Journal of Plant Biology, February 2006, 49(1) : 34-43

Accumulation of Sweet Protein monellin is Regulated by the *psb*A 5'UTR in Tobacco Chloroplasts

Kyung Hee Roh[1], Kong-Sik Shin[1], Yeon-Hee Lee[1], Seok-Cheol Seo[1], Hyo-Guen Park[2], Henry Daniell[3], and Seung-Bum Lee[1]*

[1]National Institute of Agricultural Biotechnology, RDA, Suwon 441-707, Korea
[2]Department of Horticultural Science, Seoul National University, Seoul 151-742, Korea
[3]Department of Molecular Biology and Microbiology, Biomolecular Science, University of Central Florida, Orlando, FL 32816-2364, USA

Post-transcriptional RNA processing and translational regulations are important steps for gene expression. To analyze the 5'UTR of *psb*A that enhances translation of the sweet protein monellin in chloroplasts, we cloned the monellin gene, with and without the *psb*A 5'UTR, into the chloroplast expression vector for chloroplast transformation. Transgenic plants were identified as being transplastomic via PCR and Southern blot analyses. We also observed non-specific recombination during tobacco chloroplast transformation. Analyses of the transcription patterns showed that intercistronic cleavage of the *psb*A mRNA 5' untranslated (UTR) region was functional at the mature stage, with the monocistronic mRNA of *monellin* increasing while its dicistronic mRNA decreased. Moreover, monellin accumulation accounted for 2.3% of the total soluble protein at the mature stage, but only 1.3% at the young stage in transplastomic lines that contained the 5'UTR of *psb*A. These results suggest that activation of the endonucleolytic cleavage of the *psb*A 5'UTR element depends on chloroplast developmental conditions, and that it enhances the accumulation of sweet protein monellin in those chloroplasts.

Keywords: chloroplast transformation, endonucleolytic activation, monellin, *psb*A 5'UTR, sweet protein, translation efficiency

Genetic transformation via the nuclear genome has conferred numerous beneficial agronomic traits to crop plants (Chung et al., 2003; Park et al., 2003; Lee et al., 2004). Several useful vaccine antigens, biomaterials, and biopharmaceuticals also have been produced in transgenic plants (Kumar and Daniell, 2004). The chloroplast transformation method offers unique advantages, including high levels of transgene expression (Kota et al., 1999), multi-gene engineering during a single transformation event (DeCosa et al., 2001), transgene containment via maternal inheritance (Daniell, 2002), and a lack of gene silencing (DeCosa et al., 2001; Lee et al., 2003). Plants with transformed plastid genomes are termed transplastomic (Maliga, 1993). Such an approach has rapidly advanced resulting in the development of chloroplast transgenic lines that confer herbicide resistance (Daniell et al., 1998) and drought tolerance (Lee et al., 2003), and produce therapeutic proteins including human serum albumin (Fernandez-San Millan et al., 2003) and somatotropin (Staub et al., 2000). Thus, transgenic chloroplasts are ideal for engineering agronomic traits, serving as bioreactors for the production of functional human and animal therapeutic proteins or biomaterials in an environmentally friendly manner.

In the chloroplasts of higher plants, gene expression during leaf development is regulated on several levels as transcription, RNA processing and stability, and translational control (Bock, 2000; Zerges, 2000; Hirose and Sugiura, 2004). In particular, post-transcriptional RNA processing of primary transcripts, including endonucleolytic cleavage (Westhoff and Herrmann, 1988; Chen and Stern, 1991; Klaff, 1995), processing of the 5' and 3' ends (Monde et al., 2000), and RNA editing (Maier et al., 1996), is an important step in the control of chloroplast gene expression. RNA stability and efficient translation are mainly mediated via the 5'/3' untranslated regions (UTRs) of chloroplast transcripts. The 3'UTRs are involved in RNA 3'-end formation and mRNA stability because of inverted repeat (IR) sequences that can fold into stem-loop structures (Adams and Stern, 1990; Srivastava et al., 2004). However, those 3'UTRs do not significantly influence translation efficiency (Eibl et al., 1999). In contrast, 5'UTRs appear to be of crucial importance for both mRNA stability and translational

*Corresponding author; fax +82-31-299-1732
e-mail seungblee@rda.go.kr, sbumlee@mail.ucf.edu

efficiency (Alexander et al., 1998; Bruick and Mayfield, 1998; Eibl et al., 1999; Zou et al., 2003).

The 5′UTR of *psb*A was chosen for this experiment because it is a well-characterized monocistronic gene that has been studied for its processing of polycistronic transcripts (Sexton et al., 1990; Hirose and Sugiura, 1997; Baginsky and Gruissem, 2002; Arai et al., 2004) and translational efficiency (Hirose and Sugiura, 1996). The 5′UTR of the tobacco chloroplast *psb*A encodes the D1 protein, a core component of Photosystem II. It includes potential ribosome binding sequences (RBS), an AU-box (Agrawal et al., 2001), an endonucleolytic site, and an upstream stem-loop element (Klaff, 1995; Hirose and Sugiura, 1996). Analyses of *psb*A 5′UTR stem-loop mutants have indicated that the correct primary sequence and secondary structure of the stem-loop are required for mRNA stabilization and translation (Zou et al., 2003). The *psb*A 5′UTR also mediates light-induced accumulation of GUS (Staub and Maliga, 1993) and enhances translation of GUS compared with the *rbc*L 5′UTR *in vivo* (Eibl et al., 1999).

Monellin was first purified (Morris and Cagan, 1972) from the African berry *Discoreophyllum cumminsii* Diels. On a molar basis, this protein is 100,000 times sweeter than sucrose (Edens and van der Wel, 1985), making it attractive to the food and beverage industry because of its "natural" and "low-calorie" traits. The annual market for high-intensity sweeteners in the US is $1 billion (Faus, 2000). Alternative and intensely sweet additives also are desirable for diabetic foods. However, despite its initial sweetness, the commercial applicability of monellin is very low because *Discoreophyllum* plants are difficult to grow naturally and its purification from the 1-cm fruit is very costly. In addition, monellin loses its sweetness due to unfolding of the tertiary structure when heated above 50°C under acidic pH (Jirgensons, 1976). It consists of two polypeptides, of 45 and 50 amino acid residues, that are associated *via* noncovalent interactions (Bohak and Li, 1976; Ogata et al., 1987). These chains, which have been completely sequenced by Kohmura et al. (1990), lack disulfide bonds, thereby causing this instability during heating. To solve this problem, Kim et al. (1989) have used several different linkers to fuse the two chains into a single chain. This redesigned monellin is more stable under temperature or pH changes, and re-natures easily after heating to 100°C at low pH.

