNA editing in the transplastomic lines

The *psbE* probe detects two major mRNAs in the transplastomic lines: a 1.1 kb tetracistronic mRNA which is also present in the wild-type plants and a 2.3 kb transcript specific for the transformed lines (Figure 3). The occurrence of the 2.3 kb band is independent of the incorporation of the mutated *psbF* gene into the chloroplast genome but is correlated with the presence of the chimeric *aadA* gene. This additional transcript is the product of readthrough transcription through the 3′ region of the *psbE* operon. It includes the *aadA* coding region (Figure 1A) and is stabilized by the 3′ untranslated region of the chimeric *aadA* gene.

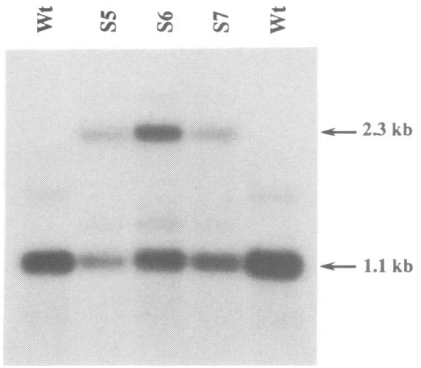

Fig. 3. RNA gel blot to detect transcripts for the *psbE* operon. Data are shown for wild type (Wt), and the S5, S6 and S7 plastid transformants. Total cellular RNA was probed with the radiolabeled PCR product obtained by amplification with primers P1 and P2 (Figure 1A). The most abundant 1.1 kb transcript spans all four cistrons of the operon. The 2.3 kb readthrough transcript is stabilized by the 3′ untranslated region of the chimeric *aadA* gene. A schematic transcription map is shown in Figure 1A. Minor visible RNA species have not been characterized.

To test if editing of the mutant *psbF* sequence occurs, cDNAs were amplified with primers P1 and P2 within the *psbE* and *psbJ* coding regions, respectively (Figure 1A). These primers amplify almost the entire *psbE* operon in both the 1.1 and 2.3 kb transcripts. Direct sequencing of the PCR products revealed identical sequences in the genomic DNA and in the cDNA, confirming that the spinach *psbF* sequence motif is not edited in tobacco plastids (Figure 4). As a control, editing of the *psbL* initiation codon was also tested. This site is normally edited in both spinach and tobacco. Data shown in Figure 4 indicate that the C to U conversion within the *psbL* start codon is unaffected by the changes introduced into the adjacent *psbF* gene.

Editing was also tested by sequencing cDNA derived from the S5-transformed line which carries the wild-type *psbF* gene due to separation of the *aadA* and the mutant *psbF* gene during the integration process. DNA sequence data for the S5 plants are identical with those obtained for wild-type plants (data not shown).

Accumulation of the novel 2.3 kb readthrough transcript in the transplastomic lines allowed us to test whether or

