S16-015
Substitution of foreign Rubiscos in the cyanobacterium, *Synechococcus* PCC7942

D Emlyn-Jones, SM Whitney, GD Price & TJ Andrews

Molecular Plant Physiology Group, Research School of Biological Sciences, The Australian National University, Canberra, A.C.T. 2601, Australia.
Fax: +61 (0)2 6125 5075
E mail: EmlynJones@rsbs.anu.edu.au

Keywords: Rubisco, cyanobacteria, recombinants.

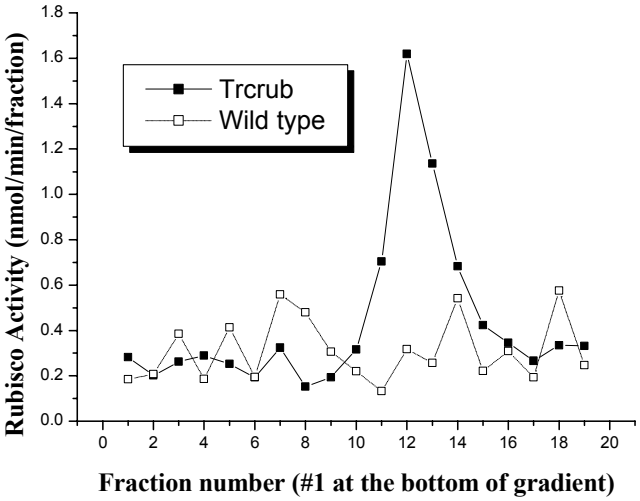
Introduction

In cyanobacteria, the genes for the central CO_2-fixing enzyme, Rubisco, can be deleted only if they are functionally replaced even when a heterotrophic carbon source is supplied. The so-called "cyanorubrum" version of *Synechocystis* PCC6803 was generated by physically replacing the cyanobacterial *rbc*LS operon with the *Rhodospirillum rubrum rbc*M gene fused to the cyanobacterial *rbc*LS promoter (Pierce *et al*. 1989, Amichay *et al*. 1993). Retarded growth and increased oxygen sensitivity were reported, presumably resulting from the inferior catalytic properties of the *R. rubrum* Rubisco as well as disruption of the carboxysome-based CO_2 concentrating mechanism (Pierce *et al*. 1989). To develop a means for screening foreign Rubiscos for ability to fold and assemble functionally and support the growth of cyanobacteria, we are generating a novel variant of this approach in *Synechococcus* PCC7942. The foreign Rubisco (from *R. rubrum* in the first instance) is encoded on a plasmid in conjunction with inactivation of the cyanobacterial *rbc*LS operon on the chromosome by replacement with an antibiotic resistance gene.

Materials and Methods

The shuttle vector, pTrcrub, was derived from the *Escherichia coli* expression vector, pTrc (Invitrogen). In this construct, expression of *R. rubrum* Rubisco was driven from a Trc promoter, which has the –35 region of the Trp promoter and the –10 region of the lac promoter. The lacI^q gene that encodes the lac repressor is also present in this vector. In the shuttle vector, pTobrub, derived from pTrcrub and pRVRR14 (Whitney & Andrews 2001), the tobacco chloroplast *rbc*L promoter, 5'-UTR and the first 42 coding nucleotides of tobacco *rbc*L are fused to the *R. rubrum rbc*M coding region. Both shuttle vectors have a *Synechococcus* PCC7942 origin of replication as well as an *E. coli* origin of replication, together with a chloramphenicol resistance gene (Price & Badger 1989). Sucrose gradients and Rubisco activity assays were performed as in Andrews (1988) except that 0.5ml of crude extracts (7.5mg ml^{-1} protein) were loaded onto each gradient and fractions collected from the base of the gradient.

