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A Heterologous Maize *rpoB* Editing Site Is Recognized by Transgenic Tobacco Chloroplasts

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Single nucleotides in plant chloroplast transcripts are edited from the genomically encoded C to U, often resulting in changes of the encoded protein sequence. Site-specific *trans*-acting factors are postulated to direct the selection of edited residues. In order to further define *cis* sequences required for RNA editing, we investigated whether two editing sites present in maize *rpoB* mRNA would be recognized by the editing machinery of transformed tobacco chloroplasts. A 93-nucleotide (nt) segment surrounding site I is sufficient to direct editing of the maize sequence in tobacco chloroplasts. However, an 86-nt segment surrounding maize site IV (which is genomically encoded as a T in tobacco) does not confer editing of this site, suggesting that *trans*-acting factors necessary for recognition of site IV are not present in tobacco. The maize sequences surrounding site I were found to compete with the endogenous *rpoB* for a depletable *trans* factor and to reduce editing of endogenous site I. The presence of exogenous maize site I was also found to decrease editing of endogenous tobacco site II, indicating that there is a shared aspect of editing for some closely spaced editing sites.

The mRNAs of plant chloroplasts and mitochondria are subject to RNA editing (reviewed in references 9, 10, 12, 13, and 16). In both cellular compartments, specific genomically encoded C's are converted to U's in mRNA transcripts, leading to RNA sequences that differ from their corresponding DNA sequences. Less frequently, a U may be edited to C at the mRNA level (15, 18). Proteins encoded by the edited mRNA are more similar to non-plant homologs than the predicted genomically encoded protein would be. A study in which a nonedited *psbF* message was expressed in transgenic tobacco produced plants exhibiting a mutant phenotype, indicating that RNA editing is essential for functional expression of some chloroplast-encoded proteins (3).

The question of how the cellular editing machinery interacts with an RNA transcript to specify precise positions for editing remains unanswered. There is no in vitro editing system available for study of chloroplast RNA editing, but the development of a biolistic system enabling the transformation of tobacco chloroplasts provides the opportunity to study chloroplast RNA editing in vivo (17). Studies have shown that a small segment of surrounding sequence is sufficient to specify the editing of some chloroplast sites (2, 6), but there are instances where even an 84-nucleotide (nt) segment surrounding an editing site does not provide sufficient information to specify editing (2). When additional copies of the *psbL* editing site were expressed in tobacco chloroplasts, editing of the endogenous message was reduced (5, 6), suggesting that *trans*-acting specificity factors present in limiting amounts are likely to be involved with site selection and editing. The presence of species-specific *trans*-acting factors also could explain the restoration of editing of a spinach *psbF* editing site expressed in tobacco protoplasts following the fusion of the tobacco protoplasts to spinach protoplasts (4). We are interested in further analysis of the *cis* sequences required to define an editing site and have used the biolistic transfer of DNA into tobacco chloroplasts to determine whether a heterologous editing site from maize can be edited in tobacco chloroplasts.

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MATERIALS AND METHODS

Plant material. Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown under sterile conditions on MS-agar medium (14) containing 30 g of sucrose per liter. Transformed lines were rooted and propagated on the same medium.

Oligonucleotides. The following oligonucleotides were used in this study: P1.1, CCGGAATGGAAATGAGGGAATGTC; Pα1.2, CAGATCTGCAACCGAACGAATACG; P2.1, TACCATGGGGGACTATAATATCAGATTGGGGAGGAAGG; Pα2.2, AATCTAGATATCTTTTGTTTTCTACTCACGCGAGCCCA; P3.1, CGCCATGGAGAGAAATTTTAGATAATGTTTCCTACCCCGA; Pα3.2, ATTCTAGATTCTTTTGACTCAATCCTCTTCTTCTCCTTAG; P4.3, GTGATCAATCAAATATTGCTAAGTCC; PC1.1, TCTTGAACAACTTGGAGCCGGGCC; PCα1.2, GAGGATAGCAAGTTCCAAATTCTGTCTCGG; PPrrn, GCCGTCGTTCAATGAGAATG; PPrrn2, AATACGAAGCGCTTGGATACAGTTGTAGGGA; PαTrps16, CTACCCCCCCTTTTGTATTTCC.

