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In vivo testing of a tobacco plastid DNA segment for guide RNA function in *psbL* editing

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Abstract A C-to-U RNA editing event creates a functional initiation codon for translation of the *psbL* mRNA in tobacco plastids. Small *trans*-acting guide RNAs (gRNAs) have been shown to be involved in editing site selection in kinetoplastid mitochondria. A computer search of the tobacco plastid genome (ptDNA) identified such a putative gRNA, a 14-nucleotide sequence motif that is complementary to the *psbL* mRNA, including the A nucleotide required to direct the C-to-U change. The critical A nucleotide of the putative gRNA gene was changed to G by plastid transformation. We report here that the introduced mutation did not abolish *psbL* editing. Since no other region of the plastid genome contains significant complementarity to the *psbL* editing site we suggest that, if gRNAs serve as *trans*-acting factors for plastid *psbL* mRNA editing, they either have only a limited complementarity to the editing site, or are encoded in the nuclear genome.

Key words RNA editing · *Trans*-acting factor · Guide RNA · *psbL* gene · Plastid transformation

Introduction

Posttranscriptional nucleotide modifications occur in both plastid (for review see Kössel et al. 1993) and mitochondrial (for review see Gray and Covello 1993; Wissinger et al. 1992) mRNAs of higher plants. RNA editing in plastids results in the conversion of cytosine to uridine, in most instances within coding regions. RNA editing may facilitate translation of the mRNAs by creating an AUG initiation codon (Hoch et al. 1991; Kudla et al. 1992) or result in restoration of codons for conserved internal amino acid residues (Maier et al. 1992a). While some of the general features of the editing process are well characterized (Kössel et al. 1993; Maier et al. 1992b; Freyer et al. 1993), little is known about the biochemical mechanisms underlying the editing reaction. The involvement of *trans*-acting factors in the recognition of editing sites has been suggested (Maier et al. 1992b; Zeltz et al. 1993; Bock et al. 1994). However, the nature of the template conferring the site specificity of the cytosine modification is still unknown.

RNA editing in the mitochondrion of kinetoplastid protozoa is directed by small mitochondrial transcripts termed guide RNAs (gRNAs) that exhibit complementarity to the mature mRNA (for review see Hajduk et al. 1993; Stuart 1993; Simpson et al. 1993). The gRNAs are capable of hybridizing to the pre-mRNA in a ribonucleoprotein complex and thereby provide the template for the subsequent modification of the mRNA (in this case uridine insertions or deletions). Similarly, gRNAs have been proposed to contribute as *trans*-acting factors to the editing reaction in higher plant plastids and mitochondria (Maier et al. 1992b). The availability of the tobacco plastid DNA (ptDNA) sequence (Shinozaki et al. 1986) made feasible a computer search for potential gRNAs in the plastid genome. This screen identified a 14-nucleotide sequence motif that is complementary to the edited *psbL* mRNA (Kudla et al. 1992), including the A nucleotide required to direct the C-to-U change. The structural features of the putative gRNA resemble those of the kinetoplast guide RNAs, including an anchor region of typically 7 to 10 nucleotides for the initial recognition of the pre-mRNA and downstream mismatch(es) to direct the nucleotide sequence alteration(s) (Hajduk et al. 1993).

We report here that mutation of the putative gRNA by plastid transformation did not abolish *psbL* editing.

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We propose that if gRNA(s) serve as *trans*-acting factors for editing of the *psbL* mRNA, they are likely to be encoded in the nuclear genome.

Materials and methods

Construction of plasmid pRB15

The ptDNA region containing the putative gRNA gene (gRNA-*psbL*) was cloned as a 5 kb *Pst*I–*Sac*II fragment (sites at nucleotide positions 36822/41891; Shinozaki et al. 1986) into a Bluescript KS + vector (Stratagene). The desired mutations were introduced in the *psaB* coding region by site-directed mutagenesis using the synthetic oligonucleotide PG1 (see below). The chimeric *aadA* gene from a progenitor of plasmid pZS197 (Svab and Maliga 1993) was excised as a 1.1 kb *Ecl*136II–*Dra*I fragment and cloned into the unique *Spe*I site between *trnfM* and *trnG* at nucleotide position 38302 (Shinozaki et al. 1986), which had been blunted by a fill-in reaction with the Klenow enzyme.

