A faster Rubisco with potential to increase photosynthesis in crops

Myat T. Lin*, Alessandro Occhialini†‡, P. John Andralojc†, Martin A. J. Parry‡ & Maureen R. Hanson†

In photosynthetic organisms, d-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the major enzyme assimilating atmospheric CO₂ into the biosphere. Owing to the wasteful oxygenase activity and slow turnover of Rubisco, the enzyme is among the most important targets for improving the photosynthetic efficiency of vascular plants. It has been anticipated that introducing the CO₂-concentrating mechanism (CCM) from cyanobacteria into plants could enhance crop yield. However, the complex nature of Rubisco’s assembly has made manipulation of the enzyme extremely challenging, and attempts to replace it in plants with the enzymes from cyanobacteria and red algae have not been successful. Here we report two plastidic tobacco lines with functional Rubisco from the cyanobacterium Synechococcus elongatus PCC7942 (Se7942). We knocked out the native tobacco gene encoding the large subunit of Rubisco by inserting the large and small subunit genes of the Se7942 enzyme, in combination with either the corresponding Se7942 assembly chaperone, RbcX, or an internal carboxysomal protein, CcmM35, which incorporates three small subunit-like domains. Se7942 Rubisco and CcmM35 formed macromolecular complexes within the chloroplast stroma, mirroring an early step in the biogenesis of Rubisco and CcmM35. Both transformed lines were photosynthetically competent, supporting autotrophic growth, and their respective forms of Rubisco had higher rates of CO₂ fixation per unit of enzyme than the tobacco control. These plastidic tobacco lines represent an important step towards improved photosynthesis in plants and will be valuable hosts for future addition of the remaining components of the cyanobacterial CCM, such as inorganic carbon transporters and the β-carboxysome shell proteins.

Rubisco catalyses the incorporation of CO₂ into biological compounds in photosynthetic organisms. During photosynthesis, Rubisco also reacts wastefully with oxygen, leading to the release of previously fixed CO₂, NH₃ and energy. Furthermore, catalysis by Rubisco is slow and very large amounts (up to 50% of leaf soluble protein, 25% of leaf nitrogen) are needed to support adequate photosynthetic rates. Some variation in the catalytic properties of Rubisco from diverse sources is apparent. Harnessing this variation has the potential to confer superior photosynthetic characteristics to specific crops and environments. C4 plants, cyanobacteria and hornworts have evolved forms of CO₂-concentrating mechanisms (CCM) that allow them to utilize forms of Rubisco that have higher catalytic rates and lower CO₂ affinity, whereas C3 plants, which lack a CCM, are constrained to express forms of Rubisco with higher CO₂ affinity but a relatively low rate of turnover. In plants, Rubisco is a L8S8 hexadecamer consisting of eight small subunits (SSU) and eight large subunits (LSU). Although the SSU genes are located in the nucleus, the LSU is encoded by the chloroplast genome, which has complicated previous attempts to engineer improvements in higher plant Rubisco.

Introduction of a CCM has been proposed as a means to improve the performance of Rubisco in C3 plant chloroplasts. In cyanobacteria and several autotrophic prokaryotes, Rubisco and carbonic anhydrase are enclosed within polyhedral microcompartments known as carboxysomes, which maintain elevated CO₂ concentrations in the vicinity of Rubisco, which both increases carbon fixation and suppresses photorespiration. However, when a tobacco transplastomic line was created in which the LSU gene, rbcL, from the cyanobacterium Synechococcus PCC6301 replaced the native tobacco rbcL, the cyanobacterial LSU did not form a functional complex with the native tobacco SSU. Although a simpler L2 homodimer Rubisco from Rhodospirillum rubrum was able to assemble inside tobacco chloroplasts, red algal Rubisco subunits failed to produce functional L8S8 complexes within chloroplasts.