Construction of plastid transformation vectors. The maize *rpoB* sequence containing editing site I was amplified by PCR with primers P2.1 and Pα2.2, which contain *Nco*I and *Xba*I restriction endonuclease sites, respectively, at their 5′ ends. The maize *rpoB* sequence containing editing site IV was amplified by PCR with primers P3.1 and Pα3.2, which contain *Nco*I and *Xba*I restriction endonuclease sites, respectively, at their 5′ ends. The digested PCR products were ligated into vector pLAA24A (20), which had been digested with the same enzymes to remove the *uidA* coding sequences (see Fig. 1). The resulting plasmids, MR210 and MR211, respectively, contain the *aadA* spectinomycin resistance gene and test sequences flanked by *trnV-rps12/7* plastid-derived sequences which direct their insertion into the inverted-repeat region of the tobacco plastid genome. Plasmids were prepared by using the Qiagen purification system and sequenced to confirm that the inserts contained the correct sequences.

Plastid genome transformation. Leaves of tobacco cultivar Petit Havana were bombarded with plasmid-coated tungsten particles by using a model PDS 1000/He Biolistic Particle Delivery System (Bio-Rad), and transformed tissue was regenerated essentially as described by Svab and Maliga (17). A 2-μg amount of plasmid DNA was precipitated on tungsten particles for each shot, and each leaf was bombarded twice. DNA was isolated from transformed tissue by the hexadecyltrimethylammonium bromide (CTAB) extraction method (8) and was analyzed for *aadA* and *rpoB* incorporation by PCR amplification.

Southern analysis. A 1-μg amount of total DNA from transformants and wild-type leaves was digested with *Eco*RI and *Eco*RV, electrophoresed, and blotted onto a positively charged nylon membrane (Amersham) by using a Turboblotter (Schleicher and Schuell). The blot was hybridized with a ^{32}P-labeled randomly primed (DECAprime II; Ambion) 350-nt genomic probe produced from PCR amplification of wild-type DNA by using primers (PC1 and PCα1.2) surrounding the vector integration site in the chloroplast genome.

Analysis of editing in chloroplast transcripts. Total RNA was isolated from transformed and wild-type leaves by using Trizol (Gibco/BRL). The RNA was treated with 1 U of RQ1 DNase (Promega) for 30 min at 37°C and reverse

