REPORT

Faithful editing of a tomato-specific mRNA editing site in transgenic tobacco chloroplasts

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ABSTRACT

RNA editing sites and their site-specific *trans*-acting recognition factors are thought to have coevolved. Hence, evolutionary loss of an editing site by a genomic mutation is normally followed by the loss of the specific recognition factor for this site, due to the absence of selective pressure for its maintenance. Here, we have tested this scenario for the only tomato-specific plastid RNA editing site. A single C-to-U editing site in the tomato *rps12* gene is absent from the tobacco and nightshade plastid genomes, where the presence of a genomic T nucleotide obviates the need for editing of the *rps12* mRNA. We have introduced the tomato editing site into the tobacco *rps12* gene by plastid transformation and find that, surprisingly, this heterologous site is efficiently edited in the transplastomic plants. This suggests that the *trans*-acting recognition factor for the *rps12* editing site has been maintained, presumably because it serves another function in tobacco plastids. Bioinformatics analyses identified an editing site in the *rpoB* gene of tobacco and tomato whose sequence context exhibits striking similarity to that of the tomato *rps12* editing site. This may suggest that requirement for *rpoB* editing resulted in maintenance of the *rps12* editing activity or, alternatively, the pre-existing *rpoB* editing activity facilitated the evolution of a novel editing site in *rps12*.

Keywords: *Nicotiana tabacum*; *Solanum lycopersicum*; plastid; evolution; *rps12*; RNA editing; plastid transformation

INTRODUCTION

RNA editing is a post-transcriptional process changing the identity of individual nucleotides in a transcript. In higher plants, editing by pyrimidine conversions occurs in both DNA-containing cell organelles, the plastids, and the mitochondria. RNA editing in plastids of higher plants proceeds exclusively by cytidine-to-uridine conversions (for review, see Bock 2000, 2001a), whereas, in hornwort chloroplasts, also “reverse” editing by U-to-C transitions has been found (Yoshinaga et al. 1996; Kugita et al. 2003). Interestingly, the RNA editing processes in plant mitochondria and plastids exhibit striking similarities (for reviews, see Hanson et al. 1996; Bock 2001a), suggesting that they originate from common evolutionary roots.

The vast majority of known plastid RNA editing sites are located within coding regions and, with only very few exceptions (Hirose et al. 1996; Kudla and Bock 1999), editing alters the coding properties of the affected triplets.

The changes brought about by mRNA editing usually result in the restoration of phylogenetically conserved amino acid residues (Maier et al. 1992a,b). Generation of transgenic plants with a noneditable version of a chloroplast gene has provided direct evidence for the functional significance of plastid mRNA editing (Bock et al. 1994).

RNA editing sites are generally believed to be recognized by the interplay between a *cis*-acting element at the RNA level and a proteinaceous *trans*-acting factor binding to the *cis*-element. The *cis*-acting element usually resides in the region immediately upstream of the editing site, as revealed by extensive analyses both in vivo and in vitro (Bock et al. 1996; Chaudhuri and Maliga 1996; Bock et al. 1997; Hermann and Bock 1999; Hirose and Sugiura 2001; Miyamoto et al. 2002; Hayes et al. 2006; Hayes and Hanson 2007). At least one site-specific *trans*-acting recognition factor is thought to bind to this *cis*-element (Chaudhuri et al. 1995; Bock and Koop 1997; Miyamoto et al. 2002, 2004). Recently, candidate site-specific editing factors have been identified for two RNA editing sites in the *ndhD* mRNA (Kotera et al. 2005; Okuda et al. 2006, 2007). The two proteins are members of the pentatricopeptide repeat (PPR) proteins, one of the largest protein families in plants, whose members appear to fulfill multifarious functions in plastid and mitochondrial gene expression.