To test whether cyanobacterial LSU and SSU can assemble into a functional enzyme within higher plant chloroplasts, we generated two transplastomic tobacco lines, named SeLSX and SeLSM35, using the biolistic delivery system, to express the two Rubisco subunits from Se7942 along with either RbcX or CcmM35, respectively. In each chloroplast transformant, three genes were co-transcribed from the tobacco rbcL promoter. Each downstream gene was preceded by an intercistronic expression element (IEE) and a Shine–Dalgarno sequence (SD) and equipped with a terminator to facilitate processing into translatable monocistronic transcripts (Fig. 1a).

The two vectors we constructed were designed to replace the tobacco rbcL gene with the foreign DNA. To determine whether all chloroplasts in each plant contained the transgenic locus rather than endogenous tobacco rbcL, we examined blots of total leaf DNA digested with restriction enzymes that would produce restriction fragment-length polymorphisms between the wild-type and transgenic loci (Fig. 1b). We found that shoots arising after two rounds on selective medium were homoplasmic for the transgene loci, lacking the fragment corresponding to the wild-type chloroplast genome. In order to verify these observations, we performed reverse transcription and PCR (RT–PCR) and observed no cDNA derived from the native rbcL transcript, whereas cDNAs produced from adaA, the selectable marker gene, and the cyanobacterial genes were detected (Fig. 1c).

To observe the expression of the cyanobacterial proteins, we extracted total leaf proteins and examined them by SDS–PAGE and immunoblots. In Coomassie-stained gels, we detected protein bands at the predicted molecular masses of 15 kDa for the LSU and 13 kDa for the SSU of the cyanobacterial Rubisco in SeLSX and SeLSM35 samples, whereas wild-type tobacco exhibited a protein of the expected and distinct SSU mass of ~15 kDa (Fig. 2a). Immunoblots probed with antibodies specific for either the cyanobacterial LSU, tobacco Rubisco, tobacco SSU or cyanobacterial CcmM35 verified the presence of cyanobacterial proteins in the two transformants and tobacco Rubisco only in the wild-type plant (Fig. 2a). Although no engineering of tobacco SSU genes was performed in the transgenic lines, tobacco SSU protein was undetectable as expected, as its stability is known to be severely affected in the absence of a compatible LSU. The absence of the tobacco SSU in the transformants also indicated that it could not form a stable complex with the cyanobacterial LSU. The estimated stoichiometry of CcmM35 per Rubisco holoenzyme in SeLSM35 transformant is about 4.5, which is consistent with the values reported for cyanobacteria (Extended Data Fig. 1).

In order to observe the configuration of the cyanobacterial Rubisco in the two transgenic lines, we examined the plant material by transmission
electron microscopy (TEM) in combination with immunogold labeling. Although the enzyme was localized to the chloroplast stroma in both transgenic lines, we observed markedly different patterns of molecular organization. In leaves of the SeLSX line, the cyanobacterial Rubisco showed a diffuse localization similar to endogenous Rubisco in wild-type tobacco (Fig. 2b, c). In contrast, in the SeLSM35 line, in which the Rubisco is co-expressed with CcmM35, the proteins were aggregated into a giant complex in each chloroplast (Fig. 2d and Extended Data Fig. 2). In Se7942, CcmM35 is translated from an internal ribosome entry site of the SSU-like domain of CcmM35 resulting in their paracrystalline arrangement in the lumen of β-carboxysomes.