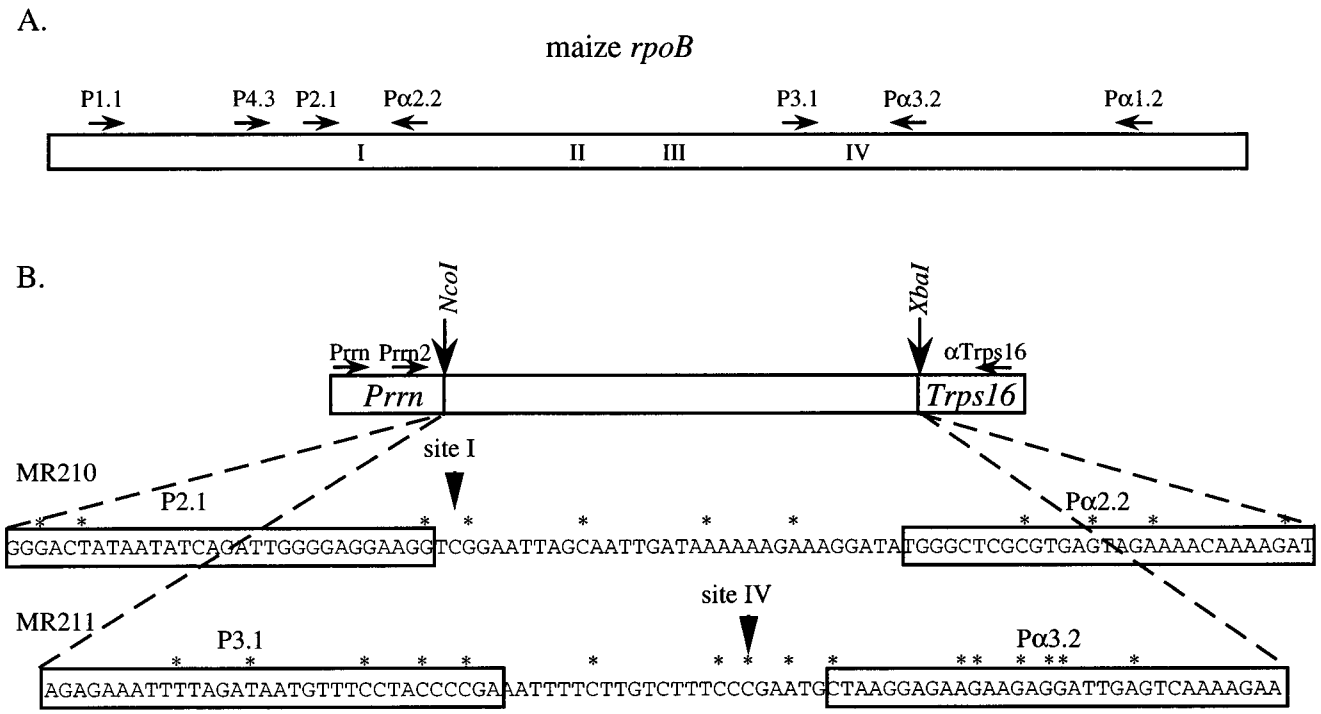

FIG. 1. Insertion of maize *rpoB* gene segments into the expression cassette of the tobacco transformation vector. (A) Schematic representation of regions of the maize *rpoB* gene showing locations of editing sites (indicated by roman numerals) and primer position and direction (indicated by arrows). Not drawn to scale. (B) Expression cassette of transformation plasmid pLAA24A (20) and maize sequences that were inserted. Asterisks indicate nucleotides that differ between maize and tobacco *rpoB*. Primer sequences are boxed, and editing sites I and IV are shown beneath vertical arrowheads.

transcribed (SuperScript II; Gibco/BRL) to make cDNA by using primer PαTrps16 for transformed sequences and primer Pα3.2 for endogenous sequences. cDNA was PCR amplified by using primers PPrrn and PαTrps16 for transformed sequences and primers P4.3 and Pα3.2 for endogenous *rpoB* sequences surrounding sites I and II. PCR products were sequenced by using the fmol DNA cycle sequencing system (Promega) with ^{33}P-labeled primer PPrrn2 for transgenic transcripts and P4.3 for endogenous sequences. PCR products were also cloned into the pCR2.1 plasmid (Invitrogen), and individual clones were sequenced by using the same primers. Endogenous *rpoB* sites I and II were sequenced in the same reaction. In order to obtain more editing information for site II, primer P2.1 was used to sequence additional endogenous and MR210 clones.

Northern analysis. A 10-μg amount of total RNA was electrophoresed on a 1.5% agarose–0.44 M formaldehyde–morpholinepropanesulfonic acid (MOPS) gel and blotted onto a positively charged nylon membrane (Amersham) by using a Turboblotter (Schleicher and Schuell). Hybridization was performed with a ^{32}P-labeled randomly primed (DECAprime II; Ambion) 950-nt probe produced from PCR amplification of wild-type DNA with primers (P1.1 and Pα1.2) which surround editing sites I to IV in *rpoB*.

Nucleotide sequence accession numbers. The GenBank accession number for tobacco *rpoB* is X12745, and that for maize *rpoB* is X73526.