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Interestingly, many of the known plastid RNA editing sites are only poorly conserved between species, indicating a remarkable evolutionary plasticity of the editing apparatus (Zeltz et al. 1993; Freyer et al. 1995, 1997; Fiebig et al. 2004). Editing sites can be easily lost by acquisition of a genomic C-to-T mutation, and loss of the editing site is usually followed by the loss of the site-specific *trans*-acting recognition factor, due to the absence of selective pressure for its maintenance. Evidence for this coevolution between editing sites and *trans*-acting specificity factors has been provided by in vitro studies (Miyamoto et al. 2002) and by plastid transformation experiments, in which heterologous editing sites were introduced into chloroplast genes: A site taken from species A and introduced into the plastid genome of species B usually cannot be edited, if species B lacks this site. For example, when the spinach *psbF* editing site was transplanted into the tobacco *psbF* gene (which naturally does not have the editing site), tobacco plastids were incapable of editing the spinach site (Bock et al. 1994; Bock and Koop 1997). Likewise, a heterologous maize editing site (from the *rpoB* transcript) remained unedited when introduced into the tobacco plastid genome (Reed and Hanson 1997). The only exception reported to date is a site within the *ndhA* gene of some but not all tobacco species: It is not present in the allotetraploid cultivated tobacco, *Nicotiana tabacum*, and one of its diploid progenitor species, *Nicotiana sylvestris*, but is present in the other diploid ancestor species, *Nicotiana tomentosiformis* (Schmitz-Linneweber et al. 2001). Nonetheless, *N. tabacum* and *N. sylvestris* possess the activity to edit the site (Tillich et al. 2006), possibly suggesting that, over short evolutionary distances, editing activities can be maintained for some time after the loss of the editing site.

We have recently conducted a comprehensive analysis of plastid RNA editing in Solanaceous plants (Kahlau et al. 2006) by comparing the editing patterns of three fully sequenced plastid genomes: tobacco (*Nicotiana tabacum*; Tsudzuki et al. 2001), deadly nightshade (*Atropa belladonna*; Schmitz-Linneweber et al. 2002), and tomato (*Solanum lycopersicum*; Kahlau et al. 2006). This comparison revealed the existence of a single tomato-specific editing site that was present neither in tobacco nor in *Atropa*. Editing in the second position of codon 74 of the *rps12* transcript changes a genomically encoded UCA serine codon into a UUA leucine codon in tomato. In tobacco and *Atropa*, a TTA leucine codon is already present at the DNA level, obviating the need for RNA editing (Fig. 1).

To gain more information about the coevolution of plastid editing sites and their specific recognition factors, we have transplanted the *rps12* editing site from the tomato into the tobacco *rps12* gene. We report that, surprisingly, this heterologous site was faithfully edited in tobacco. We provide a possible mechanistic explanation for this finding, which may have general implications for the evolution of RNA editing sites in plant organelles.

FIGURE 1. Construction of a plastid transformation vector to introduce the tomato *rps12* editing site into the tobacco *rps12* gene. (A) Map of the *rps12* region in the inverted repeat region of the tobacco plastid genome (ptDNA). Genes above the lines are transcribed from the left to the right; genes below the line are transcribed in the opposite direction. Location of the *rps12* site edited in tomato is indicated by an arrowhead. The map depicts an AvrII fragment, the restriction enzyme employed for RFLP analysis (Fig. 2). The location of the RFLP probe is also indicated. (B) Physical map of the plastid transformation vector (pDK112) constructed to integrate the tomato *rps12* editing site into the tobacco plastid genome by homologous recombination. The selectable marker gene for chloroplast transformation, *aadA* (Svab and Maliga 1993), was inserted into the intergenic spacer between the 3' portion of *rps12* and the *trnV* gene. Note that, due to the large distance between the *aadA* selection marker and the point mutation to be introduced, homologous recombination can be resolved in between the two genetic changes, resulting in transformants that harbor the *aadA* but lack the tomato *rps12* editing site. Relevant restriction sites used for cloning are indicated; sites lost by ligation of heterologous ends are shown in parentheses. The tomato restriction fragment containing the editing site is shown in gray. It exchanged the corresponding tobacco fragment (MscI/PstI). The mutation in exon II creating the tomato-specific *rps12* editing site by a T-to-C exchange is marked. Plastid transformation and selection for spectinomycin resistance produces two types of transplastomic lines: Homologous recombination at sites R1 and R3 results in integration of both the selectable marker gene *aadA* and the *rps12* editing site, whereas recombination at sites R1 and R2 integrates only the *aadA* into the plastid genome, but not the editing site. (C) Partial sequence alignment of the *rps12* region containing the mRNA editing site in tomato. Sequences are shown for three Solanaceous species: tomato (*Solanum l.*), tobacco (*Nicotiana t.*), and deadly nightshade (*Atropa b.*). Note complete sequence identity with exception of the editing position in tomato (lowercase c). The codon changed from serine to leucine by RNA editing in tomato is boxed; the editing event is indicated by the C-to-U conversion. The encoded amino acid sequence is shown in the one-letter code below the nucleotide sequence.

