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# A comparison of the effectiveness of the promoters and 5' regions of two plastid genes in directing the synthesis of Rubisco small subunits in tobacco plastids.

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## Introduction

Plastid transformation techniques allow the large subunit of the photosynthetic $CO_2$-fixing enzyme, Rubisco, to be manipulated in higher-plant chloroplasts (Whitney *et al*., 1999). This capability has been extended to Rubisco's small subunit (SSu), which is normally encoded in the nucleus, by transferring its gene to the plastome under the regulatory control of the plastid *psb*A promoter and 5' untranslated sequence (5'UTR) (Whitney and Andrews, 2001). Although plastome-encoded small subunits were synthesised and assembled into Rubisco correctly, the amounts produced were small despite an abundance of the *rbcS* transgene. Whitney and Andrews (2001) proposed that the scarcity of assembled plastid-synthesised small subunits might have been caused by inefficient translation of the plastid *rbcS* mRNA or impairment of folding or assembly of the transplastomic small subunits into the Rubisco complex.

To establish whether the *psb*A promoter/5'UTR sequence impairs the translation of *rbcS*, we transformed an analogous *rbcS* transgene (complete with a 3' hepta-His-encoding sequence and with or without the transit presequence) equipped with the plastid *rbc*L promoter/5'UTR into the inverted repeats of the tobacco plastome. The amounts of the *rbcS* mRNA and His-tagged small subunits that assembled into Rubisco were compared with those obtained with the previous *psb*A-regulated constructs.

## Materials and Methods

***Plasmid construction.*** A plasmid for transforming the tobacco plastome with a *rbc*S gene under the control of the plastid *rbc*L/5'UTR sequence was derived through modifying the transforming plasmid, pSSuH (Whitney and Andrews, 2001). This plasmid, pRp3SHis, had the *psb*A promoter and 5'UTR sequence in pSSuH replaced with their *rbc*L counterparts. Furthermore, the *rbc*S coding sequence was modified to encode three residues from the N terminus of the large subunit translationally fused to residue 2 (Glu) of the mature small subunit (Figure 1). All transforming plasmids were equipped with the same 3' hepta-histidine-encoding sequence and the *psb*A terminator sequence.

***Plastid transformation.*** The pRp3SHis plasmid was transformed into *Nicotiana tabacum* (L. cv Petit Havana) through microprojectile bombardment and a transformed plantlet was selected by growth on media containing spectinomycin (500 $\mu g\ ml^{-1}$) as described (Svab and Maliga, 1993). After regenerating three more times on selective medium, a homoplasmic transformant was confirmed by DNA blot analysis of *Hind*III-digested leaf DNA probed with

the tgf probe (Figure 1) conjugated to alkaline-phosphatase as described (Whitney *et al*., 2001). The regenerated Rp3SHis plantlet was transferred to soil and grown to seed in an air-conditioned glass house at 25ºC. Flowers were pollinated artificially with pollen from non-transformed tobacco. Subsequent analyses were performed on a $T_1$-generation Rp3Shis transformant, a non-transformed control, and $T_3$-generation tpSSuH4 and SsuH2 transformants (Whitney and Andrews, 2001).

***Analyses.*** The amount of *rbcS* transcript and transplastomic small subunits assembled into Rubisco were compared in the 2nd, 4th and 6th leaf (counting downwards from the apical meristem) from physiologically similar plants (12 cm in height) grown under the same growth conditions (see Figure 3a). RNA blots of total leaf RNA extracted using the Tri reagent (Sigma) were performed as described (Whitney and Andrews, 2001) and the blots were probed as described above. Rubisco was partially purified from individual leaves by PEG precipitation (10 to 20% w/v) as described (Whitney *et al*., 1999). The precipitated protein was solubilized in 2 ml of buffer (50 mM *N*-[2-hydroxyethyl]piperazine-*N'*-3-propanesulfonic acid (HEPES)-NaOH, pH 8.0, 0.1 mM EDTA) and dialysed for 2 h at 4ºC against the same buffer before adding glycerol to 15% (v/v). Using a Rubisco preparation from Rp3SHis,His-tagged Rubisco was affinity purified using NiNTA-agarose (Qiagen) as described (Whitney and Andrews, 2001). Rubisco content was measured in the samples by [$^{14}$C]2'-carboxyarabinitol-1,5-bisphosphate (CABP) binding (Whitney *et al*., 1999) after activating in buffer containing 20 mM $MgCl_2$ and 20 mM $NaHCO_3$ for 30 min at 25ºC. Using NuPAGE 4 to 12% Bis-Tris gels (Novex), equal amounts of total Rubisco (14 pmol) were separated and the content of His-tagged small subunits was measured on an immunoblot using the Penta-His antibody (Qiagen) as described (Whitney and Andrews, 2001).