The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes. The cyanobacterial mutant lacking CcmM58 produces large electron-dense bodies of 300–500 nm with a rectangular cross-section composed of SSU-like domains of CcmM35 resulting in the paracrystalline arrangement in the lumen of β-carboxysomes.
The fact that both transgenic lines could grow autotrophically indicated that active cyanobacterial Rubisco has assembled. We measured the carboxylase activities of the cyanobacterial Rubisco in the leaf homogenates at room temperature using ribulose bisphosphate (RuBP) and several concentrations of radiolabelled sodium bicarbonate (NaH$_{14}$CO$_3$). The assays were performed in the presence of 10 mM, 20 mM and 50 mM NaH$_{14}$CO$_3$, which at pH 8.0 would generate dissolved CO$_2$ concentrations of approximately 125 μM, 250 μM and 640 μM, respectively. The carboxylase activity of Rubisco in the tobacco control did not increase upon increasing the CO$_2$ concentration, confirming that the native enzyme was already saturated at 125 μM of dissolved CO$_2$ (Fig. 4). In contrast, cyanobacterial Rubisco displayed greater carboxylase activity at higher CO$_2$ concentrations, with a rate of catalysis which exceeded that of the tobacco enzyme at each CO$_2$ concentration. Our measured kinetic values are consistent with the reported rate and Michaelis constants for CO$_2$ ($\sim 3$ s$^{-1}$ and 10.7 μM for tobacco and $\sim 12$ s$^{-1}$ and 200 μM for the enzyme in Synechococcus PCC6301, respectively$^{23,24}$). We confirmed that the carboxylase activities detected in our samples were specific to Rubisco, as they were entirely dependent on the presence of RuBP and were inhibited by CABP$^{25}$ (Extended Data Fig. 3). The high carboxylase activities detected in the transformants are consistent with the absence of interference by tobacco SSU in the assembly of bona fide cyanobacterial Rubisco in the chloroplasts. Furthermore, both transgenic lines exhibited high Rubisco activities despite differences in its intra-organellar organization.

We included RbcX in one of our chloroplast transformation vectors because it has been shown to enhance the assembly of the LSU core complex before formation of the final hexadecameric complex$^4$. However, Se7942 lacking RbcX suffered no defect in growth rate or Rubisco activity$^{26}$. As line SeLSM35 lacks RbcX but has active Rubisco, evidently Se-RbcX is not essential for the assembly of functional cyanobacterial Rubisco in chloroplasts. CcmM35, through its SSU-like domains, might assist in the assembly of cyanobacterial Rubisco in SeLSM35 in the absence of RbcX.

The transgenic plants described here are absolutely dependent on the cyanobacterial Rubisco for carbon fixation. If the oxygenation reaction of cyanobacterial Rubisco can be suppressed and the local CO$_2$ concentration in the vicinity of the enzyme can be raised by further engineering, CO$_2$ assimilation may be enhanced, and the necessity to divert so much fixed nitrogen into this enzyme may be diminished. Recently, we demonstrated that the shell proteins of β-carboxysomes could form structures similar to empty microcompartments in the chloroplast stroma$^27$. Introduction of the carboxysome shell proteins, the required internal proteins, and appropriate transporters into transgenic plants containing cyanobacterial Rubisco is predicted to result in significantly enhanced photosynthetic performance in vascular plants$^{28}$. This report, demonstrating that cyanobacterial Rubisco can assemble into active enzyme in a C3 plant and support autotrophic photosynthesis, is an important step towards the introduction of a complete and functional CCM into the chloroplasts of vascular plants.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 17 June; accepted 14 August 2014.

Published online 17 September 2014.

---

Figure 3 Phenotype of the wild-type and transplastomic tobacco lines. Plant were grown at atmospheric CO$_2$ level about 9,000 p.p.m. a-e, Pictures showing 6-week-old wild-type (a), SeLSX (b), and SeLSM35 (c); and 10-week-old SeLSX (d) and SeLSM35 (e) tobacco lines grown in the same conditions. Scale bars, 5 cm.

Figure 4 Carboxylase activities at different $^{14}$CO$_2$ concentrations. CO$_2$ fixation by crude leaf homogenates from tobacco lines expressing cyanobacterial Rubisco (SeLSX and SeLSM35) and wild-type tobacco (WT). The rates of carboxylase activity (mol CO$_2$ fixed per mol active sites per s) at each point of the curves are the means ± standard deviation of the 2, 4 and 6 min data obtained in two independent assays at different CO$_2$ concentrations (125 μM, 250 μM, 640 μM).