Plastid transformation and regeneration of transgenic plants

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. Young leaves were bombarded with plasmid DNA-coated tungsten particles using the DuPont PDS1000He biolistic gun (Kanevski and Maliga 1994). Selection for spectinomycin resistance was carried out on RMOP regeneration medium containing 500 mg/l spectinomycin dihydrochloride. Resistant clones were subjected to a second round of regeneration to obtain homoplasmic tissue. Plastid transformants were identified by resistance to spectinomycin and streptomycin (500 mg/l each) on RMOP medium (Svab and Maliga 1993). Shoots from lines identified as plastid transformants were subsequently rooted on MS medium containing 30 g/l sucrose.

Isolation of nucleic acids

Total cellular DNA was extracted as described (Mettler 1987). Total cellular RNA was isolated using the TRIzol reagent (GIBCO BRL) following the instructions of the manufacturer. The substrate for the reverse transcription reactions was obtained by treatment of the isolated RNA with proteinase K and DNase I (Kudla et al. 1992).

DNA gel blot analysis

Restriction enzyme-digested total cellular DNA was electrophoresed on 0.8% agarose gels and transferred to nylon membranes (Amersham) according to standard protocols (Sambrook et al. 1989) and using the PosiBlot Transfer apparatus (Stratagene). Hybridization was carried out in Rapid Hybridization Buffer (Amersham) at 65° C with [^{32}P] dCTP labeled probes generated by random priming.

cDNA synthesis and polymerase chain reaction (PCR) amplification

Proteinase K and DNase I treated RNA was reverse transcribed as described (Kudla et al. 1992). PCR amplification of DNA and cDNA samples was carried out following standard procedures (30 cycles at 92° C for 1 min, 56° C for 2 min and 72° C for 1.5 min). The *psbE* operon sequences were amplified with the PE1 and PE2 primers. The *psaB* gene portion including the putative gRNA gene was amplified with the PB1 and PB2 primers.

DNA sequencing

The PCR products were separated in 2% agarose gels and isolated from gel slices using the Geneclean II kit (BIO 101, La Jolla). The linear templates were directly sequenced by a modified chain termination reaction (Bachmann et al. 1990) adding the detergent Nonidet P-40 (final concentration 0.5%) to the buffers and reaction mixes of Sequenase kit 2.0 (USB). Sequencing primer PE3 (based on internal sequences of the *psbL* gene) was used to test for *psbL* editing; primer PB1 (derived from *psaB* sequences) was used to verify the gRNA mutations.

Oligonucleotide primers

The following oligonucleotides were employed:

PG1 5′-CGATTGGGGCGTGGCCATAAGGGCCTTTAC-GACACAATCAATAATTCG-3′
PE1 5′-CCTTCCCTATTCATTGCGGGTTGG-3′
PE2 5′-GGAATCCTTCCAGTAGTATCGGCC-3′
PE3 5′-CCCCAGTAGAGACTGGTACG-3′
PB1 5′-CCCAAGGGGCGGGAACTGC-3′
PP2 5′-CCCAGAAAGAGGCTGGCCC-3′

Results and discussion

Identification of the putative *psbL* gRNA

A gRNA for tobacco *psbL* editing is expected to meet the following criteria: (i) significant complementarity to the sequence motif surrounding the editing site to allow specific recognition and (ii) a mismatch between an A nucleotide in the complementary region of the gRNA and the C within the initiation codon for the gRNA to direct the C-to-U modification. The DNA sequence of the tobacco plastid genome (Shinozaki et al. 1986) was analyzed for sequences that fulfill these criteria, using the University of Wisconsin GCG sequence analysis software package. The homology search was restricted to the region from −20 to +20 with respect to the edited nucleotide. The length of the sequence used in the search was 15 or 10 nucleotides. The possibility of G-to-U base pairing was also considered. All possible sequences between −20 and +20 were tested. No sequence motif that meets the requirements outlined above was found in the tobacco plastid genome, except for one region within the *psaA* operon (Fig. 1A, C). The putative gRNA sequence (*gRNA-psbL*) is located on the antisense strand of the *psaB* coding region (strand A of ptDNA), while *psbL* is encoded in strand B, and is part of a different (*psbE*) transcription unit. Given the size of the tobacco plastid genome, 155.8 kb, the observed complementarity of 14 nucleotides between the putative gRNA sequence and the *psbL* editing site is unexpected. More importantly, the single mismatch

between the C in the unedited *psbL* initiation codon and the A in the putative gRNA could direct the C-to-U editing reaction.