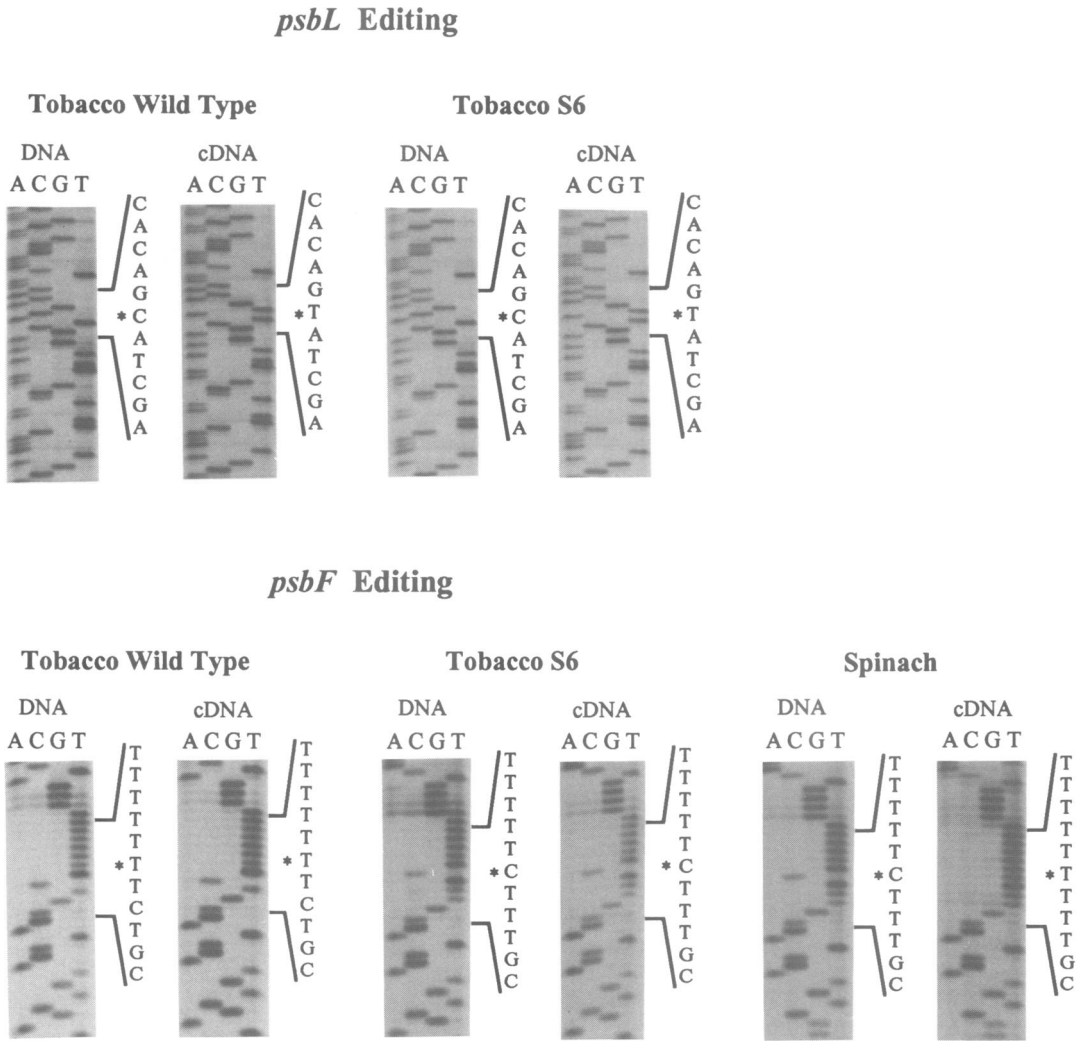

Fig. 4. DNA sequence of PCR-amplified fragments to test for editing of *psbL* (top row) and *psbF* (bottom row) mRNAs. DNA and cDNA sequences are shown alongside each sample. The nucleotide at the editing position is marked by an asterisk. Data are shown for wild-type and transplastomic (S6) tobacco and spinach. The *psbL* mRNA is fully edited, as indicated by a T residue in the cDNA sequence instead of the C residue in the genomic DNA sample. The *psbF* mRNA is fully edited in spinach (T in the cDNA sequence instead of C in the genomic DNA), but is not edited in the transplastomic tobacco (C in both genomic and cDNA). In the wild-type tobacco there is a T residue in the genomic and cDNA at the same position.

not the editing of the *psbL* site in this longer mRNA is equally efficient. The cDNAs in this case were amplified with primers in the *psbE* and *aadA* coding regions, thus excluding the 1.1 kb mRNA from amplification. The complete C to U conversion at the editing site within the *psbL* initiation codon indicates that the 2.3 kb pentacistronic mRNA is an equally efficient substrate for RNA editing (data not shown). This contrasts with the finding that stable unprocessed mRNAs are a less efficient substrate for mitochondrial RNA editing in a rice cytoplasmic male sterile line (Iwabuchi *et al.*, 1993).