A single-chain monellin was previously introduced via nuclear transformation to enhance the flavor and sweetness of tomato and lettuce; however, its expression was very low (Penarrubia et al., 1992). Recombinant single-chain monellin was then purified on a large scale from yeast, and compared with the plant-derived monellin. Unfortunately, in those efforts, the yeast-derived monellin purification began with more contaminating yeast protein and the final freeze-dried monellin was not of good appearance (Kim and Lim, 1996). Here, we constructed two kinds of chloroplast expression vectors that harbor the single-chain *monellin* gene, with and without the 5′UTR of *psb*A. Our main goal in transformation was to determine whether the 5′UTR of *psb*A confers the processing of dicistronic transcripts and translational efficiency to the sweet protein *monellin* gene *in vivo*.

MATERIALS AND METHODS

Chloroplast Expression Vectors

We constructed pLDMon (6.2 kb) by inserting the single-chain *monellin* 295-bp *Eco*RI fragment into the multiple cloning site of the pLD vector (Daniell et al., 1998). For the pLDUTRMon vector, we PCR-amplified the 83-bp sequence of the *psb*A5′UTR without the promoter, using tobacco chloroplast DNA as a template. Primers included: 5′-TCGCAGGCCTAAAAAGCCTTCCATTTTC-3′ and 5′-CCATGGTACGTAGTAAAATCTTGGTTTATTTA-3′ for *psb*A 5′UTR; and 5′-GGAGGGATTTATGGGCGAGTGGGAAA-3′ and 5′-TTATGGTGGTGGGACTGGACCGTT-3′ for the single-chain *monellin*. The fusion with single-chain *monellin* was made at the *Nco*I site placed at the 3′ end of the *psb*A5′UTR and then inserted into the pLD vector as an *Eco*RI fragment.

Bombardment and Regeneration

Sterile tobacco (*Nicotiana tabacum* cv. Petit Havana) leaves were bombarded using the biolistic device (PDS-1000/He; Bio-Rad, USA), as described (Lee et al., 2003). They were then cut into small pieces and transferred to an RMOP medium (Svab et al., 1990) containing spectinomycin (500 mg L^{-1}). After regeneration through second selection, the plants were rooted in an MS basal medium containing spectinomycin (500 mg L^{-1}) and transferred to pots in a greenhouse.

PCR Analysis

PCRs were conducted with *Ex Taq* polymerase

(Takara, Japan) to identify the putative transgenic plants as transplastomic lines, using the following primers: (3P) 5′-AAAACCCGTCCTCAGTTCGGATTGCC-3′ and (3M) 5′-CCGCGTTGTTTCATCAAGCCTTACGG-3′. All PCR reactions were performed at 94°C for 30 s, 65°C for 30 s, and finally 72°C for 2 min. The PCR products were separated on 0.8% agarose gels.

Southern Blot Analysis

Total DNA was extracted from the leaves of transformed and untransformed plants using the DNeasy Plant Maxi kit (Qiagen, USA). Total DNA (2 mg) was digested with *Eco*RV and *Bgl*II. DNA fragments were separated overnight by electrophoresis (30 V) in a 0.8% agarose gel, then transferred to nylon membranes (Hybond-N^+; Amersham, UK) under alkaline conditions (0.5 N NaOH). DNA fragments were revealed by hybridization with ^{32}P-labeled probes. The *monellin* probe was amplified by PCR with the primers described above, while the *aad*A probe was digested by *Cla*I/*Not*I of pLDMon. The 0.81-kb flanking probe was digested by *Bam*HI/*Bgl*II of pLDMon. Theses probes were labeled with [^{32}P]CTP by the oligolabeling procedure (Ready-to-go; Amersham) at 37°C for 1 h. Afterward, the membranes were washed with 2X SSC/0.1% SDS and 0.2X SSC/0.1% SDS solutions, both at 65°C.

Northern Blot Analysis

Total RNA was extracted from leaves of transformed and untransformed plants (Trizol reagent; Invitrogen, USA), then denatured with sample buffer (2.2 M formaldehyde, 50% formamide, and 0.5X MOPs buffer) for 10 min at 65°C. RNAs (3 μg) were separated over 2 h by electrophoresis (30 V) on 1.5% agarose/formaldehyde gels in MOPS buffer before being transferred to the nylon membranes under 20X SSC. The probes were labeled with [^{32}P]CTP using the Ready-to-go oligolabeling procedure. Probes for these experiments were obtained as described previously for the Southern analysis.

SDS-PAGE and Immunoblot Analysis

Transformed and untransformed leaves (100 mg) were ground in liquid nitrogen and resuspended in 500 μL of protein extraction buffer [0.1 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 18% sucrose, and 40 mM 2-mercaptoethanol]. The protein was quantitated with the Bio-Rad (USA) DC protein assay kit (BSA standard). Leaf extracts were boiled in 5X SDS-PAGE sample buffer [150 mM Tris-HCl (pH 6.8), 30% glycerol, 1.2% SDS, 1.8% bromophenol blue, and 15% 2-mercaptoethanol] and electrophoresed (50 V) in a 15% polyacrylamide gel. The separated proteins were either stained with Coomassie Brilliant Blue G-250 (0.05% Coomassie Brilliant Blue R-250, 10% glacial acetic acid, and 30% methanol) or transferred to a PVDF membrane (Bio-Rad) for immunoblotting at 50 V for 4 h, using a transfer buffer (25 mM Tris-HCl, 150 mM glycine, and 20% methanol). The primary antibody (rabbit polyclonal anti-monellin; Takara, Japan) was used at a 1:5000 dilution, followed with horseradish peroxidase conjugated rabbit IgG (Pierce, USA) at a 1:10000 dilution. Stable peroxide solution (SuperSignal West Pico Chemiluminescent Substrate; Pierce, USA) was used for detection.