RESULTS

Integration of two maize *rpoB* sites into the tobacco chloroplast genome. The *rpoB* gene encodes the β subunit of a chloroplast-specific, DNA-dependent RNA polymerase (1). Four C-to-U editing sites have been defined for maize *rpoB* (19). PCR products spanning maize plastid *rpoB* editing sites I and IV (Fig. 1) were digested with *Nco*I and *Xba*I and ligated into the vector pLAA24A (20), which had been cut with the same enzymes to remove the *uidA* coding sequences from the plasmid (Fig. 1B). The resultant plasmids were named MR210 (93 nt surrounding site I) and MR211 (86 nt surrounding site IV). There are 11 nucleotide differences between the maize sequence surrounding editing site I and the corresponding tobacco sequence (Fig. 1B) and 16 differences between the maize and tobacco sequences surrounding editing site IV (Fig. 1B). Endogenous site I is edited in tobacco, but site IV does not exist in tobacco because a T is genomically encoded at this position in the tobacco chloroplast genome. In plasmids MR210 and MR211, the *rpoB* test sequences are surrounded by regulatory regions from the chimeric *Prrn* operon promoter (*Prrn*) and 3′ region of the *rps16* ribosomal protein gene (*Trps16*) (Fig. 1B). The *Nco*I restriction site at the 5′ end of each insert contains an AUG start codon in the correct reading frame; thus, each construct contains a translatable minitransgene.

Transformation and selection of transplastomic lines. Bombardment of tobacco leaves with DNA-coated tungsten particles was followed by selection on spectinomycin-containing regeneration medium. Homologous recombination between sequences in MR210 or MR211 and chloroplast DNA results in integration of *aadA* and test sequences into the chloroplast genome (Fig. 2A). The chimeric *aadA* gene encodes aminoglycoside 3″-adenyltransferase and causes resistance to spectinomycin and streptomycin in both bacteria (7) and tobacco chloroplasts (17). Successive rounds of selection on spectinomycin-containing medium produce transformed plants that express *aadA* resistance and the test sequences. From a sample of 50 leaves bombarded with the two constructs, 40 spectinomycin-resistant clones were selected. Amplification of total DNA with primers specific for *aadA* sequences confirmed that only five of these had incorporated the *aadA* gene (data not shown). These results reveal a greater incidence of development of spontaneous spectinomycin resistance during the selection process than has been previously reported (5, 17). One plant transformed with MR210 and two plants independently transformed with MR211 were chosen for further study.

Figure 2 shows a DNA blot in which total leaf DNA digested with *Eco*RI and *Eco*RV was probed with a 350-nt fragment homologous to sequences surrounding the plasmid integration

