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Expression of complementary RNA from chloroplast transgenes affects editing efficiency of transgene and endogenous chloroplast transcripts

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ABSTRACT

The expression of angiosperm chloroplast genes is modified by C-to-U RNA editing. The mechanism for recognition of the ~30 C targets of editing is not understood. There is no single consensus sequence surrounding editing sites, though sites can be grouped into small 'clusters' of two to five sites exhibiting some sequence similarity. While complementary RNA that guides nucleotides for alteration has been detected in other RNA modification systems, it is not known whether complementary RNA is involved in chloroplast editing site recognition. We investigated the effect of expressing RNA antisense to the sequences −20 to +6 surrounding the RpoB-2 C target of editing, which is a member of a cluster that includes the PsbL-1 and Rps14-1 sites. Previous experiments had shown that chloroplast *rpoB* transgene transcripts carrying only these 27 nt were edited *in vivo* at the proper C. Though transcripts carrying sequences −31 to +60 surrounding the RpoB-2 sites were edited in chloroplast transgenic plants, transcripts carrying the −31 to +62 region followed by the 27 nt complementary region were not edited at all. In contrast, a similar construct, in which the C target as well as the preceding and subsequent nucleotides were mismatched within the 27 nt region, was efficiently edited. The presence of any of the four transgenes carrying RpoB-2 sequences in sense and/or antisense orientation resulted in reduced editing at the PsbL-1 site. Chloroplast transgenic plants expressing the three different antisense RNA constructs exhibited abnormal growth and development, though plants expressing the 92 nt sense transcripts were phenotypically normal.

INTRODUCTION

Angiosperm chloroplast RNAs are modified by RNA editing. In tobacco, 34 different C-to-U editing events have been detected (1,2). Editing in chloroplasts usually results in the encoding of an amino acid that is more conserved evolutionarily than the amino acid predicted from the unedited codon (3,4). While most changes in protein sequence resulting from editing have not yet been analyzed for their functional significance, at least in several cases, the presence of an amino acid predicted from unedited mRNA results in an abnormal chloroplast protein (5–7). In tobacco chloroplasts, editing efficiency is high; almost all transcripts exhibit 70–100% conversion of C to U at known editing sites (8). The function of chloroplast RNA editing is presently thought to be a correction mechanism for T-to-C mutations that have accumulated in the chloroplast genome (9), rather than as a mechanism to generate protein diversity, as occurs in other C-to-U editing systems (10–12).

Most Cs in chloroplast transcripts are not altered to Us, so there must be an unidentified mechanism that allows specificity of the selection of C targets for editing. Analysis of transcripts carrying various amounts of sequence surrounding editing sites in transformed chloroplast genomes, or by editing assays *in vitro*, indicates that a relatively small amount of sequence upstream and downstream of the C target is needed to confer specificity. Transcripts can be edited that carry <100 nt surrounding the editing target, and typically sequences ~30 nt 5′ and 10 nt 3′ of the edited C are sufficient for proper editing (13–19).

When all sequences immediately 5′ and 3′ to edited Cs in tobacco are aligned, no conserved *cis*-elements can be detected. However, it is possible to group most of the known editing sites into 'clusters'—pairs and trios of sequences that exhibit some modest sequence similarity. Evidence that the similar sequences are functionally significant comes from the results of competition experiments *in vivo*. When either the tobacco RpoB-2 editing site or the NdhF-2 editing site is quite highly expressed in transgenic chloroplasts, the editing efficiency of

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the corresponding sites in endogenous *rpoB* and *ndhF* transcripts is impaired, presumably because of competition between the overexpressed transgene transcripts and the endogenous transcripts for a limiting site-specific factor. However, competition is not limited to *rpoB* and *ndhF* transcripts, but also occurs with editing sites carrying similar sequences. Overexpression of RpoB-2 impairs editing of PsbL-1 and Rps14-2, while high levels of NdhF-2 result in reduced editing of NdhB-3 and NdhD-1 (20). This has led to the hypothesis that a single *trans*-factor or a family of closely related *trans*-factors may operate on all members of an editing site cluster.

While analysis of RNA editing *in vitro* presently implicates the existence of protein site-specific recognition factors, the involvement of *cis*- or *trans*-acting RNAs has not been ruled out. Furthermore, the structural requirements for editing of a C within a transcript are not understood. We decided to perturb the structure of an editing substrate by producing transgenic chloroplasts that express sequences antisense to the region immediately surrounding the edited C at the RpoB-2 editing site. We had previously observed that either a 27 nt or a 92 nt sequence surrounding the RpoB-2 C target of editing was sufficient to encode editing *in vivo* (16). We made two constructs in which the 27 nt sequence in antisense orientation was attached to the 3′ end of the 92 nt RpoB-2 sequence. We also expressed a small RNA carrying the 27 nt sequence in antisense orientation, not fused to the 92 nt RpoB-2 sequence. Our analysis of the resultant transgenic plants indicates that antisense RNA affects editing efficiency of transgene transcripts and can also affect editing of endogenous transcripts from genes within the RpoB-2 editing site cluster. The antisense RNA did not affect the abundance of *rpoB* transcripts or encoded protein. However, for unknown reasons, growth and development of plants expressing the antisense RNAs was abnormal.

MATERIALS AND METHODS

Oligonucleotides

The following oligonucleotides (Integrated DNA Techologies, Coralville, IA) were used in this study:

rpoB-anti-up, AACCGCCATGGATTCTGATCTTCCTCCCCAAT
rpoB-anti-down, AACCGATTCTAGAATCAGATTGGGGAGGAA
anti27bub-up, AAATCGAATTCCCCTCTTCCTCCCCAATCTG
anti27-down, GCCTGTTCTAGAATCAGATTGGGGAGGAAG
anti27-up, AAATCGAATTCTGATCTTCCTCCCCAATCTGA
rpoB92-down, GAATTCGATTTTTTGTTTCCTACTTACACGAGC
rpoB92-up, CAGGACCATGGGGCACCATAATATCAGATTGGGGAGG
Trps16α3.1, CTACCCCCCTTTTTGTATTTCCTTAATTTATTTCC;
PPrrn2, AATACGAAGCGCTTGGATACAGTTGTAGGGA;
mzB5.1, TCACCTATTCTTCCGAATTATATGTATCCGCG;
PC1.1, TCTTGAACAACTTGGAGCCGGGCC;
PCα1.2, GAGGATAGCAAGTTCCAAATTCTGTCTCGG;
FpsbL, TACCGTCTTTTTTTTGGGATC; and RpsbL, ATTTTGTTCGTTCGGGTTTGA.