ELISA Assay

Total soluble protein (tsp) was collected and quantitated as described for SDS-PAGE and immunoblot assays. They were diluted to 100 ng with a coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6), and loaded into each well (Reacti-Bind 96-Well EIA Plates; Pierce). After incubation overnight at 4°C, the plate was cleansed in washing buffer (PBS with 0.05% Tween 20). The remaining absorption sites were blocked by adding 300 μL of blocking buffer (1% BSA in PBS/0.05% Tween 20) to each well overnight at 4°C. In addition, 100 μL per well of the rabbit polyclonal anti-monellin was diluted at 1:1000 with blocking buffer (1% BSA in PBS-T). After incubation overnight at 4°C, the plate was washed as described above, and 100 μL per well of the horseradish peroxidase conjugated rabbit IgG, diluted to 1:10000 with blocking buffer (1% BSA in PBS-T) was added. After incubation for 1 h at 37°C, the unbound conjugate was released by washing the plate with buffer. Then, 5 mg of OPD (o-phenylenediamine) was dissolved in 10 mL of substrate buffer (0.05 M citric acid and 0.05 M Na-phosphate; pH 5) to a concentration of 0.5 mg mL^{-1}, and 2 μL of 30% H_2O_2 was added per mL of substrate. To each ELISA well, 100 μL of OPD substrate solution was added before incubation for 40 min. The reaction was stopped by adding 100 μL of 3 M sulfuric acid, and the wells were read at 490 nm (VICTOR^3; Perkin Elmer, USA).

Analysis of Maternal Inheritance

We examined maternal inheritance by analyzing

antibiotic resistance as described by Staub and Maliga (1993). Seedling phenotypes were determined by plating surface-sterilized seeds on an MS medium containing 500 mg L^{-1} of spectinomycin.

RESULTS

Transplastomic Lines Carrying psbA 5'UTR-monellin Fusion Gene or monellin Gene

The single-chain *monellin* gene (Fig. 1), with 94 amino acid residues, was engineered by joining the C-terminal residue of the B chain (B50E) with the N-terminal residue of the A chain (A1R). These chains are found in monellin, a sweet protein isolated from *D. cumminsii* (Higginbotham, 1979; Kim et al., 1989; Kohmura et al., 1990; Spadaccini et al., 2001). Because translation in plastids initiates at methionine, we incorporated the translational start codon into the *monellin* coding sequence to produce the engineered protein. Two chloroplast transformation vectors were designed, with and without *psb*A 5'UTR (Fig. 2). Here, we chose the basic pLD vector, developed by Daniell et al. (1998) for chloroplast transformation. In the plasmid pLDMon, the *aad*A gene, which confers spectinomycin resistance, and the *monellin* gene are transcribed as a dicistron from the plastid *Prrn* promoter. In the pLDUTRMon, the *psb*A 5'UTR is inserted immediately upstream of the *monellin* coding sequence and downstream of the *aad*A gene, using the *Nco*I site.

We confirmed integration of the foreign gene cassette into the chloroplast genome by PCR-screening of the primary shoots (data not shown). The strategy utilized one primer (3P) that anneals to the native chloroplast genome adjacent to the point of integration, while a second primer (3M) lands on the *aad*A gene (Lee et al., 2003). This PCR product cannot be obtained in untransformed plants. Confirmed transformants were subjected to a second round of spectinomycin selection to achieve homoplasmy. After being rooted in the presence of spectinomycin, they were transferred to pots for further characterization.

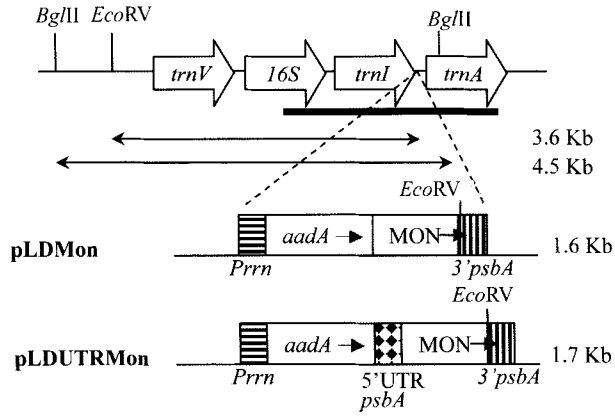

Figure 2. Construction of chloroplast expression vectors. Regions for homologous recombination are underlined in native chloroplast genome. The 295-bp *monellin* is driven by *Prrn* promoter upstream of *aadA* gene that confers spectinomycin resistance. Numbers on right indicate fragment size when native or transgenic chloroplast DNA was digested with *Eco*RV and *Bgl*II. Arrows within boxes show direction of transcription. *aadA*, aminoglycoside 3'-adenylyltransferase; *Prrn*, 16S rRNA promoter; UTR, untranslated 5' region of plastid *psbA* photosynthetic gene.

Southern blot analysis demonstrated the stable maintenance of integrated transgenes in the T_1 generation (Fig. 3). When the total genomic DNA was digested with *Bgl*II and probed with *monellin*, we observed 6.1-kb, 8.0-kb, and 6.2-kb fragments only in the transgenic lines (Fig. 3a). Contrary to nuclear transformants, one can predict the size of fragments detected in chloroplast transgenic lines through Southern blot analysis, depending on the restriction enzyme used, because the chloroplast genome of tobacco has now been sequenced entirely (Shinozaki et al., 1986; Wakasugi et al., 1998). Based on this logic, the 6.1-kb and 6.2-kb fragments corresponded to the detected size, which included the flanked (4.5 kb) and integrated (1.6 kb of pLDMon or 1.7 kb of

Native *monellin*

Chain A: REIKGYEYQL[10] YVYASDKLFR[20] ADISEDYKTR[30] GRKLLRFNGP[40] VPPP

Chain B: GEWEIIDIGP[10] FTQNLGKFAV[20] DEENKIGQYG[30] RLTFNKVIRP[40] CMKKTIYEEN[50]

Single chain *monellin*

GEWEIIDIGP[10] FTQNLGKFAV[20] DEENKIGQYG[30] RLTFNKVIRP[40] CMKKTIYEEN[50] REIKGYEYQL[60] YVYASDKLFR[70] ADISEDYKTR[80] GRKLLRFNGP[90] VPPP

Figure 1. Amino acid sequences of native monellin and single-chain monellin.