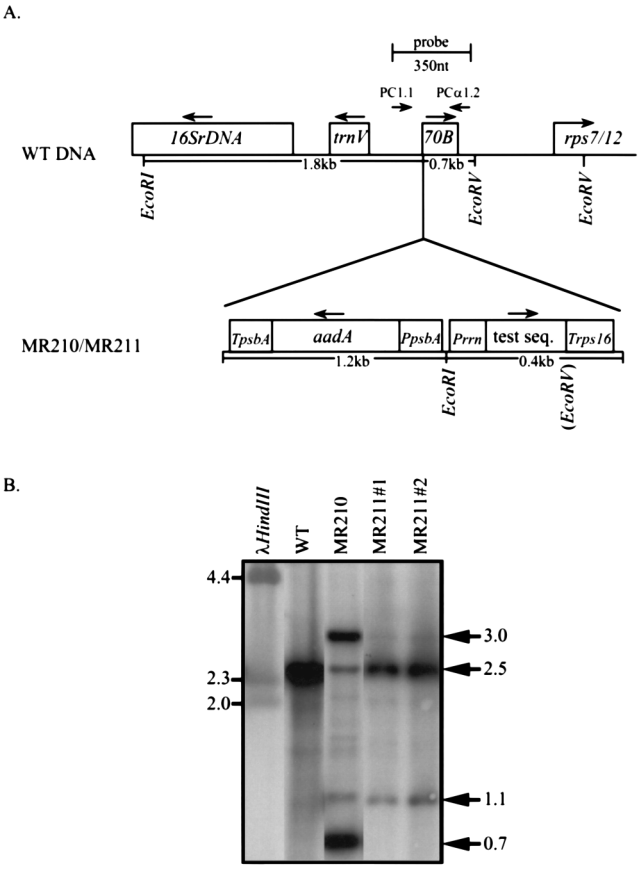

FIG. 2. Analysis of DNA from transplastomic tobacco plants. (A) Schematic representation of the wild-type (WT) plastid genome and the targeted MR210/MR211 fragment, not drawn to scale. MR210, but not MR211, contains an *Eco*RV site. (B) DNA was isolated from leaves of control and transformed plants and digested with *Eco*RI and *Eco*RV. DNA gel blots were probed with 350 nt of plastid genome sequence surrounding the plasmid integration site. Sizes of lambda DNA digested with *Hin*dIII are indicated on the left in kilobases. The small amount of 1.1-kb fragment present in MR210 is the result of an incomplete *Eco*RV digest at the site contained in the test sequence.

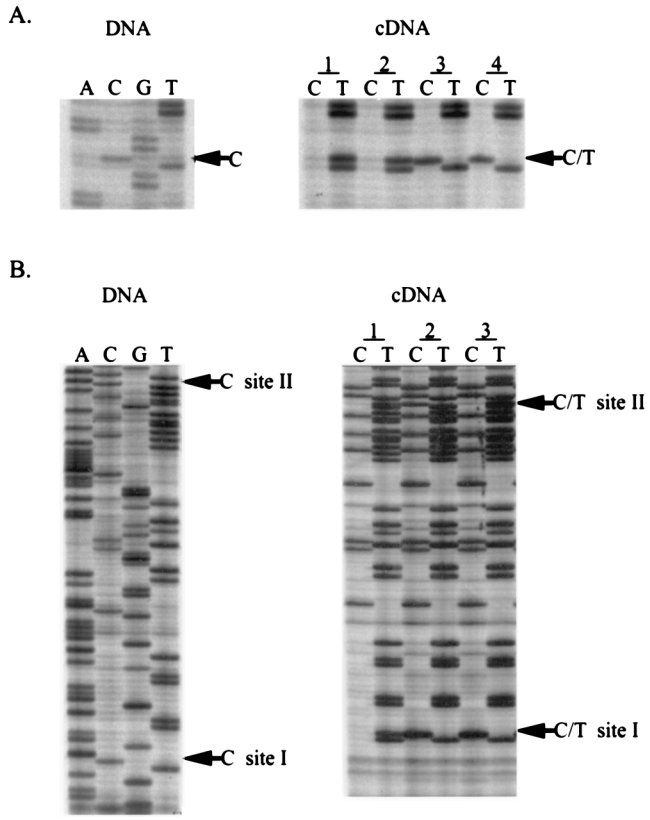

FIG. 3. Editing of *rpoB* in transgenic tobacco plants. Editing sites are indicated by arrowheads. (A) Transgenic maize *rpoB* sequences expressed in an MR210 tobacco plant. Samples 1 and 2 are edited; samples 3 and 4 are unedited. (B) Endogenous *rpoB* sequences expressed in an MR210 tobacco plant. Site I is edited in sample 1 and unedited in samples 2 and 3. Site II is edited in samples 1 and 3 and unedited in sample 2.

site in the chloroplast genome. The transgenic plant transformed with MR210 shows novel hybridization signals at 0.7 and 3 kb, as well as a small amount of residual wild-type signal at 2.5 kb indicating that the plants are not entirely homoplasmic after 5 months of selection on spectinomycin-containing regeneration medium. The plants transformed with MR211 show a large amount of wild-type signal as well as novel signals at 1.1 and 3 kb. In spite of the retention of untransformed chloroplast genomes after 3 months of selection, we were able to analyze RNA expression from the MR211 transgene because the test sequences were specifically amplified with PCR primers homologous to the 5′ and 3′ regions of the transformed sequences.