Modification of the putative *gRNA-psbL* sequence *in vivo*

In order to test *gRNA-psbL* for function, we changed the critical A nucleotide to G in a cloned ptDNA fragment by site-directed mutagenesis so that the gRNA would no longer direct C-to-U editing (Fig. 1). To facilitate the identification of plants that carry the altered gRNA sequence, two additional point mutations were introduced 6 and 15 nucleotides downstream of the gRNA mutation to generate a *Bgl*I site as a screenable restriction fragment length polymorphism (RFLP) marker (Fig. 1B, C). Each of the three nucleotide substitutions is silent and does not affect the translation of the *psaB* mRNA. Subsequently, a spectinomycin resistance (*aadA*) gene was inserted into the ptDNA fragment in the intergenic spacer between the $tRNA^{fMet}$ and the $tRNA^{Gly}$ genes to yield plasmid pRB15. In plasmid pRB15, the *aadA* gene is flanked by 1.5 and 3.6 kb of ptDNA. The mutant gRNA sequence is in the larger flanking fragment, 1.9 kb away from the *aadA* gene (Fig. 1B).

Bombardment of tobacco leaves with tungsten particles coated with pRB15 plasmid DNA, followed by selection for spectinomycin resistance, yielded plastid transformants in which the *aadA* gene had integrated into the plastid genome by two homologous recombination events via the flanking ptDNA sequences. In a sample of 25 bombarded leaves, 123 independent spectinomycin-resistant clones were obtained. Plastid transformation was confirmed in each of the 20 tested clones by resistance to streptomycin and spectinomycin, a characteristic of plants expressing *aadA* in plastids (Svab and Maliga 1993). The mutant gRNA sequence was introduced into the tobacco plastid genome with the *aadA* gene provided the recombination took place downstream of the mutant gRNA sequence (Fig. 1B). Therefore, 20 transplastomic lines were screened, in a PCR-amplified DNA sample, for the mutant *gRNA-psbL* by testing for the presence of the introduced *Bgl*I RFLP marker. Five clones carried the additional *Bgl*I site while the other 15 lines had the wild-type sequence lacking the diagnostic restriction site (data not shown). Three clones each with (Nt-pRB15-G1, Nt-pRB15-G7, Nt-pRB15-G15) and without the gRNA mutation (Nt-pRB15-G17, Nt-pRB15-G18, Nt-pRB15-G20) were selected for further study, and purified to homoplasmy by a second round of plant regeneration on spectinomycin-containing medium. Homoplasmy was verified by DNA gel blot analysis utilizing the transplastomic *Bgl*I RFLP marker (Fig. 2). Incorporation of the point mutation that would affect gRNA function was verified by sequencing PCR-amplified DNA (data not shown).