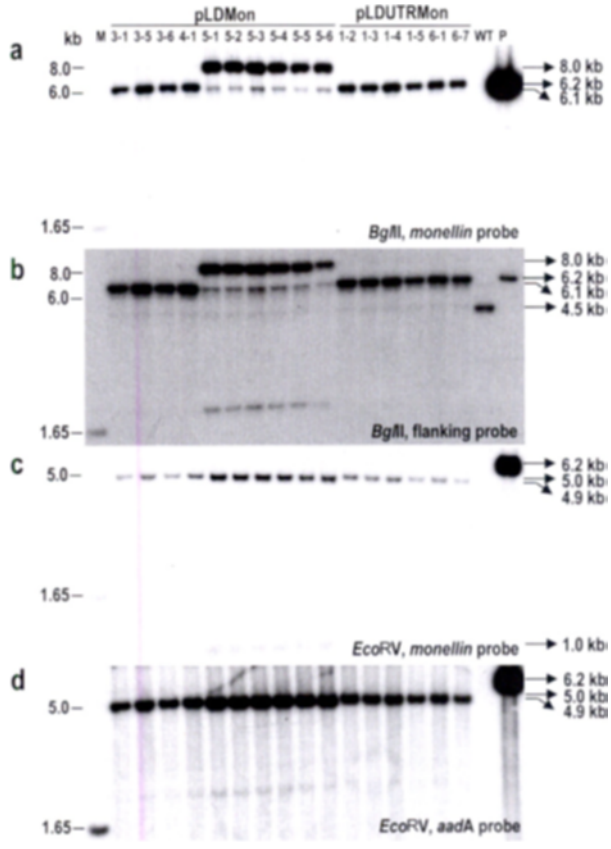

Figure 3. Integration of transgene cassettes into chloroplast genome investigated by Southern blot analyses. **(a, b)** *Bgl*II-digested genomic DNA with *monellin* and flanking sequences probe. **(c, d)** *Eco*RV-digested genomic DNA with *monellin* and *aadA* probe. Transgenic plant Lines 3, 4, and 5 were transformed with pLDMon; Lines 1 and 6 with pLDUTRMon. pLDMon as a positive control digested with *Eco*RV. WT, wild-type tobacco plant; *aadA*, aminoglycoside 3′-adenylyltransferase; *Prrn*, 16S rRNA promoter; UTR, untranslated 5′ region of plastid *psbA* photosynthetic gene.

pLDUTRMon) regions. However, 8.0-kb fragments also were detected in some of the transgenic plants. Such fragments may occur when both flanked sides include undesirable foreign DNA or when the *monellin* gene is integrated into the nuclear genome. Therefore, to identify whether these unexpected transgenic lines were nuclear transformants, we re-probed the blot with the chloroplast flanking sequences (Fig. 3b). In that analysis, all transgenic lines appeared to be transplastomic. In addition, the lack of a 4.5-kb hybridization fragment, which corresponds to the size of intact chloroplast flanking sequences digested by *Bgl*II, confirmed homoplasmy.

To verify whether more than one copy of the transgene cassette was integrated into the plastid genome of this line, total genomic DNA was digested with *Eco*RV and hybridization was performed. In those digested transgenic lines, 4.9-kb and 5.0-kb fragments were detected, indicating that one copy had been integrated (Fig. 3c). However, another 1.0-kb fragment was detected in the same transgenic lines that exhibited the 8.0-kb fragments shown in Fig. 3a and 3b. Because the size of the pLDMon transgene cassette is 1.6 kb, we could not determine whether two copies of the transgene cassette had been integrated. Therefore, to investigate differences between the detected and the expected fragments, the blot was re-probed with the *aadA* probe. Surprisingly, this probe identified only 4.9-kb and 5.0-kb hybridization fragments in the transgenic lines (Fig. 3d). Thus, we suggest that the transgenic lines that showed only one fragment with the *monellin* probe when digested with *Eco*RV demonstrated single-copy integration of the transgene cassette into the plastid genome. Here, we observed non-specific recombination during tobacco

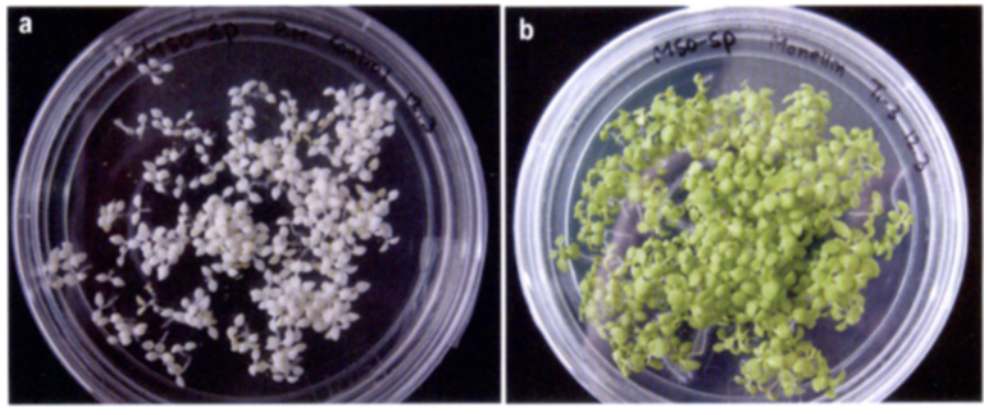

Figure 4. Chloroplast transgenes are maternally inherited. Seeds derived from either wild-type tobacco plants **(a)** or transplastomic lines **(b)** were plated onto MS medium containing 500 mg L^{-1} spectinomycin dihydrochloride. Resistant seedlings are uniformly green whereas sensitive seedlings are bleached white.

chloroplast transformation.

Seedling tests on the T_1 progeny from transgenic plants showed stable resistance to up to 500 mg L^{-1} spectinomycin, a trait maternally inherited (Fig. 4). Resistant seedlings (transplastomic plants) were green, whereas the sensitive seedlings (wild-type tobacco) were white. Uniform spectinomycin resistance in the self-fertile seed progeny also indicated that *aad*A was maintained in plants grown to maturity in the greenhouse.