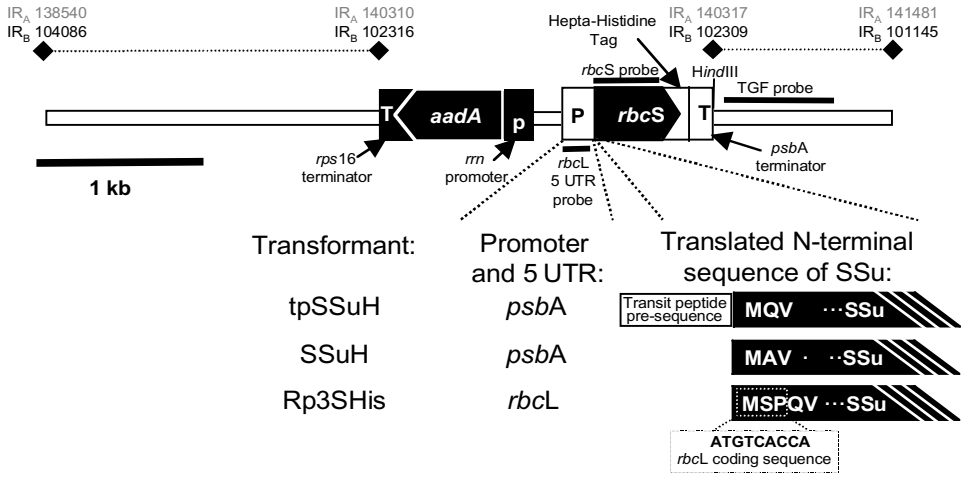


**Figure 1** A diagramatic representation of the transformation plasmids used to insert *rbcS* genes into the inverted-repeat regions of the tobacco plastid genome. Numbering (Shinozaki *et al*., 1986) corresponds to flanking plastome sequence. The annealing sites of the tgf, *rbc*S and *rbc*L 5'UTR probes are shown.

## Results and Discussion

### *Insertion of a rbcL-regulated rbcS gene into the tobacco plastome.*

The plasmid, pRp3SHis, was transformed into the tobacco plastome and a single transformant (from 10 bombardments) was identified by PCR analysis using primers that annealed to the *aad*A and *rbcS* genes (data not shown). The transformant was successively regenerated

through to homoplasmicity as determined by a DNA blot where no trace of uninterrupted wild-type plastome sequence could be detected (Figure 2a).

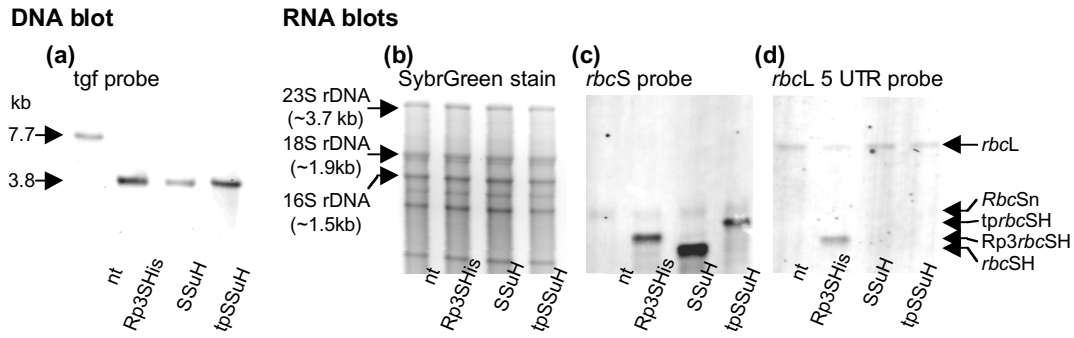


**Figure 2** DNA and RNA blot analyses of homoplasmic transformants Rp3SHis, SSuH, tpSSuH and a non-transformed (nt) control.

(**a**) Blot of *Hind*III-digested leaf DNA probed with the tgf sequence. (**b**) Separated total RNA (10 µg lane$^{-1}$) extracted from the fourth mature leaf of 12 cm-high plants and stained with Sybr Green. (**c**) Blot of the same RNA probed with *rbc*S or (**d**) *rbc*L 5'UTR sequence. The positions of ribosomal rDNA's and the mRNAs for *rbc*L, nucleus-encoded *Rbc*S (*Rbc*Sn) and plastid-encoded *rbc*S with (tp*rbcS*H) and without (Rp3*rbc*SH and *rbc*SH) the transit presequence are shown.

*The plastome encoded rbcS transgenes were abundantly transcribed.*

When RNA blots were hybridised with the *rbc*S sequence, the chloroplast *rbc*S message in all three transformants was at least 10-fold more abundant than the nuclear *Rbc*S message (Figure 2c). As the Rp3SHis transformant contained the 5'UTR from the plastid *rbc*L gene, the probe for this region hybridised to both the *rbc*L and Rp3*rbcS*H mRNAs (Figure 2d). The band densities indicated that message for the *rbcS* transgene was more than twice as abundant as the endogenous *rbc*L mRNA. This might be due to the doubled dose of the *rbc*S transgene in the inverted-repeat regions; however, the amount of message produced by the *rbc*S transgenes in the SSuH and tpSSuH transformants under the direction of the *psb*A promoter/5'UTR never exceeded two-thirds of the abundance of the endogenous *psb*A mRNA (Whitney and Andrews, 2001), despite the same gene-doubling phenomenon.