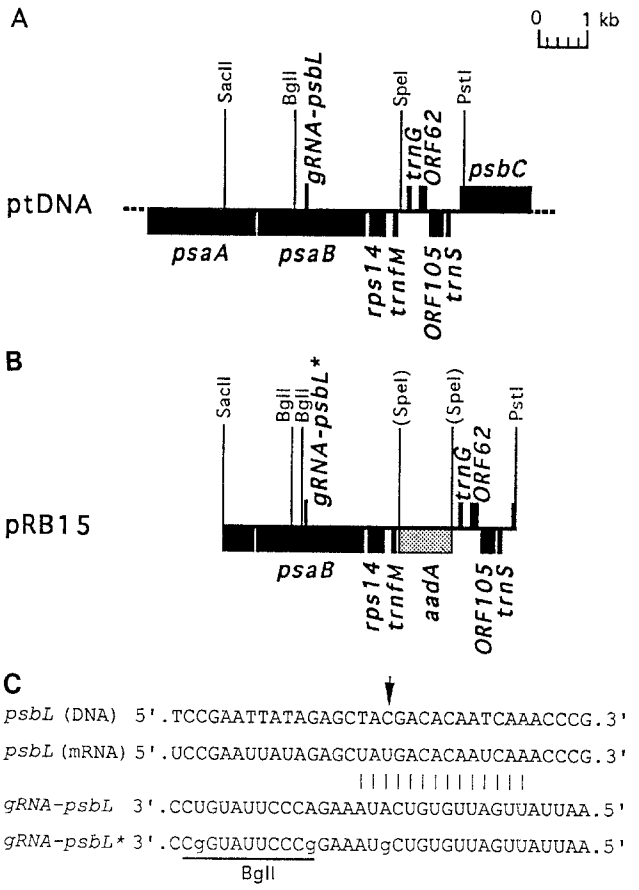


Fig. 1A–C Experimental strategy to test *gRNA-psbL* for function. **A** Map position of *gRNA-psbL* in the tobacco *psaA* operon. Genes below the line are transcribed from left to right, genes above the line are transcribed in the opposite direction. Based on Shinozaki et al. (1986). **B** Map of the plastid DNA (ptDNA) fragment in plasmid pRB15. Note the spectinomycin resistance (*aadA*) gene in the *trnfM-trnG* intergenic region. The novel *Bgl*I restriction site linked to the mutated gRNA sequence (*gRNA-psbL**) provides a screenable restriction fragment length polymorphism (RFLP). **C** Partial sequence alignment of the region surrounding the *psbL* editing site and the region containing the putative *gRNA-psbL*. The edited position in *psbL* is marked by a *vertical arrow*. Note that the putative gRNA shows complementarity to the edited *psbL* mRNA, and the mismatch at the edited position of the genomic *psbL* sequence. Mutations in *gRNA-psbL** are in *lowercase letters*. The novel *Bgl*I restriction site is *underlined*.

The observed frequency of 25% linkage between the *aadA* gene and the RFLP marker 1.9 kb away provides further evidence that long regions of homologous DNA can be efficiently integrated into the plastid genome by genetic recombination (Staub and Maliga 1992).

Editing of *psbL* mRNA in the Nt-pRB15 transplastomic lines

The plastid *psbL* gene encodes the L peptide of photosystem II, and is part of the *psbE* operon. The operon

contains the *psbE*, *psbF*, *psbL* and *psbJ* open reading frames, all four encoding photosystem II polypeptides (for review see Vermaas and Ikeuchi 1991). The *psbE* operon is transcribed as a 1.1 kb tetracistronic mRNA, which is not subject to processing (Haley and Bogorad 1990). To test whether or not the mutation in the putative *gRNA-psbL* abolishes *psbL* editing, cDNAs were derived from the *psbE* operon transcript of the three clones that carry the gRNA gene mutation. The cDNAs were amplified using a *psbE* operon-specific primer pair: a 5′ primer derived from the *psbE* gene sequence and a 3′ primer derived from the *psbJ* gene sequence (Kudla et al. 1992; Bock et al. 1993). The 589 bp PCR product includes the entire *psbL* coding region. Direct sequencing of the amplification product revealed virtually complete editing of the *psbL* initiation codon in the wild type and in the three transplastomic plants containing the mutated gRNA sequence (Fig. 3). These data indicate that the identified sequence motif does not encode a functional gRNA to direct editing of the *psbL* mRNA. In view of the absence from the tobacco plastid genome of any other region with significant complementarity to the *psbL* editing site we propose that plastid-encoded gRNAs either do not exist or have only a very limited complementarity to the editing site. Plastid-encoded *trans*-acting factors that are different from a kinetoplast-type gRNA, or function via non-Watson-Crick base pairing still remain an option. The presence of nuclear-encoded gRNAs that are imported into the plastid compartment seems to be an attractive alternative. The import of gRNAs into plastids could occur by a mechanism that is similar to the import of tRNAs into plant mitochondria (Small et al. 1992).