Construction of plastid transformation vectors

A 92 nt portion of the RpoB-2 coding region (−31/+60 nt relative to the edited C) was amplified by PCR using the primers rpoB92-up and rpoB92-down, which contained 5′ restriction sequences for NcoI and EcoRI, respectively. DNA containing 27 nt antisense to the sequence −20/+6 relative to the edited C was synthesized by annealing the primers anti27-up and anti27-down and extending with *Pfu* polymerase to give the product 27α-1. The primers contained 5′ restriction sequences for EcoRI and NcoI, respectively. The primers anti27bub-up and anti27bub-down were used to synthesize an EcoRI/NcoI flanked antisense fragment (27α-2) with a 3 nt mutation at the complement of the editing site and the upstream and downstream nucleotides (TGA→CCC). The antisense fragments were each ligated to the 92 nt minigene PCR product (Figure 1). The resulting fragments were digested with NcoI and XbaI and ligated to the chloroplast

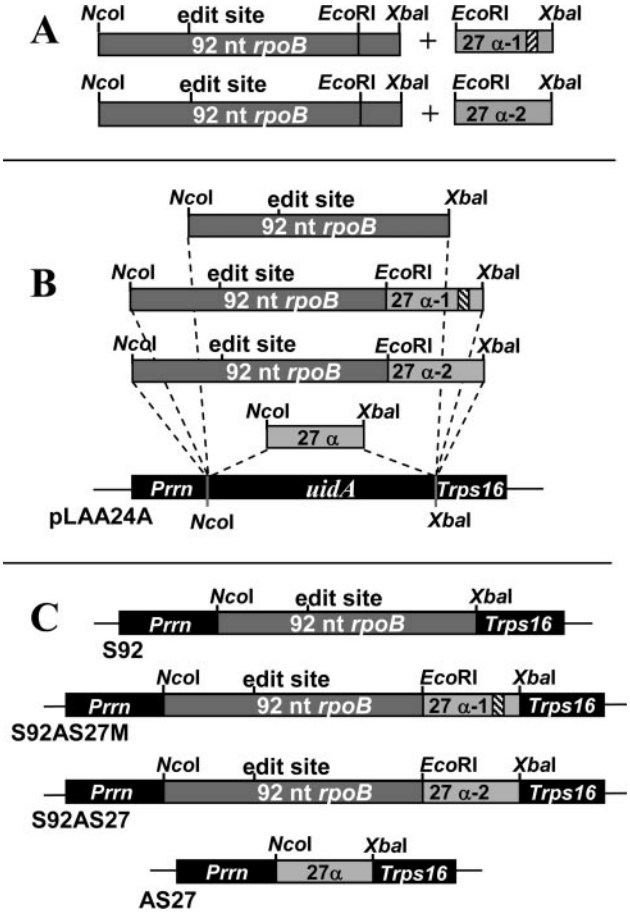

Figure 1. Construction and molecular cloning of altered versions of the sequences surrounding the tobacco RpoB-2 site. (**A**) Ligation of 92 bp rpoB-2 minigene to 27 bp antisense fragment. The hatched area on fragment 27α represents a 3 nt mutation. (**B**) Insertion of the sense/antisense ligations, a 92 bp sense fragment, and a 27 bp antisense fragment into chloroplast transformation plasmid pLAA24A. (**C**) Expression cassettes of transformation plasmids S92AS27, S92AS27M and AS27.

transformation plasmid pLAA24A (21), which had been digested with the same enzymes to remove the *uidA* coding sequences (Figure 1B and C) . The resulting plasmids CH27α and CH27α-b 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. An additional transformation construct containing the 27 nt antisense sequence between the NcoI and XbaI sites (CHα) was made by annealing and extending primers rpoB-anti-up and rpoB-anti-down, digesting the product with NcoI and XbaI, and ligating it into NcoI/XbaI digested pLAA24A. Prior to bombardment, all plasmids were sequenced to confirm that the inserts contained the correct sequences. Large-scale preparations of the plasmids made with the Qiagen purification system.

Plastid genome transformation and tissue culture

Seedlings of tobacco cultivar Petit Havana were grown on MS-agar medium (22) in 100 × 25 mm Petri dishes. Rooted 14-day-old seedlings were bombarded with plasmid-coated tungsten particles by using a Bio-Rad model PDS1000/He Biolistic Particle Delivery System. Transformed tissue was regenerated essentially as described by Svab and Maliga (23). An aliquot of 2 μg of plasmid DNA was precipitated on tungsten particles for each shot, and each plate was bombarded twice. DNA was isolated from transformed tissue by the CTAB (hexadecyltrimethylammonium bromide) method (24) and was analyzed for *aadA* incorporation by PCR amplification.

Electron microscopy

A piece of young leaf tissue, ~0.5 cm in diameter, was excised from shoots growing on RMOP medium (23). The tissue was excised from between the veins and away from the edge of the leaf and sliced into ~1 mm pieces with a scalpel. Diced tissue was fixed immediately in 2.5% glutaraldehyde/2.5% formaldehyde in 0.1 M cacodylate buffer with 0.2 M sucrose and 2 mM CaCl2, pH 7 in a vacuum dessicator for 15–30 min at room temperature, followed by overnight at 4°C. Samples were washed three times with buffer for 10–30 min, then post-fixed in 1% OsO_4 in buffer overnight at 4°C. After three 10 min washes in buffer, followed by three times in sterile double distilled water for 10 min, samples were dehydrated with increasing concentrations of ethanol and infiltrated in a step-wise manner with Spurr's low viscosity resin over 2 days before being embedding in flat embedding molds in pure resin.

After polymerization at 70°C overnight, ultrathin sections (~50–70 nm) were cut using a diamond knife on an LKB Ultratome Nova and mounted on formvar-coated copper grids. Sections were stained by immersion in 5% uranyl acetate in 50% ethanol for 1 h 20 min, rinsed in 50% ethanol and, dried and stained with Reynold's lead citrate for 3–4 min. Samples were viewed and digitally photographed in an FEI Morgagni transmission electron microscope.

Chlorophyll fluorescence

In vivo chlorophyll *a* fluorescence measurements were obtained using a Hansatech FMS2 modulated fluorometer (Hansatech Instruments, Ltd) interfaced to a desktop computer. Measurements of Φ_{PSII} and dark-adapted F_v/F_m were accomplished using standard techniques (25). Plantlets were dark adapted for 15 min prior to measurement of F_v/F_m (three consecutive measurements at 60 s intervals). Samples were then illuminated with either limiting (50 μmol photons m^{-2} s^{-1}) or saturating (500 μmol photons m^{-2} s^{-1}) light from the FMS2 until steady state photosynthesis was established (typically 8–12 min for limiting illumination and 4–6 min for saturating illumination). This was followed by three consecutive measurements of Φ_{PSII} at 30 s intervals. All data are reported as means and SDs from the three replicate samples on six plantlets.

DNA blot analysis

Total DNA (1 μg) from transformed or wild-type leaves was digested with BamHI, electrophoresed and blotted onto a positively charged nylon membrane (Nytran+, Amersham) 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 using primers (PC1.1 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 using the RNeasy Plant Mini Kit (Qiagen). Contaminating DNA was removed by a 30 min treatment with DNA-free reagent (Ambion). cDNA was synthesized by reverse transcription (Omniscript, Qiagen) using degenerate hexamers. The cDNA was PCR amplified using primers PPrrn2 and Trps16α3.1 for transformed sequences and primers mzB5.1 and mzBα3.2 for endogenous *rpoB* sequences surrounding the editing site.

Poisoned primer extension (PPE) was performed on RT–PCR products to determine editing extent. The amplification product was quantified on an agarose gel and then subjected to a primer extension reaction using radiolabeled oNP90. An aliquot of 100 ng of oNP90 was radiolabeled with 100 μCi of [γ-^{32}P]ATP, using 10 U of T4 kinase (BRL) for 2 h at 37°C. The oligonucleotide was separated from the non-incorporated [γ-^{32}P]ATP using a NucTrap column (Stratagene). A 20 μl PPE reaction contained 80–100 ng of RT–PCR template, 5 ng of labeled oNP90, 2 μl of 10× buffer (BRL), 0.6 μl of 50 mM $MgCl_2$ (BRL), 0.2 μl Platinium *Taq* polymerase (5 U/μl; BRL), 50 μmol each of dATP, dTTP and dCTP (BRL), and 500 μmol ddGTP (Amersham Pharmacia Biotech). The reaction was amplified in a thermocyler (PTC-100; MJ Research) under the following conditions: 94°C for 2 min, followed by 50 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 15 s. The reaction was stopped by adding 5 μl of DNA sequencing stop solution (Promega; Madison, WI), and then heated at 72°C for 2 min. An aliquot of 5 μl of each sample was loaded on a 12% acrylamide sequencing gel (SequaGel; National Diagnostics), and electrophoresed in 1× TBE for 2 h. The gel was exposed to a phosphorimager screen for 2 h and was then scanned using a Storm 840 (Amersham Molecular Dynamics).