Transcription and Translation Is Regulated by the psbA 5′ UTR

Transcription patterns were analyzed for the transgene integrated into the chloroplast genome. Total RNAs from young and mature leaves were hybridized with ^{32}P-labeled gene probes. Two distinct transcripts were detected in transplastomic plants, using the *monellin* probe, whereas no transcript was found in the wild-type plants (Fig. 5). The 1500 nt of transcripts corresponded to the dicistronic mRNA of *aad*A-*monellin* transcribed from the *Prrn* promoter. Another 700 nt of transcripts were observed only in the transplastomy lines with pLDUTRMon, suggesting that this transcript could be the *monellin* monocistron. The stop codon of the *aad*A gene used here for the chloroplast transformation vector was removed to produce a multicistron as the operon. Thus, that 700 nt of mRNA was supposedly the monocistron cleavage from the *aad*A-*monellin* dicistron because the *psb*A mRNA 5′ untranslated region includes an endonucleolytic cleavage site (positions -49 to -48) (Klaff, 1995).

In young leaves, the *aad*A-*monellin* dicistron was detected as a major band, whereas the *monellin* monocistron showed low intensity (Fig. 5b). However, the *monellin* monocistron of transplastomy with pLDUTRMon was abundant in mature leaves. Furthermore, the *monellin* monocistron of transplastomic Line 6-7 was more strongly detected than was the *aad*A-*monellin* dicistron, indicating processing from the dicistron transcribed from the *Prrn* promoter (Fig. 5c). To identify whether the cleavage was due to the *psb*A mRNA 5′ UTR element, another northern blot was performed, this time using the *aad*A probe. Only the *aad*A-*monellin* dicistron was detected, and degradation products of the *aad*A transcript were observed in all transplastomic plants, which were abundant in transplastomy with pLDUTRMon (Fig. 5b, 5c). When the two developmental stages were compared, 2- to 10-fold higher mRNA levels of the *monellin* monocistron was observed in mature leaves of transplastomy with pLDUTRMon (Fig. 5c).

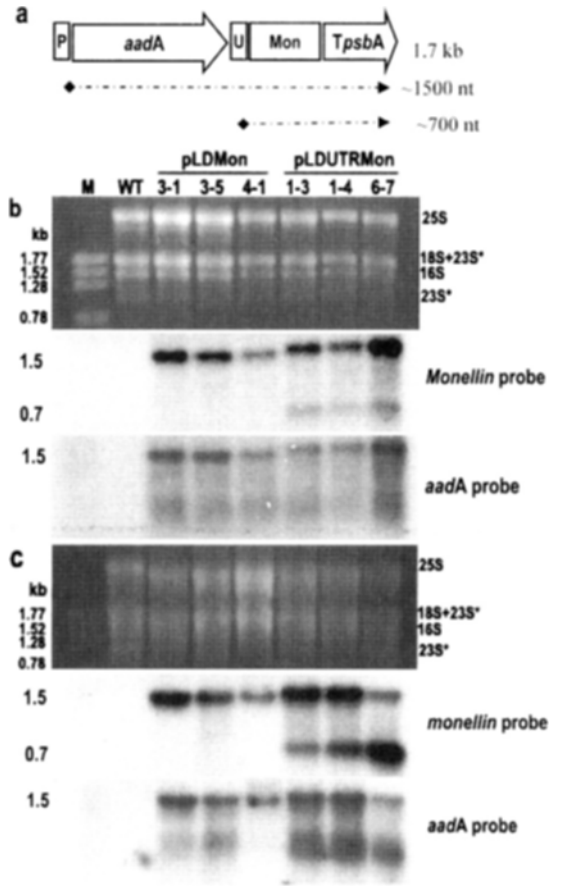

Figure 5. Two distinct transcription patterns from transgenic plants at different developmental stages, and accumulation of *monellin* in transgenic chloroplasts. **(a)** Expected transcripts of *monellin*. **(b, c)** Northern blot analysis was performed with total RNA extracted from leaves. Three mg RNA of each sample was analyzed by hybridization using probes shown at right. rRNAs (each top panel) were visualized by ethidium-bromide staining. Transgenic Lines 3 and 4, pLDMon; transgenic Lines 1 and 6, pLDUTRMon. Northern blot analysis at **(b)** young stage and **(c)** mature stage. WT, wild-type tobacco plant; UTR, untranslated 5′ region of plastid *psb*A photosynthetic gene. Known or deduced transcript sizes, marked on left, were derived by comparison to RNA ladder. Asterisks show *in vivo* breakdown products of 23S rRNA.

Expression of monellin in transgenic plants was monitored by immunoblot analysis (Fig. 6). Natural monellin, used as a positive control, was detected at around 5 kDa, corresponding to a molecular mass of 44 amino acid residues (A chain) and 50 amino acid residues (B chain), and binding to the antibody raised against it (Kim et al., 1989). Expression of single-chain monellin (11 kDa), which corresponded to the sum of

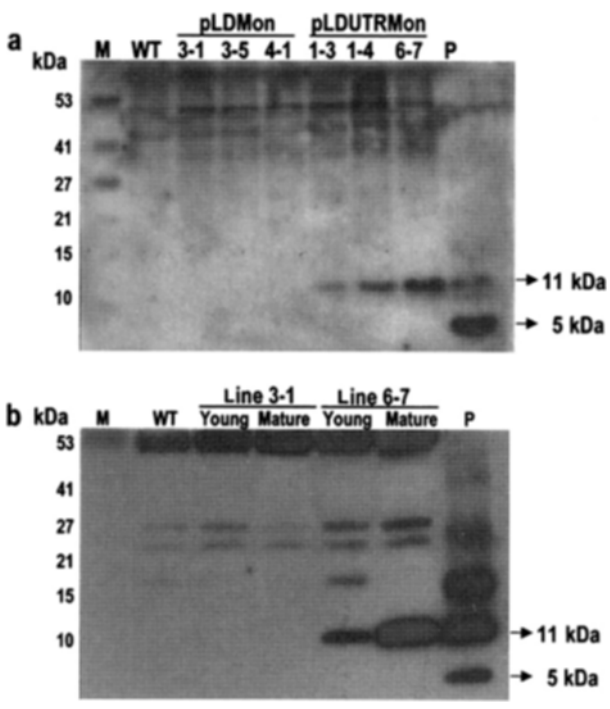

Figure 6. Western blot analyses of monellin transgenic chloroplasts. **(a)** Total soluble protein from transformed plants with pLDMon (Lines 3-1, 3-5, 4-1) and pLDUTRMon (Lines 1-3, 1-4, 6-7) was extracted at mature stage; 2 μg protein was used for immunoblot analysis. **(b)** Total soluble protein (600 ng) from transformed plants with pLDMon (Line 3-1) and pLDUTRMon (Line 6-7) was extracted at young and mature stages. Antibodies were used at 1:5000 dilution. Molecular mass standards are shown at left. P, 200 ng pure natural monellin; WT, wild type; UTR, untranslated 5′ region of plastid *psbA* photosynthetic gene.

the two chains (A and B) of natural monellin, was detected only in transgenic lines that harbored the 5′UTR of *psb*A. These transgenic lines showed levels of expression similar to those for transcription of the *monellin* monocistron (Fig. 6a). Abundant translation of monellin was observed in mature leaves of the transplastomic line with pLDUTRMon (Fig. 6b). Therefore, these results suggest that this translation was developmentally regulated by the 5′UTR of *psb*A.