---

1. Andersson, I. & Backlund, A. Structure and function of Rubisco. Plant Physiol. Biochem. 46, 275–291 (2008).
2. Whitney, S. M., Houtz, R. L. & Alonso, H. Advancing our understanding and capacity to engineer nature’s CO$_2$-sequestering enzyme, Rubisco. Plant Physiol. 155, 27–35 (2011).
3. Pary, M. A. J. et al. Rubisco activity and regulation as targets for crop improvement. J. Exp. Bot. 64, 717–730 (2013).
4. Zarzycki, J., Axen, S. D., Kinney, J. N. & Kerfeld, C. A. Cyanobacterial-based approaches to improving photosynthesis in plants. J. Exp. Bot. 64, 787–798 (2013).
5. McGrath, J. M. & Long, S. P. Can the cyanobacterial carbon-concentrating mechanism increase photosynthesis in crop species? A theoretical analysis. Plant Physiol. 164, 2247–2261 (2014).
6. Price, G. D. et al. The cyanobacterial CCM as a source of genes for improving photosynthetic CO2 fixation in crop species. *J. Exp. Bot.* **64**, 753–768 (2013).

7. Whitney, S. M., Baldet, P., Hudson, G. S., Andrews, T. J. & Form, I. Rubisco from non-green algae are expressed abundantly but not assembled in tobacco chloroplasts. *Plant J.* **26**, 539–547 (2001).

8. Kanevski, I., Maliga, P., Rhoades, D. F. & Gutteridge, S. Plastome engineering of ribulose-1,5-bisphosphate carboxylase/oxygenase in tobacco to form a sunflower large subunit and tobacco small subunit hybrid. *Plant Physiol.* **119**, 133–142 (1999).

9. Saschenbrecker, S. et al. Structure and function of RbcX, an assembly chaperone for hexadecameric rubisco. *Cell* **129**, 1189–1200 (2007).

10. Long, B. M., Badger, M. R., Whitney, S. M. & Price, G. D. Analysis of carboxysomes from Synechococcus PCC7942 reveals multiple Rubisco complexes with carboxysomal proteins CcmM and CcaA. *J. Biol. Chem.* **282**, 29323–29335 (2007).

11. Cameron, J. C., Wilson, S. C., Bernstein, S. L. & Kerfeld, C. A. Biogenesis of a bacterial organelle: the carboxysome assembly pathway. *Cell* **155**, 1131–1140 (2013).

12. Chen, A. H., Robinson-Mosher, A., Savage, D. F., Silver, P. A. & Polka, J. K. The bacterial carbon-fixing organelle is formed by shell envelopment of preassembled cargo. *PLoS ONE* **8**, e76127 (2013).

13. Parry, M. A. J., Andralojc, P. J., Mitchell, R. A. C., Madgwick, P. J. & Keys, A. J. Manipulation of Rubisco: the amount, activity, function and regulation. *J. Exp. Bot.* **54**, 1321–1333 (2003).

14. Zhu, X. G., Long, S. P. & Ort, D. R. Improving photosynthetic efficiency for greater yield. *Annu. Rev. Plant Biol.* **61**, 235–261 (2010).

15. Dhirgra, A., Portis, A. R. & Daniell, H. Enhanced translation of a chloroplast-expressed RbcS gene restores small subunit levels and photosynthesis in nuclear RbcS antisense plants. *Proc. Natl Acad. Sci. USA* **101**, 6315–6320 (2004).

16. von Caemmerer, S., Quick, W. P. & Furbanck, R. T. The development of C4 rice: current progress and future challenges. *Science* **336**, 1671–1672 (2012).

17. Whitney, S. M. & Andrews, T. J. Plastome-encoded bacterial ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) supports photosynthesis and growth in tobacco. *Proc. Natl Acad. Sci. USA* **98**, 14738–14743 (2001).

18. Maliga, P. & Tungsutchat-Huang, T. Plastid transformation in *Nicotiana tabacum* and *Nicotiana sylvestris* by biolistic DNA delivery to leaves. *Methods Mol. Biol.* **1132**, 147–163 (2014).

19. Zhou, F., Karcher, D. & Bock, R. Identification of a plastid intercistronic expression element (IEE) facilitating the expression of stable translatable monocistronic mRNAs from operons. *Plant J.* **52**, 961–972 (2007).

20. Drechsel, O. & Bock, R. Selection of Shine–Dalgarno sequences in plastids. *Nucleic Acids Res.* **39**, 1427–1438 (2011).