A restriction assay for editing extent was also performed on the RT–PCR products of the transgene transcripts. First strand

cDNA was synthesized from DNase-treated RNA (2 μg) primed with 20 pmol of a degenerated hexamer (BRL) using the Omniscript RT kit (Qiagen). The transgene cDNA was then amplified by PCR with the oligonucleotides Trps16α3.1 and PPrrn2. The resulting PCR products were cloned in the pCR2.1-TOPO vector (Invitrogen). Single white colonies were selected and cultured overnight at 37°C. Aliquots of each culture (10 μl) were transferred to an individual well of a 96-well PCR microtiter plate. Each well was supplemented with 40 μl of a PCR mix [5 μl of 10× buffer (BRL), 1.5 μl of 50 mM $MgCl_2$ (BRL), 0.5 μl Platinium *Taq* polymerase (5 U/μl, BRL), 1 μl of 10 mM dNTP, 1 μl of 10 μM Trps16α3.1, 1 μl of 10 μM PPrrn2 and 30 μl water]. A PCR was then performed with a 58°C annealing step (PTC-200; MJ Research) and 10 μl of the PCR product was digested with 5 U of Sau3AI (New England Biolabs). After 6 h at 37°C the digest reaction was loaded on a 4% NuSieve agarose gel (FMC Bioproducts) and electophoresed in 1× TBE.

RNA blot analysis

An aliquot of 10 μg of total RNA was electrophoresed on a 1.5% agarose–0.44 M formaldehyde–MOPS gel and blotted onto Hybond-N+ (Amersham) with a Turboblotter (Schleicher and Schuell). Hybridization was performed with sense and antisense oligonucleotide probes corresponding to the 27 nt sequence, labeled using the KinaseMax system (Ambion).

Immunoblot analysis

Polyclonal antibodies were prepared in rabbit by Sigma-Genosys against a synthetic oligopeptide from *rpoB* gene sequence (RIWARVSRKQKISI) that was conjugated to keyhole limpet hemocyanin.

Total protein was obtained from shoots growing in culture on RMOP medium (23) in Petridishes. Protein was extracted with homogenization buffer (50 mM Tris–HCl and 1 mM EDTA) in the presence of a plant protease inhibitor cocktail (Complete, Roche) and 0.1% (v/v) Triton X-100. Protein was quantified according to a bovine serum albumin standard curve using the Bio-Rad Protein Assay kit.

For immunodetection of RPOB, protein extracts were subjected to SDS–PAGE and were subsequently transferred to membranes according to the manufacturer's specifications (Bio-Rad). Membranes were incubated with primary antibody and secondary (horseradish peroxidase conjugated goat-anti-rabbit; Amersham) antibody at a 1:500 dilution and a 1:50 000 dilution, respectively. Protein bands were detected using the SuperSignal West Dura Extended Duration Substrate according to the manufacturer's specifications (Pierce Biotechnology).

Semi-quantitative RT–PCR

Total RNA was isolated from transformed and wild-type leaves using the RNeasy Plant Mini Kit (Qiagen). Contaminating DNA was removed by a 30 min treatment with DNA-free reagent (Ambion). cDNA was synthesized from 1 μg template RNA by reverse transcription (Omniscript, Qiagen) using degenerate hexamers. The cDNA was PCR amplified using the Qiagen PCR Master Mix kit. The primers PPrrn2 and Trps16 3.1 were used to amplify transformed sequences and the primers FpsbL and RpsbL were used to amplify the endogenous *psbL* sequences. PCR cycling (94°C, 30 s; 50°C, 30 s; and 72°C, 30 s) was stopped at 25 and 40 cycles. Amplicons were then run on a 1% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

Nucleotide sequence accession numbers

The GenBank accession number for tobacco *rpoB* is X12745.

RESULTS

Construction of chloroplast transgenes carrying the rpoB-2 editing site

Of the four edited Cs identified in the tobacco *rpoB* gene, we chose RpoB-2 for analysis because of our prior work with chloroplast transgenes expressing wild-type and altered RpoB-2 sequences surrounding the edited C (16,20,26). We synthesized four different coding regions for incorporation into the vector pLAA24A (21), which was digested with NcoI and XbaI to remove the *uidA* coding sequences and to introduce the chimeric constructs.

Three constructs contained 92 nt of coding region encompassing the tobacco rpoB-2 editing site. The 92 nt tobacco sequence (S92) corresponds to the 92 nt maize RpoB-2 sequence previously incorporated into transgenic tobacco line MR210 by Reed *et al*. (26). The maize 92 nt RpoB-2 was edited in tobacco transgenics, therefore, we expected that the tobacco RpoB-2 S92 nt sequence would also be edited. Because Reed and Hanson (16) had observed that transgene transcripts carrying only −20 to +6 sequence at the RpoB-2 site were edited *in vivo*, we expected this region to be particularly important for recognition of the C target of editing at RpoB-2. In the S92AS27 and S92AS27M construct (Figure 1), a 27 nt antisense (AS) sequence was attached to the 3′ end of the 92 nt fragment. The 27 nt sequence in S92AS27 is perfectly antisense to nucleotides −20 to +6 surrounding the C target of editing within the 92 nt coding region, while in S92AS27M, the 27 nt sequence carries a 3 nt mismatch at the C target and at the preceding and subsequent nucleotide. A third construct contained only the −20 to +6 sequence in antisense orientation (Figure 1). Hypothetical secondary structures were predicted by GeneQuest and MFold and are diagrammed in Figure 2. In the construct with the 3 nt mismatch, while it is likely that nt −1 to −20 are base paired with the attached antisense sequence, the following region at +2 to +6 is less likely to be base paired.

Cloning into the NcoI site results in the presence of an AUG and therefore a potentially translatable transgene; however, the transcript is out-of-frame with respect to *rpoB*, and only very short polypeptides are predicted. The three constructs containing the S92 sequence predict only 5−6 amino acid polypeptides if translation occurs, while the AS27 construct predicts a polypeptide of 10 amino acids if translated.

Production of chloroplast transgenic plants

Young tobacco seedlings growing in culture were bombarded with DNA-coated tungsten particles and selected on spectinomycin-containing regeneration medium (23). A number of initial transformants were detected with each construct. Successive rounds of selection resulted in identification of

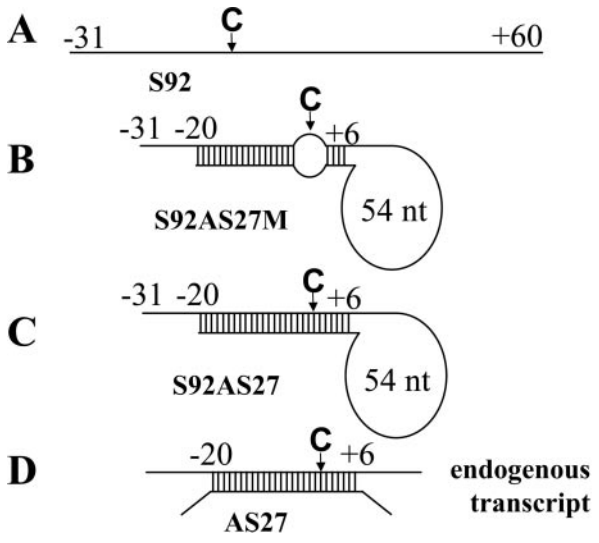

Figure 2. Hypothetical structures formed by transgene transcripts. (**A**) The 92 nt *rpoB* transgene is not expected to form a strong secondary structure. (**B**) The 27 nt antisense portion of the S92AS27 transgene transcript is complementary to the −20/+6 region of the editing minigene, except that a 3 nt mutation in the antisense sequence results in a mismatch at the position of the edited C and the two flanking nucleotides. (**C**) The S92AS27m 27 nt antisense portion is the perfect complement of the −20/+6 region of the editing minigene. (**D**) Expected interaction of the antisense transgene transcript with the endogenous *rpoB* transcript. Diagrams are not drawn to scale.

three lines carrying S92AS27M and two lines carrying either S92AS27 or AS27 that appeared to be homoplasmic upon chloroplast DNA analysis. One homoplasmic line carrying each construct was chosen for further detailed analysis.