ELISA assays were performed to monitor the accumulation of monellin in transplastomic lines at different developmental stages (Fig. 7). The results were considered positive at A_{490}. Monellin was expressed in all transgenic plants, even though its level of accumulation varied. In the transplastomic line with pLDUTRMon, monellin accumulation was up to 2.3% of tsp at the mature stage and 1.3% of tsp at the young stage. In contrast, the transplastomic line with pLDMon showed an accumulation of only as much as 1.5% of

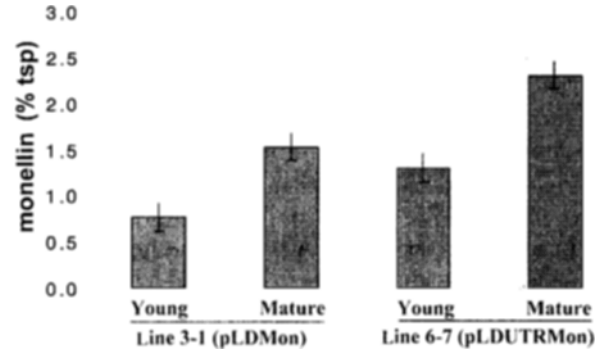

Figure 7. ELISA assay of monellin in transgenic chloroplasts at different stages. Total soluble protein from transplastomic Line 3-1 (with pLDMon) and Line 6-7 (with pLDUTRMon) was extracted at young and mature stages. Expression levels are indicated as percentage of total soluble proteins. Data are mean values of three replicates. Each well contained 100 ng protein. Rabbit polyclonal anti-monellin antibody was diluted at 1:1000.

tsp at the mature stage and 0.8% of tsp at the young stage.

DISCUSSION

These experiments demonstrate that endonucleolytic cleavage of the *psb*A 5′UTR element is activated by developmental conditions in the tobacco chloroplast. This is also the first report of an undesirable DNA fragment being integrated into a chloroplast genome via chloroplast transformation. Eibl et al. (1999) observed the loss of integrated transgenes during the procedure for transmission into the T_1 generation, and suggested that the 90-bp plastome fragment between the *uid*A and the *aad*A cassettes may serve as a homologous sequence for recombination events. Homologous recombination occurs occasionally in plant cells (Kavanagh et al., 1999; Li and Li, 2004); this mechanism also has been studied intensively in *Chlamydomonas reinhardtii* (Gumpel et al., 1994; Fujitani and Kobayashi, 2003). However, this phenomenon is largely unknown in the chloroplasts of higher plants, and may be due to intermolecular recombination with copies of the wild-type chloroplast genome during segregation.

Post-transcriptional processing of the *monellin* monocistron was observed from the *aad*A-*monellin* dicistron in transplastomic Line 6-7 with pLDUTRMon. This result suggests processing at the 5′UTR level, as the additional 700-nt transcripts were found only in the transplastomic lines with the construct containing the *psb*A 5′UTR, but not in those trans-

formed with the construct that lacked the *psb*A 5'UTR. Likewise, when the northern blots were hybridized with the *monellin* probe, the amount of monocistronic transcript was dramatically increased in transplastomic Line 6-7 at the mature stage, while that of the dicistronic transcript decreased compared with Lines 1-3 and 1-4 (Fig. 5). When probed with the *aad*A gene, the *aad*A transcript was highly susceptible to degradation. Moreover, *aad*A monocistron transcript was totally absent in pLDMon and was seen in pLDUTRMon only when protected by the *psb*A 3' UTR stabilizing sequences. The correlation found between *monellin* monocistron and protein accumulation sharply contrasts with reports from several previous studies, in which no such relationship was observed between transcript abundance and translation in transgenic chloroplasts.

The endonucleolytic and intercistronic cleavage of the *psb*A 5'UTR *in vivo* first reported here is an important step for mRNA stability and translation. Most chloroplast genes from higher plants are organized in clusters and are primarily co-transcribed as polycistronic forms and endonucleolytically processed into monocistronic mRNAs, during which some of the transcripts are edited and/or spliced (Barkan, 1988; Ruf et al., 1994). Among 130 chloroplast genes, approximately 76 are transcribed as dicistronic or polycistronic transcription units. Most tRNA genes as well as five others (*ndh*F, *psb*A, *psb*M, *psb*N, and *rbc*L) also are transcribed as monocistronic transcription units (Sugita and Sugiura, 1996). The endonucleolytic cleavage site (-49/-48) of the *psb*A 5'UTR has been proposed for an *in vitro* degradation system (Klaff, 1995). Hirose and Sugiura (1997) have also reported that *psa*C/*ndh*D dicistronic mRNA is not functional, and that the intercistronic cleavage is a prerequisite for both *ndh*D and *psa*C translation *in vitro* assays. In contrast, the GUS gene that is integrated by plastid transformation into the chloroplast genome downstream of the *rbc*L gene is transcribed together with *rbc*L, and the transcript is mainly accumulated as dicistronic mRNAs from which GUS is efficiently translated in tobacco chloroplasts (Staub and Maliga, 1995).