RESULTS AND DISCUSSION

Integration of the tomato *rps12* editing site into the tobacco plastid genome

The plastid *rps12* gene encodes ribosomal protein S12, an essential component of the small subunit of the prokaryotic 70S ribosome (Alksne et al. 1993). In higher plant plastids, the *rps12* gene consists of three exons and exhibits an unusual split structure. Exon 1 is located in the large single-copy region of the chloroplast genome, far away from exons 2 and 3, which are located in the inverted repeat region (Fig. 1A). Joining of exons 1 and 2 occurs by trans-splicing which generates mature *rps12* mRNA from two separately transcribed pieces (Zaita et al. 1987; Kohchi et al. 1988). The tomato-specific *rps12* editing site (Kahlau et al. 2006) is located in exon 2 and represents the only nucleotide in which the tomato exon 2 sequence deviates from the corresponding tobacco sequence (Fig. 1; data not shown).

To introduce the tomato *rps12* editing site into the tobacco *rps12* gene, we transplanted the corresponding tomato sequence into a cloned tobacco plastid DNA fragment containing *rps12* exons 2 and 3 (Fig. 1). To facilitate the selection of chloroplast transformants, the selectable marker gene *aadA* (Svab and Maliga 1993) was integrated into the adjacent intergenic spacer between the *rps12*–*rps7* operon and the *trnV* gene (Fig. 1B). The *rps12* exons 2 and 3 are part of a complex transcription unit (Koller et al. 1987; Zaita et al. 1987; Hildebrand et al. 1988), which precludes integration of the *aadA* marker in closer proximity to the editing site. Thus, in plastid transformation vector pDK112 (Fig. 1B), the mutation creating the editing site is ~1.7 kb away from the selectable spectinomycin resistance gene *aadA*. This rather large distance between mutation and marker gene is expected to result in frequent resolution of homologous recombination in between the two genetic changes, thus separating the *aadA* marker from the mutation (Bock et al. 1994; Bock and Maliga 1995; Hager et al. 2002). Consequently, two different types of chloroplast transformants are expected to be obtained: (1) transformants carrying only the *aadA* but not the tomato *rps12* editing site and (2) transformants having incorporated both the *aadA* gene and the mutation in *rps12* (Fig. 1).

Biolistic bombardment of young leaves from aseptically grown tobacco plants with plasmid pDK112-coated gold particles was followed by selection of primary spectinomycin-resistant cell lines. Successful chloroplast transformation was verified by tests for double resistance on medium containing two aminoglycoside antibiotics, spectinomycin, and streptomycin (Svab and Maliga 1993; Bock 2001b), and further confirmed by PCR assays using *aadA* gene-specific primers (Bock et al. 1996; data not shown). Chloroplast transformants were purified to homoplasmy by passing them through additional regeneration cycles under antibiotic selection to eliminate residual copies of the

wild-type chloroplast genome. Homoplasmy of the transplastomic lines was finally confirmed by RFLP analysis and transgene inheritance assays (Fig. 2A,B). Maternal inheritance of the antibiotic resistances confirmed transgene localization in the plastid genome; lack of segregation in the T1 generation (Fig. 2B) proved homoplasmy of the transplastomic lines.

Due to incomplete linkage between the selectable marker gene and the point mutation creating the tomato-specific editing site, the two expected types of plastid transformants were obtained. DNA sequencing of PCR products generated with *rps12*-specific primers revealed that 10 out of

FIGURE 2. (Legend on next page)

12 transformants carried only the *aadA* marker, but not the *rps12* editing site. However, two transformants could be isolated that harbored also the point mutation in *rps12*, creating the tomato-specific editing site (lines Nt-pDK112-1B and Nt-pDK112-3B; Fig. 2).