To determine homoplasmicity, i.e. whether the transgene was incorporated into all chloroplast genomes the total leaf DNA was digested with BamHI, electrophoresed, transferred to nylon membrane and probed with a 350 nt fragment corresponding to the integration site in the plastid genome. In the wild-type DNA, a prominent signal is detected at 3.2 kb that is absent in the three homoplasmic transformed lines, which exhibit signals at 3.8 and 0.8 kb due to the integration of the transgenes (Figure 3).

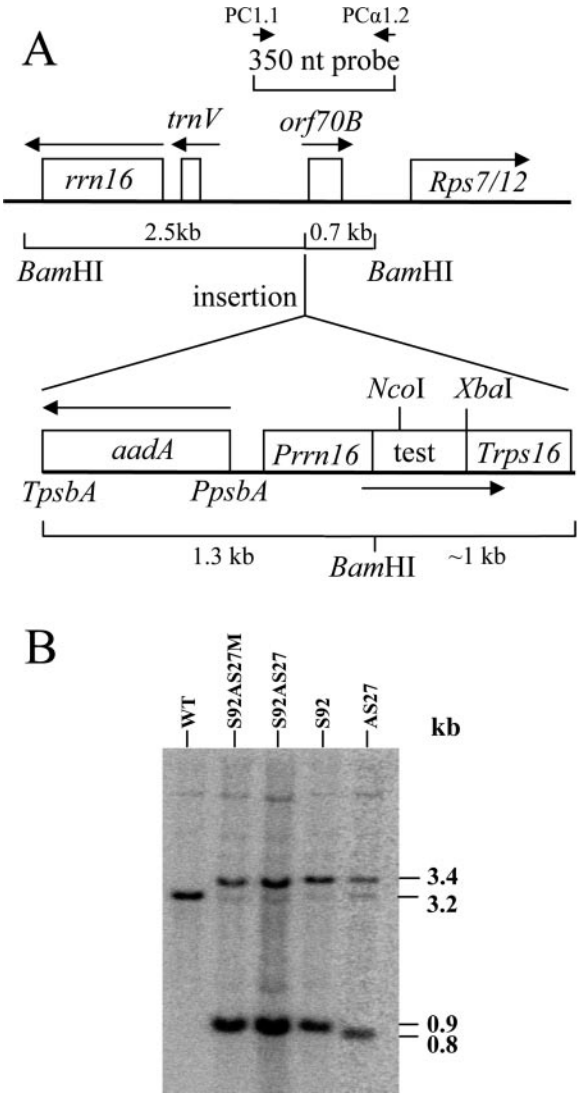

Figure 3. Analysis of DNA from transplastomic shoots (**A**) Schematic representation of the wild-type plastid genome and the targeted transformation plasmid expression cassettes (not drawn to scale). (**B**) DNA was isolated from leaves of transformed and control plants and digested with BamHI. DNA blots were probed with a 350 bp PCR product spanning the plasmid integration site. Fragment sizes are indicated on the right in kb.

Phenotype of chloroplast transgenic plants

S92 transgenic plants were phenotypically normal (Figure 4A). These plants were able to root in the MS medium and could be transferred to soil, where they grew to flowering and seeds were obtained. Homoplasmic lines containing S92AS27M exhibited smaller shoots and leaves in culture (Figure 4B), but a plantlet rooted normally and was grown to flowering. Though the plant was male sterile, seeds could be obtained by using a wild-type tobacco plant as male parent. Homoplasmic lines containing S92AS27 exhibited small shoots with short internodes and thick, narrow, disorganized leaves and elongated trichomes (Figure 4C). AS27 lines had the same abnormal phenotype as line S92AS27, but the phenotype is more pronounced in plants containing the AS27 construct (Figure 4D). Lines containing S92AS27 or AS27 did not form roots on MS medium; after treatment with auxin, small thick roots formed, but shoots would not thrive on soil. Independent homoplasmic lines containing each of the four constructs exhibited the same phenotype. The abnormal phenotypes of S93AS27 and AS27 shoots were not evident until sufficient rounds of selection had occurred to produce homoplasmic plants.

Leaf tissue of the two lines with the most severe phenotype, S92AS27 and AS27, were analyzed further by electron microscopy. Both lines exhibited abnormal chloroplasts with prominent starch granules and disorganized thylakoid membranes in comparison with wild-type tobacco growing on the same culture medium (Figure 5). The starch accumulation phenotype is typical of plants with disrupted chloroplast function that utilize sucrose from the medium as an energy source.

Analysis of transgenic and endogenous *rpoB* transcripts

Total RNA was extracted from shoots of the four homoplasmic transgenic lines and wild type growing in culture on RMOP

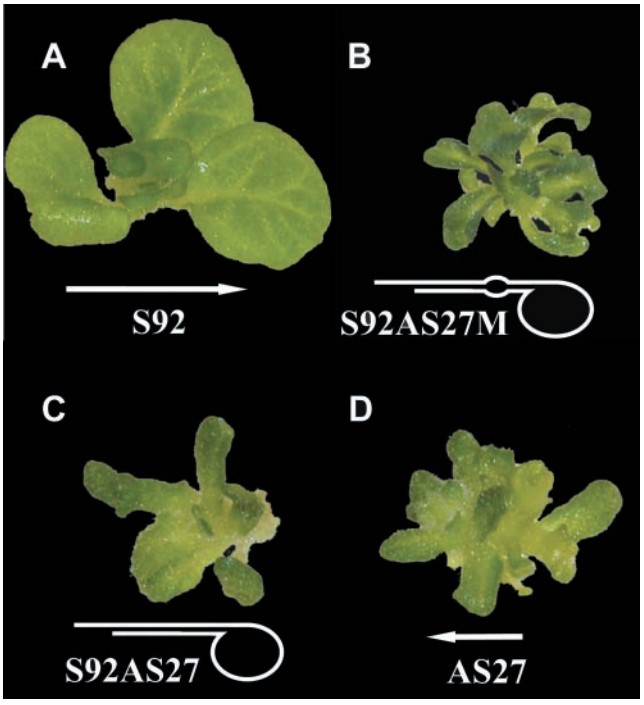

Figure 4. Shoot phenotypes of transplastomic lines. (**A**) A phenotypically normal shoot expressing a 92 nt *rpoB* editing minigene transcript. (**B**) A plantlet carrying S97AS27M has small leaves with normal venation. (**C**) A plantlet carrying S92AS27 is small with thick, disorganized leaves and elongated leaves. (**D**) A line carrying AS27 has the same phenotype as the S92AS27, but the abnormalities are more pronounced.

medium (23). Following electrophoresis, blots were probed with an oligonucleotide complementary to the −20 to +6 region in sense or in antisense orientation (Figure 6A and B). The probe for RpoB-2 sense transcripts readily detected signals in the lines containing S92, S92AS27 and S92AS-27M, but did not detect the endogenous *rpoB* transcripts at the exposure shown, as those transcripts are much less abundant. Transgene transcript abundance appeared the greatest in S92 and less in S92AS27M and S92AS27. As expected, no signals were seen in AS27 or in WT, because those lines do not carry the 92 nt coding region in a highly expressed transgene. The endogenous transcript was detected with a double-stranded probe that does not contain the −20 to +6 region. The amount and sizes of endogenous *rpoB* transcripts appear to be approximately the same in all five lines (Figure 6C).

