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Bacterial Rubisco supports the photosynthetic growth of tobacco.

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Introduction

The CO_2-fixing enzyme, Rubisco, can be manipulated in tobacco by plastid transformation (Kanevski et al. 1999; Whitney et al., 1999). Some Rubiscos from non-green algae have more efficient combinations of kinetic properties than the higher plant enzyme (Read and Tabita 1994; Uemura et al. 1997; Whitney et al. 2001) but attempts to co-express their subunits in chloroplasts have not produced functional enzymes because of folding or assembly problems (Whitney et al., 2001). Even replacement of the tobacco Rubisco large subunit with sunflower large subunit did not yield a hybrid enzyme capable of supporting the growth of the plant. Although the hybrid enzyme assembled, incompatibility between the sunflower large subunits and the tobacco small subunits crippled its activity (Kanevski et al., 1999).

In order to establish whether any foreign Rubisco can support tobacco photosynthesis, we resorted to the dimeric Form II Rubisco from *Rhodospirillum rubrum* that lacks small subunits and is known to assemble in prokaryotic foreign hosts (Goloubinoff et al., 1989; Pierce et al., 1989). Substitution of this Rubisco into tobacco yielded fully autotrophic and reproductive plants but induced a requirement for CO_2 enrichment.

Materials and Methods

The pLEV1 plastid transformation plasmid (Whitney et al. 1999) was modified to replace tobacco *rbc*L with an operon containing the *R. rubrum rbc*M gene linked via a 37-bp synthetic intergenic region containing a ribosome-binding sequence to an *aad*A gene that confers spectinomycin resistance (Fig. 1). This plasmid, pRubLev14, has the 5' end of the *rbc*M-*aad*A operon fused downstream of the first 42 nucleotides of *rbc*L coding sequence via a *Nde*I site that inserts an additional histidyl residue (Fig. 1a). The plasmid was biolistically transformed into *Nicotiana tabacum* L. cv Petit Havana and spectinomycin resistant plantlets were selected as described (Svab and Maliga 1993) in air containing 5% CO_2 (v/v). Four resistant plantlets were obtained from 20 bombardments and two independent plastome transformants, and their subsequent homoplasmicity, were confirmed by DNA blot analysis of restricted leaf DNA probed with alkaline-phosphatase-conjugated DNA probes (APBiotech), detected flourimetrically with AttoPhos (Promega). These tobacco-*rubrum* transformants (tr1 and tr3) were grown in 5 l pots of soil in a artificially lit (200 µmol quanta m^{-2} s^{-1}) growth cabinet in air containing 2.5% CO_2 (v/v) using a 14 h photo-period (25ºC / 17ºC). The plants set seed after artificially pollinating with pollen from non-transformed tobacco. The subsequent T_1 generation of tr1 transformants, and non-transformed controls, were germinated and grown under the same growth conditions. Samples were taken from leaves of similar physiological ages of non-transformed and tobacco-*rubrum* plants (see Fig. 2). Total leaf RNA was extracted using the Tri reagent (Sigma), separated by electrophoresis, blotted

(Whitney and Andrews 2001) and the blots probed as described above. Rubisco content and carbamylation status were measured by [^{14}C]2'-carboxyarabinitol-1,5-bisphosphate (CABP) binding (Whitney et al. 1999) and protein content was measured using the Coomassie Plus (Pierce) dye-binding method. SDS-PAGE, immunoblot and ^{35}S-Methionine pulse-chase analyses on leaf samples were performed as described (Whitney and Andrews 2001). Photosynthetic gas exchange was measured with young, fully expanded leaves of physiologically similar 28 (control) and 42 (t*r*1) day-old plants as previously described (Whitney et al. 1999).