Interestingly, when still being heteroplasmic, the transplastomic lines with the *rps12* mutation showed evidence of active gene conversion and/or recombination between wild-type plastid genomes and transgenic genomes. This was discovered by direct sequencing of PCR products covering the *rps12* gene. The sequencing revealed a mixed population of transgenic plastid genomes which all carried the *aadA*, but were heterogeneous with respect to the presence of the point mutation in *rps12*. Consequently, purification of these lines to homoplasmy and segregation of the two transgenic genome types allowed isolation of transplastomic lines with and without the *rps12* mutation from one and the same primary transformed clone. For example, line Nt-pDK112-1B is homoplasmic for the tomato (editing site-containing) allele of *rps12*, whereas line Nt-pDK112-

1A is homoplasmic for the tobacco *rps12* allele lacking the editing site. Both lines stem from one and the same transformation event (primary transformant Nt-pDK112-1). Similarly, line Nt-pDK112-3B has the editing site and line Nt-pDK112-3A does not. Gene conversion between a transplastome with the *aadA* and the tomato editing site and a wild-type genome provides a likely explanation for obtaining these two transplastomic genome types and is in line with the earlier finding that gene conversion operates at high activity in plastids (Khakhlova and Bock 2006).

Analysis of tobacco plastid transformants carrying the tomato-specific *rps12* editing site

As the *rps12* exons 2 and 3 are part of an operon which is transcribed as a polycistronic mRNA, it was important to verify that neither insertion of the *aadA* selection marker nor the point mutation introduced into exon 2 affect transcription of the operon or stability of the *rps12* mRNAs. We, therefore, comparatively analyzed accumulation of *rps12* transcripts in wild-type and transplastomic tobacco lines (Fig. 2C). No significant difference was found in transcript pattern and mRNA accumulation levels between the wild type, the transplastomic lines only having the *aadA* marker and those additionally carrying the *rps12* point mutation indicating that integration of the *aadA* and introduction of the editing site do not negatively affect synthesis or stability of *rps12* transcripts.

Homoplasmic transplastomic tobacco plants with the tomato editing site in *rps12* displayed no discernable phenotype when grown in in vitro culture or under greenhouse conditions (Fig. 2B; data not shown). This tentatively suggested that either the tomato site can be edited in tobacco or the site remains unedited and this is functionally irrelevant in that the S12 protein can function with either serine or leucine in the amino acid position corresponding to the codon containing the editing site.

RNA editing of a tomato-specific editing site in transplastomic tobacco

We next wanted to determine whether the tomato *rps12* editing site can be processed by the tobacco editing machinery. To this end, we synthesized cDNA and directly sequenced the amplified cDNA population. As a control, the corresponding DNA amplification products were also sequenced. Surprisingly, the tomato-specific RNA editing site turned out to be fully edited in transplastomic tobacco (Fig. 3), indicating that tobacco plants possess the capacity to edit this foreign site. This suggests that the site-specific *trans*-acting recognition factor required for *rps12* editing is present in tobacco chloroplasts, even though the editing site does not exist in tobacco.

Maintenance of the editing capacity in the absence of the target site can be fortuitous in that, despite the loss of the

FIGURE 2. Analysis of plastid transformants generated with vector pDK112. (A) Southern blot confirming plastid transformation and assessing homoplasmy of transplastomic lines. Digestion with the restriction enzyme *AvrII* produces an ~7.0-kb fragment in the wild type and an ~8.2-kb fragment in all transplastomic lines. This size difference corresponds to the size of the *aadA* cassette. Note that line Nt-pDK112-3B is still heteroplasmic as evidenced by the presence of both the 7.0-kb wild-type fragment and the 8.2-kb fragment specific to the transplastome. This line was purified to homoplasmy by an additional regeneration round under antibiotic selection (not shown). (B) Confirmation of homoplasmy of transplastomic lines by seed assays and lack of a mutant phenotype in tobacco plants carrying the tomato-specific *rps12* mRNA editing site. Seeds from the wild-type and the selfed transplastomic lines Nt-pDK112-1B and Nt-pDK112-6A were germinated on medium with spectinomycin (500 mg/L). The progeny from the two transplastomic lines shown here is uniformly resistant to spectinomycin demonstrating complete absence of wild-type genome copies (Svab and Maliga 1993; Bock 2001a,b). As a control, untransformed wild-type seedlings are shown which are sensitive to spectinomycin and bleach out rapidly after germination. Transplastomic line Nt-pDK112-1B carries both the *aadA* selection marker and the tomato *rps12* editing site, whereas line Nt-pDK112-6A harbors only the *aadA*, but lacks the tomato editing site and thus has the unchanged wild-type tobacco *rps12* sequence. Plants from the two lines are phenotypically identical also at later developmental stages (not shown), indicating that introduction of the tomato editing site into the tobacco plastid genome does not result in a mutant phenotype. (C) Northern blot to analyze *rps12* transcript pattern and RNA accumulation in transplastomic tobacco lines. Total cellular RNA was electrophoresed under denaturing conditions and hybridized to a radiolabeled PCR product covering all three exons of *rps12*. In addition to the ~1.5-kb fully processed dicistronic *rps12-rps7* mRNA, the probe recognizes several other RNA species which represent incompletely spliced transcripts and other unprocessed or partially processed precursor transcripts. mRNA accumulation and transcript patterns are indistinguishable between the wild-type and the Nt-pDK112 transplastomic plants, indicating that neither integration of the selectable marker gene *aadA* upstream of the *rps12-rps7* operon (Fig. 1) nor introduction of the point mutation creating the *rps12* editing site (in lines Nt-pDK112-1B and Nt-pDK112-3B) affect transcription and/or mRNA accumulation.