To examine transcripts further, we performed semi-quantitative RT–PCR using RNA isolated from wild-type and the four transgenic lines (Figure 7). The amount of *psbL* transcript appears to be approximately the same in all five lines. Transgene transcript could be detected in all four transgenic lines, but the abundance of the S92AS27M and S92AS27 transcript appear to be lower than that of the S92 transcript, a result consistent with the RNA blot. This RT–PCR experiment also reveals that the AS27 transcripts accumulate in transgenic chloroplasts.

Analysis of RpoB protein in wild-type and transgenic lines

We were interested in determining the amount of RpoB protein present in the transgenic lines, because of the possibility that the abnormal phenotypes could result from interference of proper translation of *rpoB* transcripts by the antisense region present in the transgenic plants exhibiting abnormalities. We produced antisera to a synthetic peptide predicted from the *rpoB* sequence.

Total leaf protein was extracted from wild-type and transgenic shoots growing on RMOP medium and electrophoresed on SDS–polyacrylamide gels. Immunoblots probed with the anti-RpoB antiserum revealed the presence of similar amounts of a protein with the mobility expected for the 120 kDa RpoB protein. As a control, the membrane was stained with Ponceau-S, revealing that each line exhibited approximately the same amount of signal at the mobility of the large subunit of Rubisco (Figure 8). Thus, the presence of antisense transgenic transcripts does not appear to affect the abundance of the RpoB protein.

Editing extent of transgenic and endogenous *RpoB* transcripts

Editing of transgene transcripts was assayed by an RT–PCR restriction digestion assay, previously used in a study of tobacco chloroplast transgenic plants containing the maize rpoB-2 editing site (26). In this assay, cDNA is synthesized using primers specific to either the transgene or endogenous transcripts, and RT–PCR products are cloned. Each clone is subjected to digestion by Sau3AI, which can distinguish whether a clone carries a C or a T at the editing site. This assay was used for analysis of the transgene transcripts because it is not sensitive to secondary structure, unlike the PPE assay. However, because the PPE assay analyzes a larger number of transcripts simultaneously, it is more accurate and precise when extensive base pairing is not an issue (27). Therefore, the PPE assay was used for the analysis of endogenous *rpoB* transcripts in the transgenic plants.

According to the digestion assay, the S92AS27M transcript was more highly edited than the S92 transcript, 74% versus 40% editing (Table 1). In contrast, the transgene transcript containing a perfect complementary antisense region was not edited in any of the transcripts analyzed. Editing of endogenous *rpoB* transcripts was affected only in the S92 line (Table 1). The finding of 60% editing of endogenous transcripts in the S92 transgenic line is expected from our previous work in which the comparable 92 nt maize sequence was expressed in tobacco (26), which resulted in 50% editing of endogenous transcripts. The reduction in the editing of endogenous transcripts is presumably due to competition by the highly expressed transgene transcript for a limiting *trans*-acting factor. The S92AS27M and S92AS27 transcripts that are present in lower abundance than the S92 transcripts (Figure 6) do not affect the editing extent of the endogenous *rpoB* transcripts. The expression of the AS27 transcript also does not significantly affect editing of endogenous *rpoB* transcripts (Table 1).

Editing extent of Cs in transgenic versus wild-type tobacco plants

Previously we observed that high-level expression of the 92 nt maize RpoB-2 sequence resulted in reduced editing not only of endogenous *rpoB* transcript editing, but also decreased editing of PsbL-1 and Rps14-1 in chloroplasts of transgenic tobacco

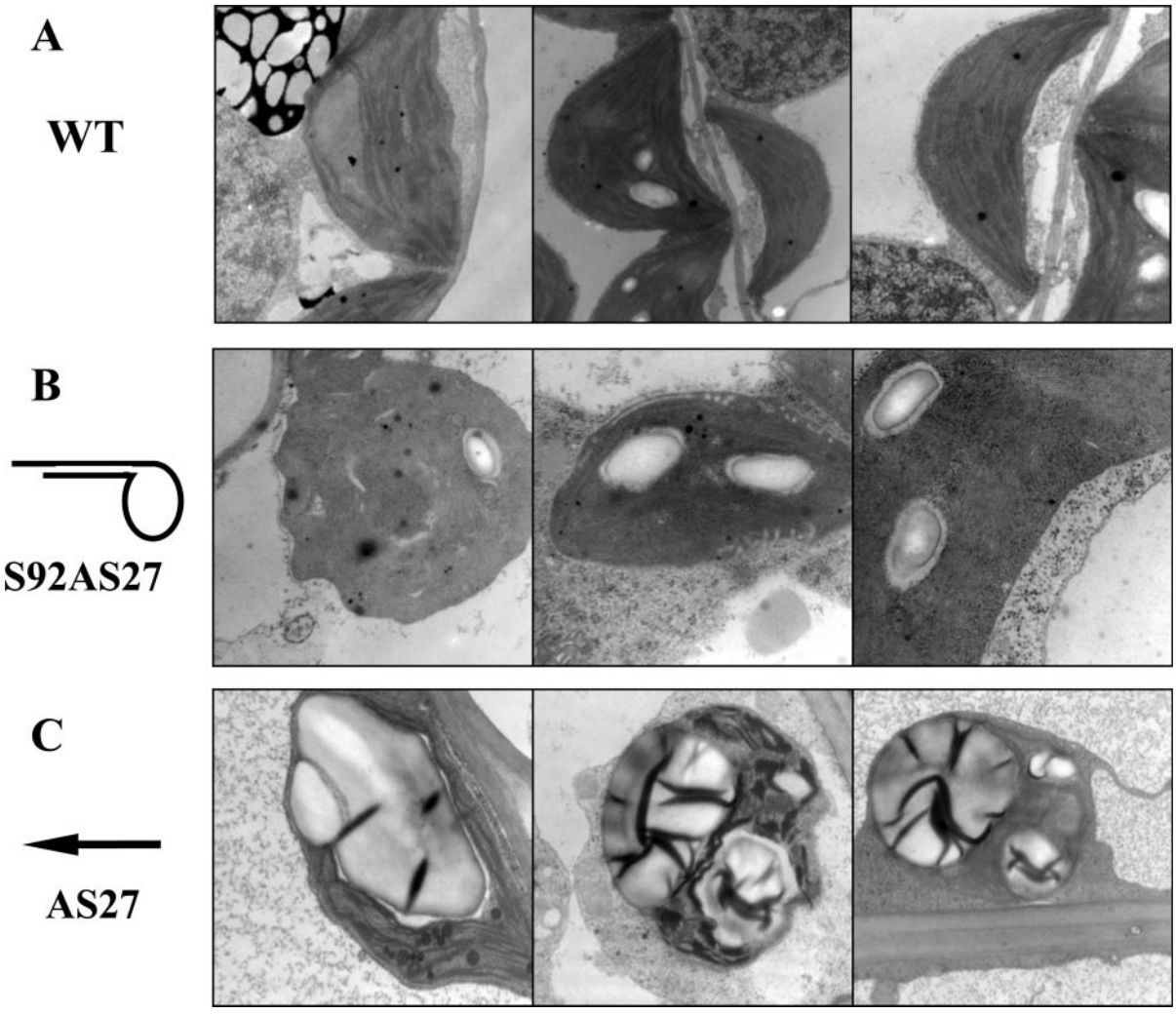

Figure 5. Electron micrographs of cells in young leaves of cultured wild-type (**A**) and transplastomic lines S92AS27 (**B**) and AS27 (**C**).