Analysis of transgenic transcripts in plastids. Total RNA isolated from leaves was reverse transcribed to produce cDNA and then subjected to PCR amplification. Editing of the transcripts expressed in transgenic MR210 and MR211 plants was evaluated by PCR-based sequencing of individual reverse transcription-PCR product clones. Endogenous *rpoB* site I is normally 100% edited in tobacco chloroplasts (data not shown). MR210 transcripts expressing *rpoB* site I exhibited 50% editing of the exogenous site, with a C present in half the 12 clones sequenced (Fig. 3A, samples 3 and 4; Table 1) and a T present in the remaining half (Fig. 3A, samples 1 and 2). Incomplete editing of the heterologous *rpoB* sequence could be due to depletion of site-specific factors involved in *rpoB* editing.

In all transformants the *rpoB* minigene was introduced as an additional copy into the inverted-repeat region of the plastid genome, with an intact endogenous *rpoB* gene present in the large single-copy region of the genome. Editing of the endogenous *rpoB* sequence in the MR210 transgenic plants was analyzed by reverse-transcription-PCR amplification with primers homologous to regions of *rpoB* not contained in the transgenic sequence. Editing of endogenous transcripts decreased in MR210 transgenic plants from the normal 100% to 50%, with 6 of the 12 sequenced clones exhibiting a C (Fig. 3B, samples 2 and 3) and the remainder exhibiting a T (Fig. 3B, sample 1). Another *rpoB* editing site is present 78 nt 3′ of site I (called site II), and it was examined to determine whether editing of this endogenous sequence was affected by the presence of transgenic site I in MR210-expressing plants. Endog-

TABLE 1. *rpoB* editing efficiency in tobacco plants

Plant	*rpoB* editing efficiency (%) at site:		
	Transgenic I	Endogenous I	Endogenous II
Wild type		100	97
MR210	50	50	74

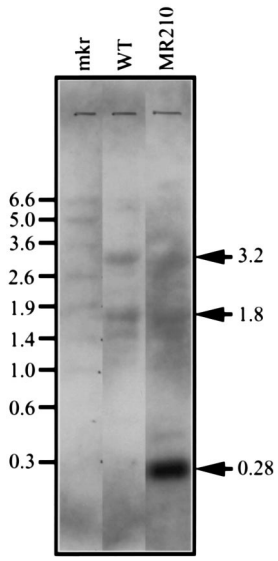

FIG. 4. RNA blot analysis of minigene RNA from transplastomic tobacco plants. Total RNA isolated from leaves of control and transformed plants was probed with a 950-nt fragment of tobacco *rpoB* containing the editing site. Approximately 10 μg of each RNA sample was run per lane. Sizes of RNA markers (Promega) are indicated on the left in kilobases. mkr, RNA size markers; WT, wild type.

enous site II is 97% edited (28 of 29 clones) in wild-type plants, but in MR210 plants, editing is decreased to 74% (20 of 27 clones). This decrease in editing is a statistically significant difference ($X^2_{0.05,1}$). No observable change in phenotype resulted from the decrease in endogenous *rpoB* editing in MR210-expressing plants. The absence of the albino phenotype, produced by *rpoB* gene deletion in tobacco (1), indicates the presence of sufficient functional RPOB in MR210-expressing plants.

Total RNA was electrophoresed on an agarose gel, blotted, and probed with a 950-nt fragment of tobacco *rpoB* that includes sequences present in MR210 (Fig. 4). MR210 exhibits a 280-nt transcript, as would be predicted from the size of the transgene. In addition, both the wild type and MR210 express the endogenous *rpoB* messages of 1.8 and 3.2 kb (11). The transgenic sequences are expressed at extremely high levels in comparison with levels of the endogenous gene.

As discussed earlier, *rpoB* site IV does not exist in tobacco because a T is genomically encoded by tobacco plastids. When the maize editing site was expressed in tobacco from MR211, no editing was detected although transcripts were present (data not shown).