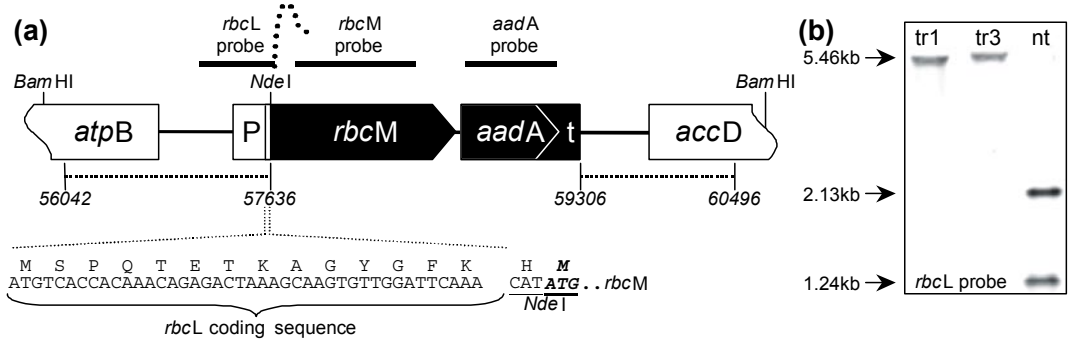

Fig. 1 Substitution of the tobacco plastome *rbc*L gene with a *rbc*M-*aad*A operon.

(**a**) Section of the large single-copy region of the tobacco-*rubrum* plastome. The dotted lines underline the flanking sequences from the plastome (Shinozaki et al. 1986) present in the transforming plasmid pRubLev14 to direct the insertional replacement of *rbc*L with the *rbc*M-*aad*A operon. The 45-bp nucleotide sequence from *rbc*L fused 5' to the *rbc*M coding sequence and the annealing positions of the *rbc*L, *rbc*M and *aad*A DNA probes are shown. P, *rbc*L promoter and 5'UTR sequence; t, *rbs*16 terminator sequence. (**b**) DNA blot of *Bam*HI-digested leaf DNA from two homoplasmic transformants (t*r*1 and t*r*3) and a non-transformed control (nt) probed with the *rbc*L sequence.

Results and Discussion

Transformation of the tobacco plastid genome.

Homologous replacement of the coding and termination sequence of tobacco *rbc*L with the *rbc*M-*aad*A bicistronic construct, still under the transcriptional and translational control of *rbc*L 5' sequences (Fig. 1a), yielded two independent transplastomic lines, t*r*1 and t*r*3, in tissue culture under an atomosphere containing 5 % (v/v) CO_2. After several further regenerations on selective medium, these became homoplasmic as judged by total elimination of the uninterrupted non-transformed plastome sequence (Fig 1b).

Developme*nt and analysis of plants grown in air containing 2.5% CO_2 (v/v).*

Transformants rooted in soil were unable to grow in air without CO_2 enrichment. Even when grown in 2.5% CO_2, the transformants developed slower than non-transformed controls grown in the same conditions. The tobacco-*rubrum* plants took 55 days to attain the same height and number of leaves as 37 day-old non-transformed plants (Fig. 2a). Although they grew more slowly, the tobacco-*rubrum* plants appeared normal and they were fully reproductive.

There was no evidence of the *rbc*L transcript in RNA blots of total leaf RNA from T_1 generation tobacco-*rubrum* plants (produced by back-crossing with wild-type pollen) (Fig. 2b). Both *rbc*M and *aad*A probes recognised the same, larger, mRNA transcript, consistent with a single bicistronic message. SDS-PAGE and immunoblot analyses of leaf protein (using antibodies to the tobacco and *R. rubrum* Rubiscos) confirmed that only the foreign bacterial Rubisco was being expressed in the transformants and that it was nearly fully soluble (data

not shown). On an area basis, leaves of the tobacco-*rubrum* plants contained 25% less soluble protein and 75% less Rubisco than comparable leaves of the non-transformed controls (Fig. 2c). However, the *R. rubrum* Rubisco was more active (carbamylated) than its tobacco counterpart under the high-CO_2 growth conditions, so the reduction in carbamylated Rubisco sites was only 66% in the transformants (Fig. 2c).