Table 1. Editing of *rpoB* transcripts at the RpoB-2 site

Plant	Transgene (%)	Endogenous (%)
Wild-type	NA	89
S92	40	60
S92AS27M	74	87
S92AS27	0	94
AS27	NA	95

A restriction digestion assay of cDNAs was used to measure editing in transgene transcripts of S92AS27M (29 of 39 cDNAs were edited) and S92AS27 (0 of 45 cDNAs were edited). PPE assays were used on all other transcripts.

plants (20). A minor effect on editing was also detected on the RpoA-1 and RpoB-1 sites (20). Editing of all other known sites in tobacco was not affected. Some sequence similarities were detected between the 5′ regions of the five sites whose editing was affected by expression of the 92 nt maize RpoB-2 sequence (20). This observation, along with a similar one in transgenic lines overexpressing the NdhF-2 editing site, is interpreted as resulting from competition for the same or similar *trans*-acting factor(s) needed for the recognition and editing of C targets of similar sequence. We used the term 'editing site cluster' for two or more sites sharing similar putative *cis*-elements.

We analyzed editing extent of the five sites in the RpoB-2 cluster and 16 other sites in the four transgenic tobacco lines and a wild-type control growing under the same conditions in culture. We used the sensitive and accurate PPE assay for these experiments, with which results are usually reproducible within 5%. The S92 construct, like the maize 92 nt RpoB-2 transgene, significantly reduced editing of PsbL-1 and Rps14-1 (Figure 9) and appears to have a minor effect on RpoA-1, but did not reduce editing of RpoB-1 (Figure 10). While none of the other transgenes affected editing of endogenous RpoB-2, their expression did significantly reduce editing of PsbL-1 (Figure 10), which encodes a component of Photosystem II (PS II).

Photosynthesis in the transgenic plants

In order to assess the effects of the expression of the transgenes on photosynthesis in the plantlets, two parameters were measured using *in vivo* chlorophyll fluorescence techniques. The first parameter, F_v/F_m, measures the maximum quantum

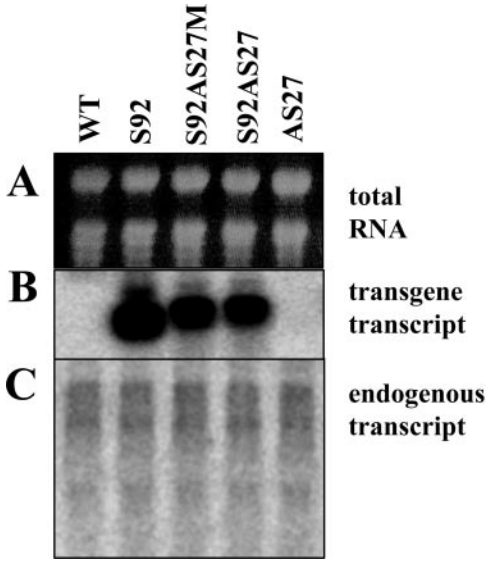

Figure 6. RNA blot analysis of minigene RNA from transplastomic shoots. Total RNA isolated from wild-type and transplastomic shoots was probed with (**A**) a single-stranded *rpoB* probe complementary to the −20/+6 region of the sense transcript, (**B**) a single-stranded *rpoB* probe complementary to the −20/+6 region of the antisense transcript, and (**C**) a double-stranded probe complementary to the endogenous *rpoB* (but not transgene) transcripts.

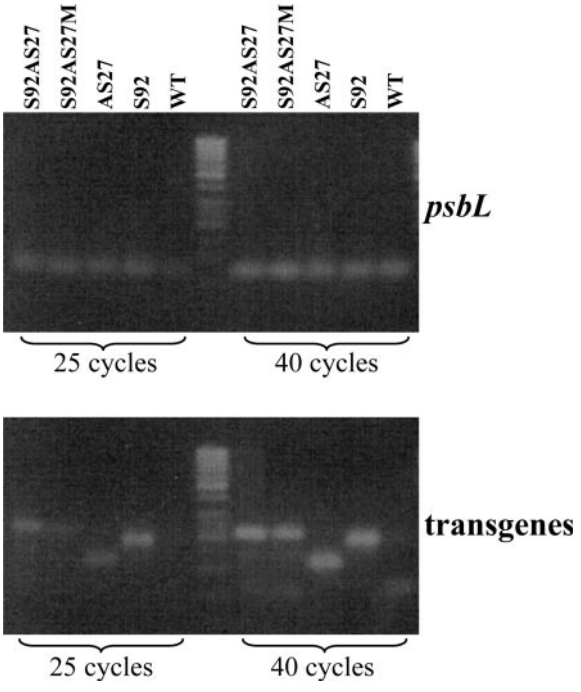

Figure 7. Semi-quantitative RT–PCR of endogenous and transgene transcripts in wild-type and transplastomic shoots. Endogenous *psbL* (upper panel) and transgene transcripts (lower panel) were amplified by reverse transcription followed by either 25 or 40 cycles of PCR. Products were separated on an agarose gel and stained with ethidium bromide. The faint band in the control WT lane is primer dimer.

efficiency of PS II. A decrease in F_v/F_m below the optimum value (typically 0.8–0.82) is an indication of accumulated photooxidative damage to PS II. As shown in Figure 11, none of the plantlets exhibited a significant decrease in F_v/F_m for growth at either 15 or 100 μmol photons m^{-2} s^{-1}. The second parameter, Φ_{PSII}, is a measure of the intrinsic quantum efficiency of PS II under a given set of experimental conditions. Typically Φ_{PSII} measured under limiting light conditions is slightly less than the optimal F_v/F_m, but its value decreases rapidly as light saturation is reached (28). Φ_{PSII} is quite sensitive to any treatment that decreases the ability of a plant to perform photosynthesis under a given set of experimental conditions (28). Figure 11 shows that when measured under limiting (50 μmol photons m^{-2} s^{-1}) light intensity, the higher light-grown plantlets exhibited Φ_{PSII} values close to the measured F_v/F_m, while the plantlets grown at the lower light intensity showed slightly lower Φ_{PSII} values. This is an indication that the 50 μmol photons m^{-2} s^{-1} treatment intensity was not truly light limiting for the low light-grown plantlets. At the higher treatment intensity (500 μmol photons m^{-2} s^{-1}), both the low and higher light-grown plantlets exhibited the typical reduction in Φ_{PSII} characteristic of plants exposed to supersaturating illumination. The measurements at both limiting

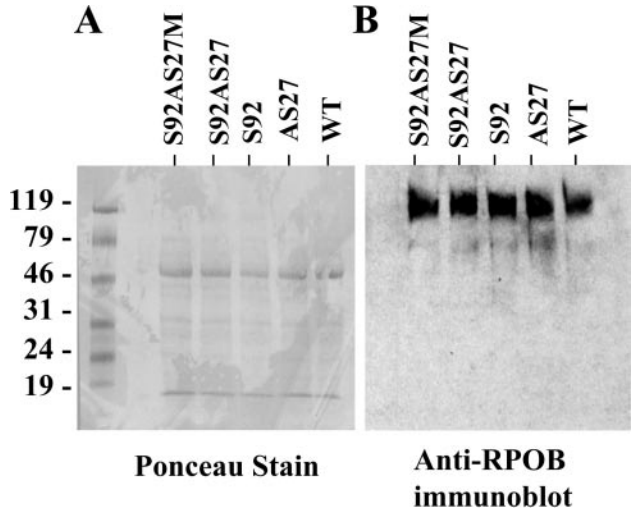

Figure 8. Immunodetection of RPOB in protein extracted from wild-type and transplastomic shoots. (**A**) Total protein was isolated from wild-type and transplastomic shoots was subjected to SDS–PAGE, transferred to a PVDF membrane, and stained with Ponceau-S to verify equal loading. Bands corresponding to Rubisco subunits are evident. (**B**) Primary antisera raised against an RPOB peptide was used to detect the 120 kDa RPOB protein.