A



B

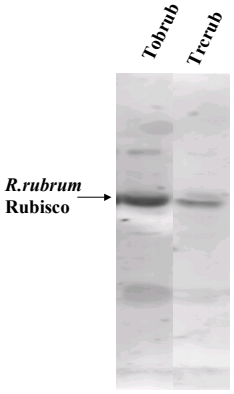


Fig. 1: *R.rubrum* Rubisco is expressed and active in *Synechococcus* PCC7942. **a)** Rubisco activity in soluble extracts from the Trcrub transformant and the wild type sedimented through sucrose gradients. A single peak corresponding to *R. rubrum* Rubisco is present in Trcrub. Most of the cyanobacterial enzyme is sedimented with the carboxysomes to the bottom of the gradient and thus not recovered. **b)** Immunoblot (using antibodies raised against *R. rubrum* Rubisco which do not detect cyanobacterial Rubisco) following SDS-PAGE analysis of soluble extracts isolated from Trcrub and Tobrub (100 µg protein per lane). Levels of *R. rubrum* Rubisco are three-to-five fold greater in Tobrub compared to Trcrub.

Results & Discussion

Expression of R. rubrum Rubisco in Synechococcus PCC7942

Plasmid pTrcrub was transformed into *Synechococcus* PCC7942 and transformants were selected with chloramphenicol. Quantitative immunoblot analysis performed on soluble extracts from transformants revealed that the *R. rubrum* Rubisco was present at levels of approximately 0.4% of the soluble protein, irrespective of the presence of IPTG (not shown). Sucrose-gradient ultracentrifugation of soluble extracts isolated from strain Trcrub revealed a large peak of Rubisco activity which was absent in the wild type (Fig. 1a). Immunoblots confirmed that this activity peak corresponded to the *R. rubrum*

Rubisco (not shown). This demonstrated that the *R. rubrum* Rubisco was expressed and active in *Synechococcus* PCC7942.

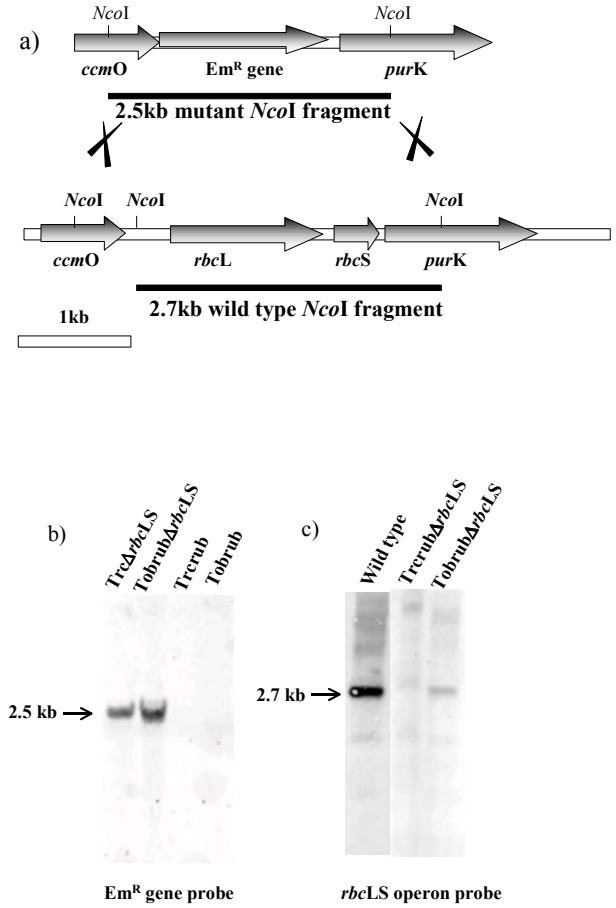


Fig. 2: Strategy for insertional inactivation of the *rbc*LS operon in *Synechococcus* PCC7942 and genomic analysis of transformants grown in 5%CO_2/95% air. **a)** The construct used for insertional inactivation by homologous recombination of the *rbc*LS operon in *Synechococcus* PCC7942, and the *Nco*I sites used in DNA blot analysis. **b)** DNA blot analysis of erythromycin-resistant transformants using the erythromycin resistance gene as probe. c) DNA blot analysis of erythromycin resistant transformants and the wild type using the *rbc*LS gene as probe. Equal loadings of DNA were used. The faint 2.7 kb signals indicate the heteroplasmic state of TrcrubΔ*rbc*LS and TobrubΔ*rbc*LS.expressed and active in *Synechococcus* PCC7942.