FIGURE 3. Analysis of mRNA editing in transplastomic tobacco plants. *rps12* DNA and cDNA sequences are shown for a transplastomic tobacco plant harboring the tomato-specific *rps12* editing site (Nt-pDK112-1B) and a control transplastomic line containing only the *aadA* selectable marker gene, but the wild-type tobacco sequence in *rps12* (Nt-pDK112-6A). The RNA editing site is marked by vertical arrows in all sequences. Note complete C-to-U conversion in the cDNA sequence of line Nt-pDK112-1B suggesting efficient recognition of the heterologous tomato-specific editing site in transplastomic tobacco plants.

editing site in tobacco, the nuclear gene for the site-specific editing factor remained functional and escaped evolutionary deterioration. Alternatively, and perhaps more likely, the gene could be maintained by selection, because *rps12* editing is not the only function of this *trans*-acting editing factor. Based on competition analysis in plants overexpressing individual editing sites, it has been suggested that some *trans*-acting editing factors may be involved in the editing of more than one site (Chateigner-Boutin and Hanson 2002). We, therefore, searched for possible alternative targets of the *rps12* editing factor by analyzing all editing sites in the tobacco and tomato plastid genomes for sequence similarity to the tomato *rps12* site. As earlier work had established that the essential *cis*-acting elements for editing site recognition reside in the region immediately upstream of the edited nucleotide (Bock et al. 1996; Chaudhuri and Maliga 1996; Bock et al. 1997; Hirose and Sugiura 2001; Miyamoto et al. 2002; Hayes et al. 2006; Hayes and Hanson 2007), we analyzed the sequence stretches ranging from position –31 nt upstream of the editing site to position +1 downstream. We used phylogenetic tree construction software to cluster all editing sites in the genome according to their sequence similarities (Fig. 4A). This analysis revealed a single editing site that clustered with the *rps12* site: the editing site in codon 667 of *rpoB*, the gene encoding the catalytic β -subunit of the *Escherichia coli*-like plastid RNA polymerase (PEP). Alignment of the two sequences confirmed their high similarity (Fig. 4B) and may suggest that, indeed, the two sites are served by the same *trans*-acting specificity factor.

Earlier work has established that introduction into the plastid genome of extra copies of an editing site can result in reduced editing efficiency at both the endogenous and the transgenic editing site, presumably due to competition for *trans*-acting specificity factors that are present in

limiting amounts (Chaudhuri et al. 1995; Chaudhuri and Maliga 1996). To determine whether or not the presence of an additional target site in *rps12* makes the *trans*-acting site-specific factor limiting for *rpoB* editing in tobacco, we also investigated the efficiency of *rpoB* editing in codon 667. When the editing efficiency was determined by directly sequencing the amplified *rpoB* cDNA populations from transplastomic plants harboring the *rps12* site and control lines carrying only the *aadA* marker gene, the *rpoB* mRNA turned out to be fully edited at the site in codon 667 (data