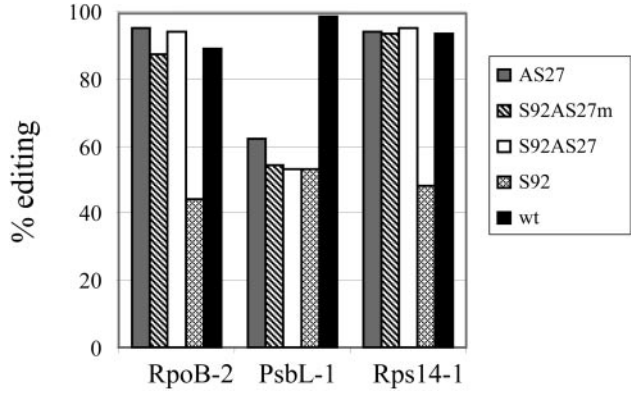

Figure 9. Editing extent of endogenous chloroplast transcripts in the rpoB-2 cluster in wild-type and transgenic plants as determined by PPE.

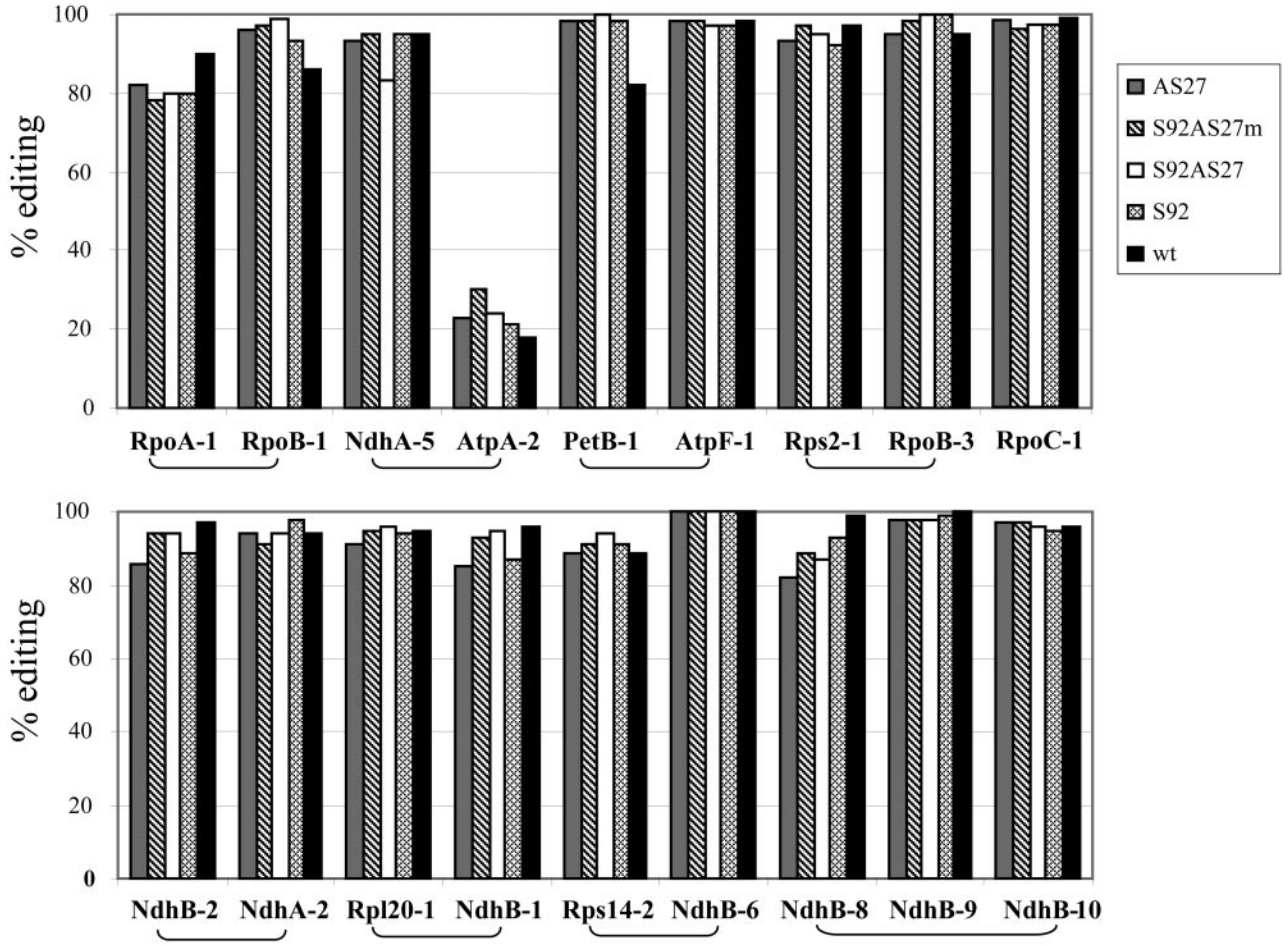

Figure 10. Editing extent of endogenous chloroplast transcripts in wild-type and transgenic plants as determined by PPE. Brackets indicate sites with common 5′ sequence elements that group the sites into a putative editing cluster (20).

and saturating intensities indicate that there are no significant differences in the intrinsic photosynthetic properties of the plantlets in spite of the abnormal appearance of some plastids and their abnormal growth.

DISCUSSION

Our results indicate that the editing of a C target in a chloroplast transgene is affected by the addition of a sequence that is complementary to the −20 to +6 region of the 3′ end of the transgene transcript. Presence of a perfectly complementary sequence on the transgene transcript completely prevented editing of the C target on the transgene transcript. This suggests that the C target of editing is not normally present as base-paired RNA in wild-type plants. This finding also indicates that it would be possible to specifically prevent editing at a C within a transcript by expressing antisense sequence on the same transcript.

While a transcript predicted to base pair from −20 to +6 around the C target was not edited, a transcript likely paired from −2 to −20 was highly edited (Table 1). Results from ultraviolet cross-linking, competition analysis, and mutagenesis studies have indicated that sequences from −6 to −20 around C targets in several species are likely to interact with *trans*-factors (13–15,19,20). Evidently the presence of complementary sequence does not prevent functioning of these *trans*-factors.

We did not detect any effect of expression of the AS27 sequence on the editing of the endogenous *rpoB* transcripts. A likely explanation is that the AS27 sequence does not base pair with the endogenous sequence *in vivo*. Possibly the endogenous transcripts are folded and/or bound with protein in such a way that the 27 nt antisense sequence present on a separate transcript is not able to interact with the complementary sequence. Furthermore, the antisense transcript could also be bound by protein, or the concentrations of the two transcripts within the viscous chloroplast stroma (29) might make it unlikely that they would happen to encounter each other. When the antisense sequence is present on the same transcript as the sense sequence, perhaps base pairing occurs immediately following transcription.