Processing of the *psb*A 5'UTR occurs just upstream of a consensus Shine-Dalgano sequence in *Chlamydomonas* (Bruick and Mayfield, 1998). It depends upon factors that mediate ribosome associations (Shen et al., 2001). Moreover, translation of the *psb*A mRNA requires RB 47, the nuclear-encoded poly (A)-binding protein in *C. reinhardtii* (Yohn et al., 1998; Kim and Mayfield, 2002), and the 43-kDa protein identified as the chloroplast homologue of the *Escherichia coli* ribosomal protein Sl in spinach (Alexander et al., 1998). The *psb*A 5'UTR enhances translation of the foreign genes and mediates light-induced activation of translation in transgenic chloroplasts (Eibl et al., 1999; Fernandez-San Millan et al., 2003; Dhingra et al., 2004). Accumulation of monellin in our transplastomic line with the construct containing the 5'UTR of *psb*A was about 2- to 3-fold higher than from the construct without the UTR (Fig. 7). Monellin accumulation was also higher in the mature stage than in the young stage for all transplastomic lines. The GUS gene fused to the *psb*A 5'UTR is expressed at levels about 100 times higher than when fused to *rbc*L and the RBS 5'UTR, as seen through *in vivo* analysis by chloroplast transformation (Eibl et al., 1999). Accumulation of HSA (Human Serum Albumin) accounts for only 0.02% of tsp when the Shine-Dalgarno sequence (SD) is used, but may be up to 11.1% of tsp when using the *psb*A 5'UTR in transplastomic tobacco plants (Fernandez-San Millan et al., 2003). Our results strongly suggest that endonucleolytic activation of the *psb*A 5'UTR enhances translational efficiency in transgenic chloroplasts, depending on their developmental condition. So far, the 5'UTR element of *psb*A is known as an important determinant of mRNA stability and translational efficiency, because of the secondary structure (Zou et al., 2003) and the ribosomal binding regions (Kim et al., 1993; Yohn et al., 1998). Here, we demonstrated that endonucleolytic activation is part of the mechanism of the *psb*A 5'UTR that affects processing and translational efficiency in tobacco chloroplasts.

ACKNOWLEGEMENTS

We thank Prof. Sung-Hou Kim of the University of California, Berkeley, USA for providing the synthetic single-chain monellin DNA. This research was supported by a basic research fund from the Rural Development Administration, Korea.

Received October 18, 2005; accepted November 28,2005.

LITERATURE CITED

Adams CC, Stern DB (1990) Control of mRNA stability in chloroplasts by 3' inverted repeats: Effects of stem and loop mutations on degradation of *psb*A mRNA *in vitro*. Nucl Acids Res 18: 6003-6010

Agrawal GK, Kato H, Asayama M, Shirai M (2001) An AU-

box motif upstream of the SD sequence of light-dependent *psb*A transcripts confers mRNA instability in darkness in cyanobacteria. Nucl Acids Res 29: 1835-1843

Alexander C, Faber N, Klaff P (1998) Characterization of protein-binding to the spinach chloroplast *psb*A mRNA 5' untranslated region. Nucl Acids Res 26: 2265-2272

Arai Y, Shikanai T, Doi Y, Yoshida S, Yamaguchi I, Nakashita H (2004) Production of polyhydroxybutyrate by polycistronic expression of bacterial genes in tobacco plastid. Plant Cell Physiol 45: 1176-1184

Baginsky S, Gruissem W (2002) Endonucleolytic activation directs dark-induced chloroplast mRNA degradation. Nucl Acids Res 30: 4527-4533

Barkan A (1988) Proteins encoded by a complex chloroplast transcription unit are each translated from both monocistronic and polycistronic mRNAs. EMBO J 7: 2637-2644

Bock R (2000) Sense from nonsense: How the genetic information of chloroplasts is altered by RNA editing. Biochimie 82: 549-557

Bohak Z, Li SL (1976) The structure of monellin and its relation to the sweetness of the protein. Biochim Biophys Acta 427: 153-170

Bruick RK, Mayfield SP (1998) Processing of the psbA 5' untranslated region in *Chlamydomonas reinhardtii* depends upon factors mediating ribosome association. J Cell Biol 143: 1145-1153

Chen HC, Stern D (1991) Specific ribonuclease activities in spinach chloroplasts promote mRNA maturation and degradation. J Biol Chem 266: 24205-24211

Chung HJ, Cho IS, Kim JH, In DS, Hur CG, Song JS, Woo SS, Choi DW, Liu JR (2003) Changes in gene expression during hairy root formation by *Agrobacterium rhizogenes* infection in ginseng. J Plant Biol 46: 187-198

Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20: 581-586

Daniell H, Datta R, Varma S, Gray S, Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat Biotechnol 16: 345-348

DeCosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the Bt *cry2Aa2* operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol 19: 71-74

Dhingra A, Portis Jr AR, Daniell H (2004) Enhanced translation of a chloroplast-expressed *Rbc*S gene restores small subunit levels and photosynthesis in nuclear *Rbc*S antisense plants. Proc Natl Acad Sci USA 101: 6315-6320

Edens L, van der Wel H (1985) Microbial synthesis of the sweet tasting plant protein thaumatin. Trends Biotechnol 3: 61-64

Eibl C, Zou Z, Beck A, Kim M, Mullet J, Koop HU (1999) *In vivo* analysis of plastid *psb*A, *rbc*L and *rpl*32 UTR elements by chloroplast transformation: Tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. Plant J 19: 333-345

Faus I (2000) Recent developments in the characterization and biotechnological production of sweet-tasting proteins. Appl Microbiol Biotechnol 53: 145-151

Fernandez-San Millan A, Mingo-Castel A, Miller M, Daniell H (2003) A chloroplast transgenic approach to hyper express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnol J 1: 71-79

Fujitani Y, Kobayashi I (2003) Asymmetric random walk in a reaction intermediate of homologous recombination. J Theor Biol 220: 359-370

Gumpel NJ, Rochaix JD, Purton S (1994) Studies on homologous recombination in the green alga *Chlamydomonas reinhardtii*. Curr Genet 26: 438-442

Higginbotham JD (1979) Protein sweeteners, *In* CAM Hough, KJ Parker, eds, Development in Sweeteners-1. Applied Science Publishers, London, pp 87-123

Hirose T, Sugiura M (1996) Cis-acting elements and trans-acting factors for accurate translation of chloroplast psbA mRNAs: Development of an *in vitro* translation system from tobacco chloroplasts. EMBO J 15: 1687-1695

Hirose T, Sugiura M (1997) Both RNA editing and RNA cleavage are required for translation of tobacco chloroplast *ndh*D mRNA: A possible regulatory mechanism for the expression of a chloroplast operon consisting of functionally unrelated genes. EMBO J 16: 6804-6811

Hirose T, Sugiura M (2004) Functional Shine-Dalgarno-like sequences for translational initiation of chloroplast mRNAs. Plant Cell Physiol 45: 114-117