FIGURE 4. Identification of an editing site in the *rpoB* gene which displays strong sequence similarity to the tomato *rps12* site. (A) Assessment of sequence similarities between editing sites using phylogenetic tree construction software. For all editing sites in the tomato plastid genome (Kahlau et al. 2006), the DNA sequence from position –31 to position +1 downstream from the editing site was used for tree construction. Only a single RNA editing site (located in codon 667 of the *rpoB* mRNA) exhibits close sequence relatedness with the tomato-specific *rps12* editing site (sites shaded) introduced into the tobacco plastid genome. Dotted lines indicate negative branch lengths. (B) Alignment of the regions immediately upstream of the tomato-specific *rps12* editing site and the *rpoB* editing site in codon 667 of tobacco and tomato. Nucleotides identical in both sequences are indicated by asterisks and shading; positions in which both sequences differ only in the purine (A or G) or the pyrimidine (T or C) are indicated by dots. The edited C is in bold and underlined.

not shown). This, together with complete editing at the heterologous *rps12* site (Fig. 3), indicates that the *trans*-acting editing factor(s) are not present in limiting amounts.

The tree construction approach taken here to identify sequence similarities between editing sites may serve as a useful tool to (1) detect editing sites with shared *cis*-acting recognition elements which may be served by identical *trans*-acting specificity factors and (2) predict editability of heterologous mRNA editing sites in plastid (and mitochondrial) genomes.

Evolutionary considerations

The editing of the heterologous *rps12* editing site from tomato in transplastomic tobacco plants and the observed high similarity of the sequences upstream of the *rps12* site and the *rpoB* site in codon 667 raise two possible evolutionary scenarios. In the “editing early” scenario, *rps12* editing could be the ancestral situation in Solanaceous plants and, during diversification of this family, the site was lost in multiple lineages, including the *Atropa* and tobacco clades. The editing activity for the *rps12* site, however, was maintained, because the same *trans*-acting specificity factor is also required for editing of a site in *rpoB* (which is present in all Solanaceae; Kahlau et al. 2006). In the alternative “editing late” scenario, *rps12* editing represents a more recent acquisition and is newly evolved in the *Solanum* clade. The birth of this novel site may have been facilitated by the possibility to co-opt a preexisting editing activity, which had evolved earlier to edit a site in *rpoB*. Preexistence of the editing activity would make a genomic T-to-C mutation in *rps12* codon 74 neutral, because it can immediately be corrected at the RNA level by editing (Covello and Gray 1993).

To distinguish between these two possibilities, we have conducted a preliminary phylogenetic analysis of *rps12* editing. Our results suggest that the editing site is not unique to tomato and is present also in other Solanaceous species, for example, *Petunia hybrida*, *Capsicum annuum*, and *Physalis alkekengi* (data not shown). More importantly, the site is also present in species outside the Solanaceae family, for example, in *Antirrhinum majus*, a species in the Scrophulariaceae, the sister family of Solanaceae, and in *Buddleja davidii*, a species in the family Buddlejaceae (data not shown). This may suggest that the “editing early” scenario represents the more likely evolutionary scenario for this site.

MATERIALS AND METHODS

Plant material

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown under aseptic conditions on agar-solidified MS medium containing 30 g/L sucrose (Murashige and Skoog 1962). Transplastomic

lines were rooted and propagated on the same medium in the presence of spectinomycin (500 mg/L).

Construction of chloroplast transformation vector pDK112

The PstI site in cloning vector pMCS5 (MoBiTec) was deleted by digestion with AvrII and XbaI and subsequent relegation generating plasmid pDK108. The *rps12-trnV* region of the tobacco plastid genome was cloned as a 2337 base-pair (bp) SacII/NcoI restriction fragment into the similarly cut pDK108, resulting in plasmid pDK109. A 770-bp MscI/PstI fragment including the tomato-specific *rps12* RNA editing site (Fig. 1B) was isolated from a cloned tomato plastid DNA fragment and replaced the corresponding fragment in pDK109, generating clone pDK110. Part of the vector backbone in plastid transformation vector pPRV111A (Zoubenko et al. 1994) was replaced with the corresponding region of pBluescript SK by ligation of two PvuII fragments, resulting in plasmid pDK111. Plasmid pDK110 was digested with AflII and SapI and ligated into the similarly cut vector pDK111, resulting in final transformation vector pDK112.