RNA with a mismatch surrounding the edited C resulted in a higher level of editing than in transgene transcripts lacking any complementary RNA near the C target of editing. However, we cannot conclude that the enhanced editing resulted from improved recognition of the C target due to an altered secondary structure. The transgene transcripts with the

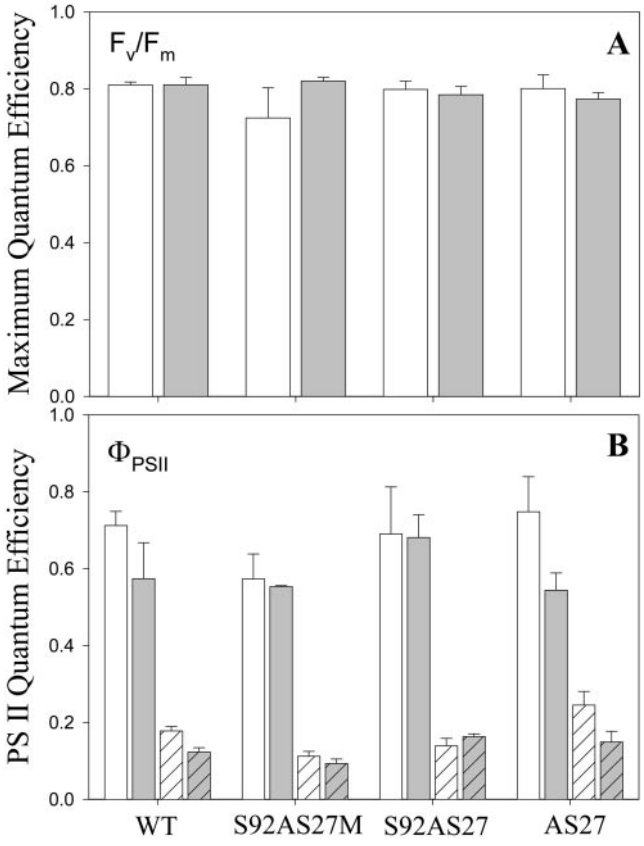

Figure 11. (**A**) Measurements of maximum PS II quantum efficiency (F_v/F_m) for plantlets grown at 15 μmol photons m^{-2} s^{-1} (gray bars) and 100 μmol photons m^{-2} s^{-1} (white bars). Data represent the mean and SD for measurements on six plantlets. (**B**) Measurements of intrinsic PS II quantum efficiency at saturating (500 μmol photons m^{-2} s^{-1}, hatched bars) and limiting (50 μmol photons m^{-2} s^{-1}, no hatch) steady state illumination for plantlets grown at either 15 μmol photons m^{-2} s^{-1} (gray bars) or 100 μmol photons m^{-2} s^{-1} (white bars). Data represent the mean and SD for measurements on six plantlets.

mismatched 27 nt complementary sequence were present in lower amount in the transgenic chloroplasts than the transcripts lacking any complementary RNA. The amount was insufficient to cause a competition effect with endogenous *rpoB* RNA. The enhanced editing could therefore be due to lower transcript abundance and less competition for a limiting *trans*-factor.

Our results are consistent with our previous hypothesis (20,30) for the existence of editing site 'clusters', in that high-level expression of a 92 nt tobacco RpoB-2 sequence reduced editing of transcripts from endogenous wild-type genes at the RpoB-2, PsbL-1 and Rps14-1 editing sites. Expression of a 27 nt sequence either perfectly or partially complementary to *rpoB* near the RpoB-2 editing site did not affect editing of endogenous *rpoB* transcripts, but did significantly reduce editing of *psbL* transcripts. The PsbL-1 editing site exhibits some sequence similarity to RpoB-2, and has been classified as a member of the RpoB-2 editing 'cluster' along with Rps14-1 (20). Overexpression of either the maize (20) or tobacco (Table 1) editing site results in decreased editing of the endogenous transcripts at RpoB-2, PsbL-1 and Rps14-1. The reduction of PsbL-1 editing by the S92AS97 and S92AS97M transgenes can be explained as possible cross-competition for a shared *trans*-acting factor, analogous to the effect of the S92 sequence, because all three of the transgenes carry the 92 nt sequence in sense orientation. If so, then PsbL-1 must be more sensitive to the competition effect than RpoB-2 or Rps14-1, because editing of RpoB-2 and Rps14-1 is affected only in the S92 line. Perhaps the affinity of the *trans*-factor for PsbL-1 is lower, making PsbL-1 less likely to be edited in the presence of large amounts of similar sequence with higher affinity for a limiting factor.

Alternatively, perhaps the effect of the three transgenes carrying the 27 nt antisense sequence does not result from a limiting amount of a factor that interacts with the sense sequence surrounding the C target of editing. The effect of the AS27 transgene on PsbL-1 cannot be explained as cross-competition, as the transgene does not contain any sense sequence at the editing site. It could be that the antisense sequence within the transgene transcripts is reducing editing of PsbL-1 by disturbing the PsbL-1 transcript's structure and preventing either recognition or editing of the C target. However, the 27 nt antisense sequence is predicted to base-pair less well with the PsbL-1 transcript than with the RpoB-2 transcript. The presence of the PsbL-1 editing site within the *psbL* start codon might make editing at this site particularly vulnerable to disruption. Another possibility is that there is an editing *trans*-factor that can bind both the sense and antisense sequence surrounding the RpoB-2 editing site.

Little information is yet available about the effect of expression of antisense RNA from chloroplast transgenes. There is no evidence for an RNA silencing mechanism in chloroplasts, though silencing of nuclear-encoded plant genes is well known (31). In our experiments, the presence of transcripts containing 27 nt antisense to *rpoB* transcripts did not appear to affect either abundance or accumulation of RpoB protein. The antisense RNA is complementary to a region within the RpoB coding region, but neither near the 5′ nor the 3′ end of the *rpoB* transcript. In an experiment with *Chlamydomonas* chloroplasts in which transgenic antisense RNA was expressed that was complementary to the 3′ end of an *atpB* gene whose transcripts were destabilized because of a mutated 3′ nontranslated region, the presence of antisense RNA resulted in increased accumulation of both the transcript and protein expressed from the mutated gene (30). In the *Chlamydomonas* experiments, the antisense RNA was longer (121 nt versus 27 nt) than in our tobacco experiments and located at the 3′ end of the *atpB* transcript rather than entirely within the coding region.

An unexpected result of introducing transgenes carrying 27 nt sequences in antisense orientation to *rpoB* into the chloroplast genome was the disturbance in growth and development. The defects in growth do not appear to result from a lack of accumulation of RpoB protein, as immunoblot assays indicate RpoB is present in normal amounts. The abnormal phenotypes of some of the transgenic lines cannot be explained by the presence of partially edited endogenous transcripts that might be translated into abnormal proteins. The S92 line contains a substantial proportion of unedited endogenous *rpoB* transcripts but is normal in appearance. Furthermore, there is no significant difference in editing extent of RpoB-2 in the abnormal plants compared with wild-type plants. Although the abnormal transgenic plants do exhibit a reduction in editing at PsbL-1 from 100% in the wild-type to 50–60%, the S92 plants,

which are phenotypically normal, also exhibit a decrease in editing of PsbL-1 to about 50%. Thus, the reduced PsbL-1 editing observed in leaves cannot explain the phenotypes of the plants expressing transcripts carrying the 27 nt antisense RNA. However, we do not know the editing efficiency of RpoB-2, PsbL-1, and Rps14-1 in meristematic cells of the abnormal transgenic plants. Conceivably, the AS27 minigene could be severely impairing editing in the meristem, affecting plastid gene expression so that development is disrupted.

The chloroplast biogenesis and morphology defects of the plants carrying complementary RNA may not be owing to an effect on editing, but owing to an unexpected interaction of the transgene transcripts with nucleic acids or proteins within the plastid that consequently impairs chloroplast function and plant development. In that case, the reduction in PsbL-1 editing in AS27 plants might not be a direct effect of AS27 expression, but instead is a stress response to the unknown dysfunction that causes the abnormal plant phenotype. Indeed, perhaps the reduction in editing of PsbL-1 in the S92AS27 and S92AS27M lines is also a stress effect. However, the reduction of both PsbL-1 and Rps14-1 editing in the S92 plants is likely to be a competition effect, given the wild-type vigor of the S92 plants.

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