In leaves of similar age, the abundance of the *rbc*S message varied over a less than two-fold range regardless of promoter/5'UTR sequence (Figures 2c and 3b). However, there was a clear difference in way that the strength of the two promoters varied during leaf development. The *rbc*L promoter was most active in young leaves while the *psb*A promoter peaked in middle-aged leaves (Figure 3b). Whether this developmental pattern of expression correlates with that of the endogenous *rbc*L and *psb*A mRNAs remains to be tested.

*Small amounts of plastid-synthesised small subunits were assembled into Rubisco.*

SDS-PAGE analysis of purified Rubisco from Rp3SHis transformants showed that plastid-synthesised, His-tagged small subunits were only a minor component of the total small subunits assembled into Rubisco. As shown previously (Whitney and Andrews, 2001), Rubisco molecules containing His-tagged small subunits could be purified using Ni-NTA affinity chromatography and densitometry indicated that the ratio of His-tagged to non-His-tagged small subunits in such preparations was 1:7 (data not shown).

The proportion of His-tagged small subunits to total small subunits in total Rubisco purified conventionally from different-aged leaves, measured using immunoblots probed with the PentaHis antibody (Figure 3c), correlated with the abundance of plastid encoded *rbcS* message (Figure 3b). However, only 0.5 to 1.6% of the small subunits were the products of the transgenes. As there is no substantial difference in the abundance of the plastid-synthesised small subunits between the three transformants, the influence of the promoter and 5'UTR elements cannot be solely responsible for the low expression level of transplastomic small subunits assembled into Rubisco.

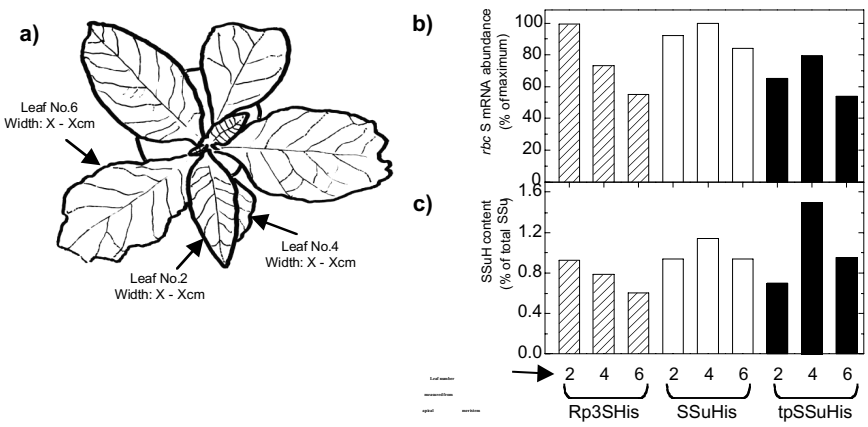


**Figure 3** Contents of *rbcS* mRNA and plastid-encoded small subunits in the transformants.

(**a**) Sketch showing the sampled leaves of the developmentally similar transformed plants. (**b**) The relative abundance of *rbc*S transcripts in the transformants during leaf development. (**c**) Content of plastid-encoded small subunits assembled into Rubisco in the transformants during leaf development.

## Conclusions

Both *psb*A and *rbc*L promoter/5'UTR elements directed the synthesis of large amounts of *rbc*S transcripts in tobacco plastids. However, substituting the *psb*A promoter and 5'UTR sequence with the corresponding sequence from *rbc*L did not improve the levels of plastid-encoded small subunits assembled into Rubisco. This could be caused by inefficient translation of the *rbc*S transgene but, if so, incorporation of the translational control region of *rbc*L (composed of the 5'UTR plus nine nucleotides of the 5' coding sequence) does not alleviate the problem. Perhaps a longer sequence from the 5' coding region of *rbc*L is required (Kuroda and Maliga, 2001). Alternatively, translation may be impaired by an inherently unfavourable secondary structure of the *rbc*S transcripts, perhaps induced by interactions with the *psb*A terminator sequence. A further alternative is that translation may not be impaired but folding and/or assembly of the plastid-synthesised small subunits is inefficient due to a restriction in their access to the chaperone-assisted pathway of Rubisco assembly optimised to accept cytoplasmically sourced small-subunit precursors from transporter complexes in the envelope membranes rather than nascent small subunits or precursors delivered from plastid ribosomes (Whitney and Andrews, 2001). Such a restriction might expose plastid-synthesised small subunits to rapid proteolytic degradation.

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