Plastid transformation and selection of transplastomic tobacco lines

Young leaves harvested from sterile tobacco plants were bombarded with plasmid pDK112-coated gold particles using the BioRad PDS1000He biolistic gun. Primary spectinomycin-resistant cell lines were selected on plant regeneration medium containing 500 mg/L spectinomycin dihydrochloride (Svab and Maliga 1993). Spontaneous spectinomycin-resistant plants were eliminated by double selection on medium containing spectinomycin and streptomycin (500 mg/L each; Svab and Maliga 1993; Bock 2001b). Putative plastid transformants were subjected to three additional rounds of regeneration on regeneration medium with spectinomycin to eliminate residual wild-type genome copies. Shoots resulting from the final round of regeneration were rooted on phytohormone-free spectinomycin-containing medium, transferred to soil, and further cultivated in the greenhouse.

For inheritance assays, surface-sterilized seeds were germinated on MS medium with spectinomycin (500 mg/L) and analyzed for uniparental inheritance of the *aadA* resistance gene used to introduce the tomato *rps12* editing site into the tobacco plastid genome. Selfed transformants and crosses with a plastid transformant as the maternal parent give rise to resistant (green) progeny, whereas seeds collected from wild-type plants yield sensitive (white) seedlings.

Isolation of nucleic acids

Total plant DNA was isolated from fresh leaf tissue samples by a cetyltrimethylammoniumbromide (CTAB)-based method (Doyle and Doyle 1990). Total cellular RNA was extracted with the TriFast reagent (Peqlab GmbH). For reverse transcription, RNA samples were treated with DNase I (Roche Diagnostics GmbH) to eliminate residual contaminating DNA.

DNA and RNA gel blot analyses

Total cellular DNA from wild-type and transplastomic plants was digested with restriction enzymes, separated in 0.8% agarose gels

and transferred onto Hybond nylon membranes (Amersham) by capillary blotting. A PCR product obtained with primers Nb0 (5'-TGCTTCTCTTCGATGGAAG-3') and P13 (5'-TTTTGGATCCTA TACATTTGTGTATTGATA-3') was radiolabeled with ^{32}P -dCTP and used as a probe in Southern blot analyses (Fig. 1A). Likewise, a radiolabeled PCR product served as a specific probe in Northern blot analyses to specifically detect *rps12* transcripts. The PCR product covers all three exons of *rps12* and was obtained with primer pair P184 (5'-CGGTTAGGATCAATCTAAACCAACCC-3') and P185 (5'-GAGCGTGAAAGGGGTCAAGAATC-3') using cDNA as a template. Hybridizations were performed at 65°C in Rapid-Hyb buffer (Amersham) following the manufacturer's protocol.

cDNA synthesis and PCR

RNA samples treated with RNase-free DNase I were reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen) following the instructions of the supplier. cDNA synthesis was primed with random hexanucleotide primers. cDNA samples were amplified with the *rps12*-specific primer pair P184/P185 in an Eppendorf thermocycler using Taq DNA polymerase (Promega) and standard protocols. The genomic *rps12* sequence was amplified with primers PSeq-rps12 (5'-GGGTGACTTATCTGTCAAC-3') and PRps12-rev (5'-ATGGTAGCCTGCTCCAGTC-3'). DNA and cDNA amplification products were purified for sequencing by electrophoresis in 1.5% agarose gels and subsequent extraction from gel slices using the Nucleospin Extract II kit (Macherey-Nagel). Direct sequencing of amplification products was performed by cycle sequencing. Oligonucleotide P184 served as sequencing primer for cDNA PCR products; DNA amplification products were sequenced with primer PSeq-rps12.

Bioinformatics analyses

Sequence similarities between putative *cis*-acting recognition elements upstream of plastid RNA editing sites were identified using phylogenetic tree construction software (MegAlign program from the Lasergene software package, DNASTAR; GATC Biotech). For each editing site, a 33-bp sequence stretch was entered, including the editing site itself, 31 bp upstream and 1 bp downstream. As the upstream *cis*-elements for editing site recognition can be somewhat variable in their distance from the edited C, the unaligned raw sequences were used for tree construction. This procedure ensured that all sequences had the edited C in the same position and no gaps were introduced through the alignment algorithm. The length of each pair of branches represents the distance between sequence pairs. Dotted lines in the phenogram indicate negative branch lengths, a common consequence of averaging. The phenogram with balanced branches averages the distances between ancestors in the tree.

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