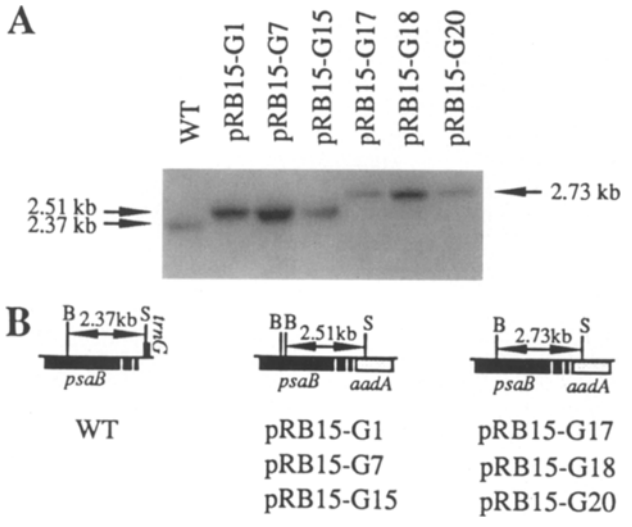
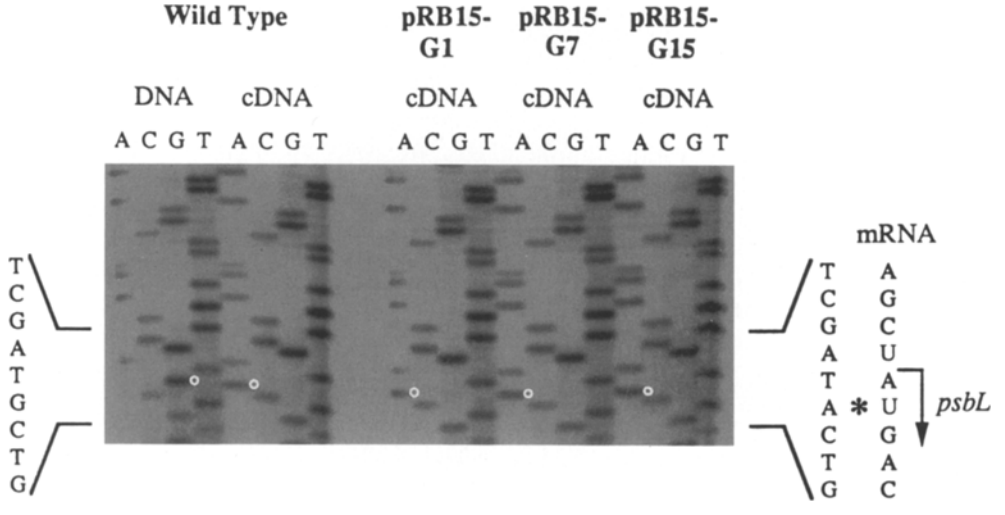


Fig. 2A, B Classification of plastid transformants. **A** DNA gel blot analysis. Total cellular DNA was digested with *Bgl*I and *Sty*I restriction enzymes and probed with a radiolabeled *Bgl*I-*Spe*I fragment containing *gRNA-psbL* (Fig. 1A). Note the 2.37 kb fragment in wild type (WT), the 2.51 kb fragment in the Nt-pRB15-G1, Nt-pRB15-G7 and Nt-pRB15-G15 clones, which is diagnostic for the presence of *aadA* and the novel *Bgl*I site, and the 2.73 kb fragment in Nt-pRB15-G17, Nt-pRB15-G18 and Nt-pRB15-G20 clones, which is diagnostic for the presence of *aadA* but lack of the novel *Bgl*I site. **B** Schematic map of the ptDNA regions yielding the diagnostic fragments shown in Fig. 2A. Restriction endonuclease recognition sites: *B Bgl*I, *S Sty*I

Fig. 3 Sequence analyses to test for *psbL* mRNA editing. Polymerase chain reaction (PCR) amplified genomic and cDNA data are shown for the wild type (WT), and cDNA data are shown for the independently transformed Nt-pRB15-G1, Nt-pRB15-G7 and Nt-pRB15-G15 clones. Due to the polarity of the sequencing primer this autoradiogram shows the sequence that is complementary to the *psbL* mRNA. The editing site in each lane is marked by an *open circle*. Note that the C-to-U conversion is practically complete in both the wild type and the three transformed lines. Note also the DNA sequence on the left, and the cDNA and mRNA sequences on the right. The edited position in the cDNA is marked by an *asterisk*



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