The lack of RNA editing leads to a mutant phenotype

RNA editing within the spinach *psbF* mRNA results in the restoration of a highly conserved phenylalanine residue that is present in the β subunit of cytochrome b559 of all photosynthetic organisms investigated to date (Bock *et al.*, 1993). Translation of the unedited *psbF* mRNA in the transplastomic tobacco plants should result in an amino acid substitution from phenylalanine to serine. Homoplasmic-transformed plants showed no visible phenotype when grown on sucrose-containing medium under low light conditions (2500 lux). When transferred to soil and kept under greenhouse conditions, the plants differed from the wild-type control by a significantly slower growth and a light-green leaf color (Figure 5A and B).

Illumination of leaves with UV light results in a strong 'high chlorophyll fluorescence' (hcf) phenotype (Figure 6; Miles, 1980), indicating a deficiency in photosynthetic electron transport. The hcf phenotype (and spectinomycin resistance) is maternally inherited in the seed progeny, as expected for a plastid trait (data not shown).

Plants from each of the three independently transformed lines carrying the spinach *psbF* editing site exhibited the hcf phenotype. Since the plants representing the nine transformed lines which carry the chimeric *aadA* gene but not the engineered *psbF* gene are indistinguishable from wild-type plants, we conclude that the lack of *psbF* mRNA editing causes the mutant phenotype.

Discussion

Testing of the spinach *psbF* RNA editing site in a heterologous *in vivo* system revealed the absence of an editing capacity for this site in tobacco plastids. Apparently, additional *cis*- or *trans*-acting factors are necessary to elicit the editing reaction. In view of the strong sequence conservation of the entire *psbE* operon in species both with and without the *psbF* editing site (Carillo *et al.*, 1986; Kudla *et al.*, 1992; Bock *et al.*, 1993), a missing *trans*-acting factor seems to be a more likely explanation. We suggest that the evolutionary loss of the *psbF* editing site allowed the subsequent loss of such a factor in the absence of selective pressure.

'Loss of function mutations' within the *psbF* gene have been shown to abolish photosystem II function completely (Pakrasi *et al.*, 1991). Although deficient in activity, the tobacco plants homoplasmic for the spinach *psbF* editing site are still photosynthetically competent. We propose that the amino acid substitution caused by the lack of editing leads to a reduced protein activity rather than a complete loss of function. The affected amino acid residue is located within a transmembrane helix and is not involved directly in the heme coordination within cytochrome b559 (Cramer *et al.*, 1990; Pakrasi *et al.*, 1991).