Jirgensons B (1976) Conformational transitions of monellin, an intensely sweet protein. Biochim Biophys Acta 446: 255-261

Kavanagh TA, Thanh ND, Lao NT, McGrath N, Peter SO, Horvath EM, Dix PJ, Medgyesy P (1999) Homeologous plastid DNA transformation in tobacco is mediated by multiple recombination events. Genetics 152: 1111-1122

Kim IH, Lim KJ (1996) Large-scale purification of recombinant monellin from yeast. J Fermentat Bioengineer 82: 180-182

Kim J, Mayfield SP (2002) The active site of the thioredoxin-like domain of chloroplast protein disulfide isomerase, RB60, catalyzes the redox-regulated binding of chloroplast poly(A)-binding protein, RB47, to the 5' untranslated region of psbA mRNA. Plant Cell Physiol 43: 1238-1243

Kim M, Christopher DA, Mullet JE (1993) Direct evidence for selective modulation of *psb*A, *rpo*A, *rbc*L and 16S rRNA stability during barley chloroplast development. Plant Mol Biol 22: 447-463

Kim SH, Kang CH, Kim R, Cho JM, Lee YB, Lee TK (1989) Redesigning a sweet protein: Increased stability and renaturability. Prot Engineer 2: 571-575

Klaff P (1995) mRNA decay in spinach chloroplasts: *psb*A mRNA degradation is initiated by endonucleolytic cleavages within the coding region. Nucl Acids Res 23: 4885-4892

Kohmura M, Nio N, Ariyoshi Y (1990) Complete amino acid sequence of the sweet protein monellin. Agric Biol Chem 54: 2219-2224

Kota M, Daniell H, Varma S, Garczynski SF, Gould F, Moar WJ (1999) Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc Natl Acad Sci USA 96: 1840-1845

Kumar S, Daniell H (2004) Engineering the chloroplast genome for hyper-expression of human therapeutic proteins and vaccine antigens. Meth Mol Biol 267: 365-383

Lee MK, Kim HS, Kim SH, Park YD (2004) T-DNA integration patterns in transgenic tobacco plants. J Plant Biol 47: 179-186

Lee SB, Kwon HB, Kwon SJ, Park SC, Jeong MJ, Han SE, Byun MO, Daniell H (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. Mol Breed 11: 1-13

Li HQ, Li MR (2004) RecQ helicase enhances homologous recombination in plants. FEBS Lett 574: 151-155

Maier RM, Zeltz P, Kössel H, Bonnard G, Gualberto JM, Grienenberger JM (1996) RNA editing in plant mitochondria and chloroplasts. Plant Mol Biol 32: 343-365

Maliga P (1993) Towards plastid transformation in higher plants. Trends Biotech 11: 101-107

Monde RA, Greene JC, Stern DB (2000) The sequence and secondary structure of the 3′ UTR affect 3′-end maturation, RNA accumulation, and translation in tobacco chloroplasts. Plant Mol Biol 4: 529-542

Morris JA, Cagan RH (1972) Purification of monellin, the sweet principal of *Dioscoreophyllum cumminsii*. Biochim Biophys Acta 261: 114-122

Ogata C, Hatada M, Tomlinson G, Shin WC, Kim SH (1987) Crystal structure of the intensively sweet protein monellin. Nature 328: 739-742

Park MC, Shin J, Kim N, Cho H, Park S, An K, Lee S, An G (2003) High-frequency *Agrobacterium*-mediated genetic transformation of Tongil rice varieties. J Plant Biol 46: 23-30

Penarrubia L, Kim R, Giovannoni J, Kim SH, Fischer RL (1992) Production of the sweet protein monellin in transgenic plants. Bio/Technol 10: 561-564

Ruf S, Zeltz P, Kssel H (1994) RNA editing of unspliced and dicistronic transcripts of the intron containing reading frame IRF170 from maize chloroplasts. Proc Natl Acad Sci USA 91: 2295-2299

Sexton TB, Christopher DA, Mullet JE (1990) Light-induced switch in barley psbD-psbC promoter utilization: A novel mechanism regulating chloroplast gene expression. EMBO J 9: 4485-4494

Shen Y, Danon A, Christopher DA (2001) RNA binding-proteins interact specifically with the *Arabidopsis* chloroplast psbA mRNA 5′ untranslated region in a redox-dependent manner. Plant Cell Physiol 42: 1071-1078

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Matsubayashi T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kato A, Tohdoh N, Shimada H, Sugiura M (1986) The complete nucleotide sequence of the tobacco chloroplast genome: Its gene organization and expression. EMBO J 5: 2043-2049

Spadaccini R, Crescenzi O, Tancredi T, Casamassimi ND, Saviano G, Scognamiglio R, Donato AD, Temussi PA (2001) Solution structure of a sweet protein: NMR study of MNEI, a single chain monellin. J Mol Biol 305: 505-514

Srivastava S, Trivedi PK, Nath P (2004) Components involved in RNA-protein interaction at the 3′ untranslated region of *rbc*L mRNA of *Populus deltoides*. Plant Sci 167: 765-772

Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat Biotechnol 18: 333-338

Staub JM, Maliga P (1993) Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the psbA mRNA. EMBO J 12: 601-606

Staub JM, Maliga P (1995) Expression of a chimeric uidA gene indicates that polycistronic mRNAs are efficiently translated in tobacco plastids. Plant J 7: 845-848

Sugita M, Sugiura M (1996) Regulation of gene expression in chloroplasts of higher plants. Plant Mol Biol 32: 315-326

Wakasugi T, Sugita M, Tsudzuki T, Sugiura M (1998) Updated gene map of tobacco chloroplast DNA. Plant Mol Biol Rep 16: 231-241

Westhoff P, Herrmann RG (1988) Complex RNA maturation in chloroplasts: The *psb*B operon from spinach. Eur J Biochem 171: 551-564

Yohn CB, Cohen A, Rosch C, Kuchka MR, Mayfield SP (1998) Translation of the chloroplast psbA mRNA requires the nuclear-encoded poly(A)-binding protein, RB47. J Cell Biol 142: 435-442

Zerges W (2000) Translation in chloroplasts. Biochimie 82: 583-601

Zou Z, Eibl C, Koop HU (2003) The stem-loop region of the tobacco psbA 5′UTR is an important determinant of mRNA stability and translation efficiency. Mol Gen Genom 269: 340-349