DISCUSSION

The determinants involved in site selection and catalysis of C-to-U RNA editing in the plastids of higher plants are not yet defined. There is accumulating evidence indicating that sequences immediately surrounding an editing site are important in determining editing of the site (2, 5, 6); however, all necessary information may not be contained in nearby sequences (2). The size of flanking sequences required to specify an editing site is site specific. Studies by Chaudhuri et al. (5) determined that a 98-nt fragment spanning the *psbL* editing site contained all the *cis* information required to specify editing, while a 35-nt segment surrounding the spinach *psbF* editing site (which is genomically encoded as a T in tobacco) was insufficient to direct editing of the heterologous site in tobacco chloroplasts (3). A much smaller fragment, from positions −16 to +5, surrounding the *psbL* editing site is sufficient to direct editing of transgenic transcripts at levels somewhat below endogenous editing levels (6). In an analysis of three pairs of editing sites in *ndhB*, Bock et al. (2) found that a −42/+42 fragment directed almost complete editing of one pair of sites, partial editing for a second pair, and no editing for the third pair.

We examined whether the editing machinery of tobacco chloroplasts could recognize and modify an editing site from another species, maize. Transcripts of maize chloroplast-encoded *rpoB* had been observed to undergo RNA editing at four sites (19). We used two different types of sites originating from maize *rpoB* in this study: a site that is present in and edited by tobacco chloroplasts (*rpoB* site I) and a second site (*rpoB* site IV) that is genomically encoded as a T in tobacco plastids and is therefore not normally an editing target in tobacco. Our minigene constructs included 93 nt (MR210) and 86 nt (MR211) surrounding the maize editing sites and were expressed in addition to the endogenous tobacco *rpoB* gene.

We report here the first instance of RNA editing of a heterologous sequence by tobacco chloroplasts. Maize transgene mRNA molecules expressed from MR210 are 50% rather than 100% edited as the sequence would normally be; however, endogenous *rpoB* sequences are also edited less efficiently than they normally would be in the transformed chloroplasts. The reduced endogenous-sequence editing and partial transgene editing are likely due to the extremely high expression of the maize *rpoB* test sequences from the Prrn promoter, which results in depletion of a common *trans* factor(s) necessary for both site I and site II editing. A factor more generally needed for RNA editing is not depleted since the start codon of *psbL* is edited normally in the MR210 plant (data not shown). When additional copies of *psbL* (5) or *ndhD* (6) editing sites were introduced into tobacco, some of the endogenous sites were shown to be edited less efficiently than is normal. That endogenous *rpoB* site II editing is also decreased in the MR210 transgenic plants is extremely interesting. It suggests that there may be a shared aspect of editing of these nearby sites; possibly the depleted factor(s) necessary for site I editing is involved in site II recognition or editing. Some transcripts in which site I is unedited have site II edited (Fig. 3B, sample 3), so there is not a 5′-to-3′ processivity requirement for editing of these two sites. This is the first example of an endogenous tobacco editing site being affected by the presence in *trans* of extra copies of a nearby editing site.

Several hypotheses can be formulated to explain the inability of transformed tobacco plants to edit maize *rpoB* site IV. It may be that the altered nucleotides surrounding the maize site are not recognized by tobacco editing machinery or, more likely, that a *trans*-acting factor(s) necessary for editing of the site has been lost from tobacco subsequently to the loss of the editing site from the genome. Bock et al. (3) reached a similar conclusion in the only other test of whether tobacco chloroplasts can edit a heterologous site that is genetically encoded as a T in the tobacco plastid genome.

Introducing a maize editing site with 11 sites of divergence from the tobacco sequence is comparable to introducing a tobacco editing site with 11 nearby nucleotides changed. The "mutations" represented by the maize site are evidently not sufficient to disrupt the editing process in transgenic plants. The multiple changes in sequence surrounding an editing site that have occurred during divergence of this monocot and dicot have not interfered with editing, which indicates that certain *cis* sequences are not essential for recognition of RNA

editing sites in tobacco plastids. It is quite likely that other heterologous sites from one species will be able to be recognized by another species, provided that both species undergo editing at the same site. Additional chloroplast transformation experiments should provide further insight into the *cis* sequence elements required for selection of chloroplast editing sites.

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