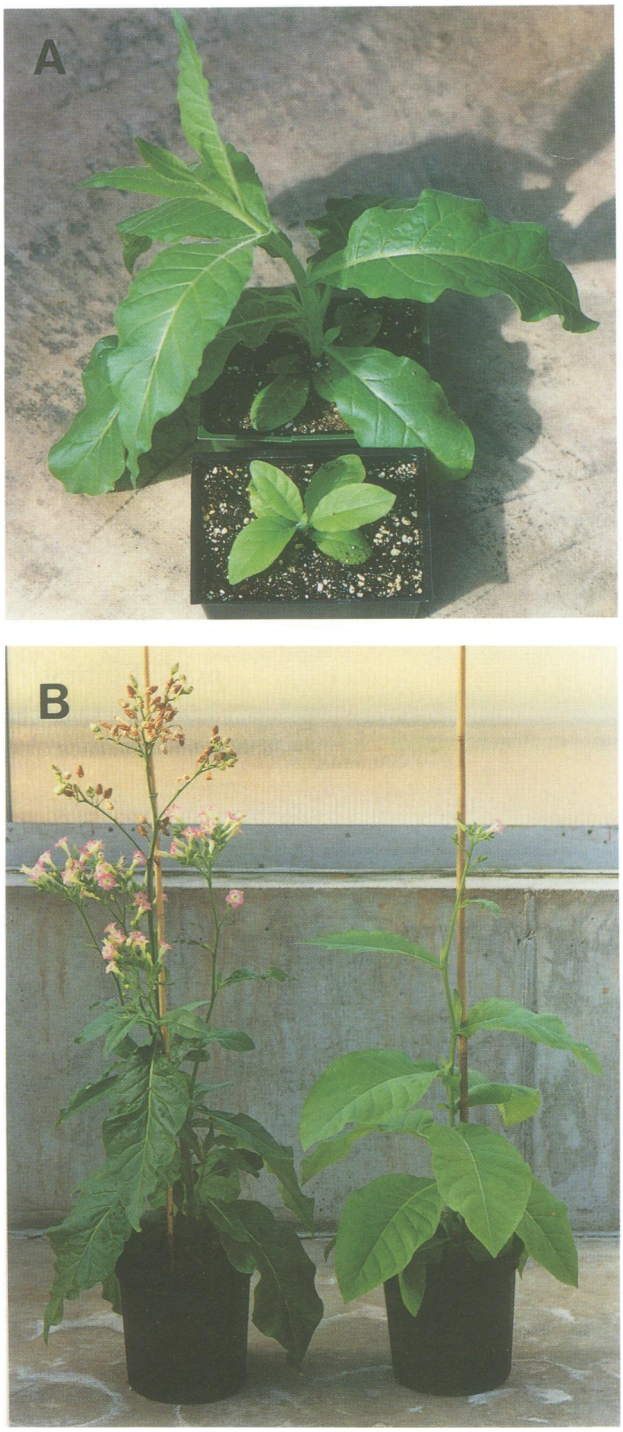

Fig. 5. The phenotype of tobacco plants carrying the spinach *psbF* editing site. (**A**) Comparison of a greenhouse-grown wild-type plant (behind) with a mutant plant (line S6; in front) 3 weeks after transfer to the greenhouse. Both plants were grown from cuttings rooted on MS medium and planted at the same time. (**B**) The same plants 3 months after transfer to the greenhouse. Note the lighter green leaf color and the delayed development of the transplastomic (right) compared with the wild-type plant (left).

The plants with the hcf phenotype accumulate a 2.3 kb transcript in addition to the wild-type 1.1 kb mRNA.

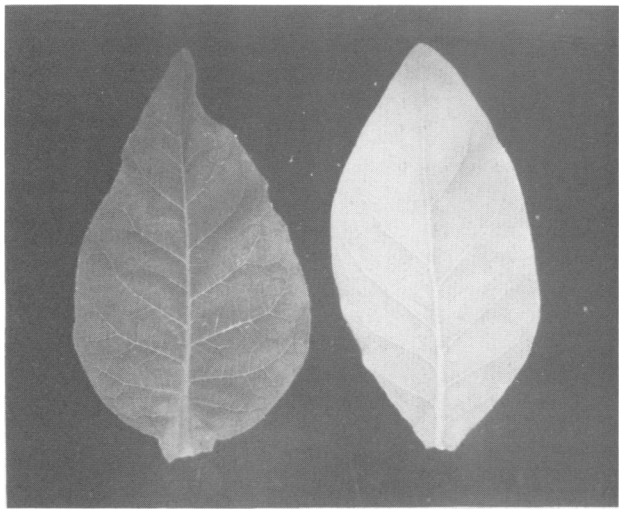

Fig. 6. The high chlorophyll fluorescence phenotype of the S6 plants. Young leaves from a wild-type (left) and a transplastomic plant (right) were illuminated with UV light (320 nm) for 2 s and photographed.

Since this is also the case for the S5-like plastid transformants which show wild-type phenotype with respect to chlorophyll fluorescence, the altered transcript pattern cannot be the cause of the hcf phenotype.

Although mRNA editing usually results in the restoration of codons for conserved amino acids, so far there has been no direct evidence confirming its functional importance for the synthesis of plant organellar proteins. Identical sequence motifs within one mRNA have been shown to undergo editing in one species while they remain unmodified, or only partially modified, in others. For example, in the case of the plastid *rpoB* mRNA, a C to U change in maize results in the incorporation of a leucine instead of a proline, while the absence of editing leads to the incorporation of a proline residue at the same site in barley without an apparent phenotypic consequence (Zeltz *et al.*, 1993). Similar examples have been reported for plant mitochondria (Covello and Gray, 1990; Wissinger *et al.*, 1990). Testing the *psbF* editing site in tobacco directly confirms the functional importance of RNA editing for the synthesis of a plant organellar protein.