In cyanorubrum strain of *Synechocystis* PCC6803, *R. rubrum* Rubisco expression was driven from the endogenous *rbc*LS promoter and the amount of *R. rubrum* enzyme predicted to be roughly equivalent to the amount of cyanobacterial enzyme (Pierce *et al.,* 1989). Cyanobacterial Rubiscos are typically present at levels of 1-2% of the soluble protein. To attain higher levels of expression of *R. rubrum* Rubisco, plasmid pTobrub, in which expression of *R. rubrum* Rubisco is driven constitutively from the tobacco *rbc*L promoter, was transformed into *Synechococcus* PCC7942. A similar construct transformed into the tobacco chloroplast leads to levels of *R. rubrum* Rubisco of 5% of total soluble protein (Whitney & Andrews 2001). Quantitative Western analysis on Tobrub transformants showed the *R. rubrum* enzyme to be present at levels three-to five-fold greater than in the Trcrub transformants (Fig. 1b).

Insertional inactivation of the Synechococcus PCC7942 rbcLS operon

The entire *rbc*LS operon (including the promoter) of the *Synechococcus* PCC7942 chromosome was replaced with an erythromycin resistance gene (Elhai & Wolk 1988) to produce the chromosome-transforming construct shown in Fig. 2a. This construct was sequenced to ensure that no errors had been introduced into the flanking *ccm*O or *pur*K genes by the PCR manipulations and then transformed into both Trcrub and Tobrub. Transformants were selected with erythromycin (15μg ml^{-1}). Resistant transformants (TrcrubΔ*rbc*LS and TobrubΔ*rbc*LS) were detected after four weeks and were restreaked and grown photoautotrophically in liquid culture with 15 μg ml^{-1} erythromycin sparged with 5%CO_2/95% air. DNA-blot analysis of transformants using the erythromycin resistance gene as a probe revealed that correct insertion of the erythromycin resistance gene had taken place (Fig. 2b). However, similar blots using the *rbc*LS operon as a probe showed that traces of the wild type *rbc*LS operon persisted in the transformants (Fig. 2c), indicating a heteroplasmic condition with both wild-type and transformed chromosomes co-existing. Growth under increasing concentrations of erythromycin (up to 1000 μg ml^{-1}) failed to drive transformants to homoplasmicity (data not shown). The cyanorubrum strain of *Synechocystis* PCC6803 could grow photoautotrophically in the presence of 5%CO_2/95% air once homoplasmic. To drive cells to this homoplasmic state, however, heterotrophic growth in 5%CO_2/95% air or photoautotrophic growth in 5%CO_2/95% N_2 was required (Pierce *et al*. 1989, Amichay *et al.* 1993). Therefore, the *Synechococcus* PCC7942 Δ*rbc*LS transformants are now being grown in the presence of 5%CO_2/95%N_2 to drive the *rbc*LS deletion to homoplasmicity.

Conclusion

Here we demonstrate that the tobacco chloroplast *rbc*L promoter and 5'UTR can drive expression of *R. rubrum* Rubisco in *Synechococcus* PCC7942 from a plasmid to levels of ~1% soluble protein. Expression of *R. rubrum* Rubisco from a similar construct in the tobacco chloroplast led to levels of ~5% total soluble protein (Whitney & Andrews 2001). This presumably highlights similarities in the transcriptional/translational machinery between chloroplasts and cyanobacteria. Indeed, it has previously been observed that expression of the chloroplast *rbc*LS operon of the non-green alga *Galdieria sulphuraria* can also occur from its own promoter in *Synechococcus* PCC7942 (unpublished observation). The generation of this new system for expressing foreign Rubiscos in *Synechococcus* PCC7942 will provide new information about folding and assembly of Rubisco and may also give new insights into the plasticity of the carbon concentrating and fixing mechanisms of cyanobacteria.

References

Amichay, D. *et al.* (1993). *Plant Molecular Biology* **23**: 465-476.
Andrews, T.J., (1988). *Journal of Biological Chemistry* **263**: 12213-12219.
Elhai J. and Wolk C. P. (1988). *Gene* **68**: 119-138.
Pierce, J. *et al.* (1989). *Proc.Natl.Acad.Sci.USA* **86**: 5753-5757.
Price & Badger (1989). *Plant Physiology* **91**: 505-513.
Whitney, S.M. & Andrews, T.J. (2001). *This Proceedings*.