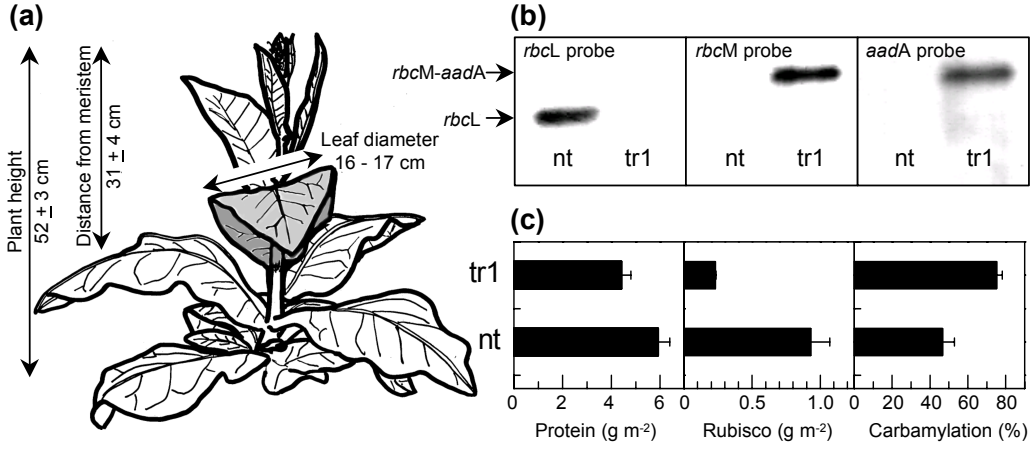

Fig. 2 rbcL and rbcM mRNA and Rubisco protein in non-transformed and tobacco-rubrum plants.

(**a**) Sketch showing growth features of physiologically similar 37 (non-transformed, nt) and 55 (T_1-generation tobacco-rubrum, tr1)day-old plants grown in air containing 2.5% (v/v) CO_2. The plant height and the position and breadth of the fifth leaf analysed (shaded) is shown. All plants had 19-20 leaves. (**b**) Blots of leaf RNA (10µg) hybridised with rbcL, rbcM and aadA DNA probes (see Fig. 1a). (**c**) The protein content, Rubisco content and carbamylation status in tr1 (n=9) and nt (n=6) leaf samples.

Pulse-chase analyses using ^{35}S-Met showed neither the bacterial Rubisco in tobacco-*rubrum* plants or tobacco Rubisco in the controls degraded appreciably during three hours after synthesis (data not shown). This apparent lack of post-translational turnover suggests the reduced abundance of the bacterial enzyme is due to a reduced abundance of its transcript and/or a reduction in its rate of translation. These questions will be addressed in future studies comparing the relative levels and rates of transcription for the *rbc*L and *rbc*M-*aad*A mRNA's.

Leaf photosynthesis

The CO_2 response of photosynthesis in leaves of tobacco-*rubrum* plants is consistent with the content and kinetic properties of *R. rubrum* Rubisco (not shown). For example, the CO_2-compensation point of the transformant leaves in air was increased 20 fold relative to wild-type to approximately 1200 µbar CO_2. When measured at 2 % (v/v) O_2, the CO_2-compensation point decreased 2.5 fold, consistent with the poorer CO_2/O_2 specificity of the bacterial Rubisco (Kane et al. 1994).

Conclusions

Although the kinetic properties of *R. rubrum* Rubisco are not suited to higher plant chloroplasts, they are sufficient to sustain full photosynthetic and reproductive growth of the tobacco-*rubrum* plants at high atmospheric CO_2 concentrations. This clearly demonstrates that the activity of a Rubisco from an entirely different phylogeny can be integrated into the chloroplast photosynthetic metabolism without prohibitive problems. This provides encouragement for the continuing efforts to integrate catalytically more efficient Rubiscos,

such as those from non-green algae, into the chloroplasts of plants. However, recent transformation efforts (Kanevski et al. 1999; Whitney et al. 2001) indicate that such an endeavor will require a greater understanding of the translational, folding and assembly requirements of the Rubiscos that are to be integrated.

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