Materials and methods

Plant material

Tobacco (*Nicotiana tabacum* cv. Petit Havana) plants were grown under aseptical conditions on agar-solidified MS salt and sucrose (30 g/l)-containing medium (Murashige and Skoog, 1962). Spinach (*Spinacia oleracea* cv. Matador) seedlings were raised on moist cellulose layers and harvested 10 days after germination.

Construction of the transformation vector pRB8

The region containing the *psbE* operon was isolated as a 2384 bp *Sal*I–*Spe*I tobacco plastid DNA fragment in a Bluescript KS$^+$ plasmid (Stratagene). The spinach *psbF* editing site was introduced into a 631 bp *Eco*RI subclone containing part of the *psbE* operon (Figure 1A) by site-directed mutagenesis using the synthetic oligonucleotide 5′-GCTGTACCTACCGTTTCTTTTTTGGGGTCAATATCAGCAATGC-3′. The mutagenized *Eco*RI fragment was sequenced and used to replace the wild-type *Eco*RI fragment in the *Sal*I–*Spe*I clone. A chimeric *aadA* gene was isolated as a 1.1 kb *Ecl*136II–*Dra*I fragment from plasmid pZS197 (Svab and Maliga, 1993) and cloned into the *Eco*RV site (position 66045 of the ptDNA; Shinozaki *et al.*, 1986) between the *psbJ* and *petA* genes (Figure 1B).

Plastid transformation and regeneration of transplastomic plants

Tobacco leaves were bombarded with DNA-coated tungsten microprojectiles using the DuPont PDS1000He Biolistic gun as described earlier (Kanevski and Maliga, 1994; Maliga, 1994). Spectinomycin-resistant shoots were selected on RMOP medium containing 500 μg/ml spectinomycin dihydrochloride. Homoplasmic shoots were obtained by a second round of regeneration on the same medium and were subsequently rooted on MS agar. Plants were tested for drug resistance by incubating leaf sections on RMOP medium containing spectinomycin and streptomycin (500 μg/ml each). Resistant leaf segments form green calli, whereas the sensitive segments bleach on this medium (Svab *et al.*, 1990).

RNA gel blot analysis

Total RNA was extracted using TRIzol (Gibco BRL). RNA was electrophoresed on formamide-containing 1% agarose gels and transferred to nylon membrane (Amersham) using the PosiBlot Transfer apparatus (Stratagene). Hybridization was carried out at 65°C in rapid hybridization buffer (Amersham), with ^{32}P-labeled probes generated by random priming (Boehringer Mannheim).

cDNA synthesis and PCR

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 was carried out as described by Kudla *et al.* (1992).

DNA and cDNA were amplified according to standard protocols (1 min at 92°C, 2 min at 56°C, 1.5 min at 72°C, 30 cycles). The P1 (5′-CCTTCCCTATTCATTGCGGGTTGG-3′) and P2 (5′-GGAATCC-TTCCAGTAGTATCGGCC-3′) primers specific for the *psbE* operon have been described (Kudla *et al.*, 1992; Bock *et al.*, 1993). The sequence of the *aadA*-specific P3 primer is 5′-CGCTCGATGACGCCAA-CTACC-3′.

DNA sequencing

The amplification products were separated in 2% agarose gels and purified using the Geneclean II kit (BIO 101 Inc., La Jolla, CA). Direct sequencing of the products was performed as described by Bachmann *et al.* (1990) using the Sequenase kit (USB) and the detergent Nonidet P-40 (Sigma). The sequencing primer 5′-GACTATAGATCGAACC-TATCC-3′ is based on sequence in the 5′ portion of the *psbF* gene.

Crosses and testing of seedling phenotypes

Wild-type, S6 and S7 plants were grown to maturity in the greenhouse. Seeds were collected after selfing and from reciprocal crosses of the transplastomic plants with wild-type tobacco. Surface-sterilized seeds were germinated on spectinomycin-containing RM medium. Resistant progeny are green, whereas sensitive seedlings are white. The hcf phenotype was tested by illuminating seedlings with UV light (320 nm).

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