21. Long, B. M., Rae, B. D., Badger, M. R. & Price, G. D. Over-expression of the beta-carboxysomal CcmM protein in *Synechococcus* PCC7942 reveals a tight co-regulation of carboxysomal carbonic anhydrase (CcA) and M58 content. *Photosynth. Res.* **109**, 33–45 (2011).

22. Long, B. M., Tucker, L., Badger, M. R. & Price, G. D. Functional cyanobacterial β-carboxysomes have an absolute requirement for both long and short sequences of the CcmM protein. *Plant Physiol.* **153**, 285–293 (2010).

23. Yokota, A. & Canvin, D. T. Ribulose bisphosphate carboxylase/oxygenase content determined with [14C]-ribulose-1,5-bisphosphate in plants and algae. *Plant Physiol.* **77**, 735–739 (1985).

24. Mueller-Gajjar, O. & Whitney, S. M. Evolving improved Synechococcus Rubisco functional expression in *Escherichia coli*. *Biochem. J.* **414**, 205–214 (2008).

25. Parry, M. A. J., Keys, A. J., Madgwick, P. J., Carmo-Silva, A. E. & Andralojc, P. J. Rubisco regulation: a role for inhibitors. *J. Exp. Bot.* **59**, 1569–1580 (2008).

26. Emlyn-Jones, D., Woodger, F. J., Price, G. D. & Whitney, S. M. RbcX can function as a Rubisco chaperonin, but is non-essential in *Synechococcus* PCC7942. *Plant Cell Physiol.* **47**, 1630–1640 (2006).

27. Lin, M. T. et al. β-carboxysomal proteins assemble into highly organized structures in *Nicotiana* chloroplasts. *Plant J.* **79**, 1–12 (2014).

Acknowledgements We thank C. Kerfeld (Michigan State University) for helpful discussion and providing us with the Se7942 genomic DNA and purified His-tagged CcmM protein. W. Li (Cornell University) for technical assistance in generating, selecting and analysing the tobacco chloroplast transformants and M. Waqar Hameed (Cornell University) for the codon-optimized cyanobacterial Rubisco genes. This material is based upon work supported by the National Science Foundation under grant number EF-1105584 to M.R.H., Biotechnology and Biological Sciences Research Council under grant number BB/I024488/1 to M.A.J.P. and the National Institute of General Medical Sciences of the National Institutes of Health under award number F32GM103019 to M.T.L. P.J.A. and M.A.J.P. also acknowledge support from the 20:20 Wheat Institute Strategic Program (BBSRC BB/J/00426X/1).

Author Contributions M.T.L. designed and generated the DNA constructs and the transgenic tobacco lines. A.O. carried out the TEM imaging, protein analyses and Rubisco activity assays. M.R.H., P.J.A. and M.A.J.P. supervised the project. All authors interpreted results and wrote the manuscript.

Author Information The nucleotide sequences are deposited in GenBank with accession numbers KM102745 and KM102746 for SeLSX and SeLSM35 tobacco lines, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.R.H. (mrh5@cornell.edu).

550 | NATURE | VOL 513 | 25 SEPTEMBER 2014

©2014 Macmillan Publishers Limited. All rights reserved
METHODS

Construction of the transformation vectors. The Se-rbcL and Se-rbcS genes with codons optimized for chloroplast translation system were designed by Muhammad Waqar Hameed and synthesized by Bioneer. Extended Data Table 2 contains the primers ordered from Integrated DNA Technologies and used in this work. The amplifications of DNA molecules were carried out with Phusion High-Fidelity DNA polymerase (Thermo Scientific). The restriction enzymes and T4 DNA ligase were also purchased from Thermoscientific.

The two tobacco chloroplast genomic loci (F1 and F2) immediately flanking the rbcL gene (base pairs 56620–57599 and 59034–60033 of NCBI Reference Sequence: NC_001879.2) were amplified from the DNA extracted from tobacco plants using the primer pairs F1for-F1rev and F2for-F2rev respectively and cloned into pCR8/GW/TOPO TA vector (Life Technologies) adding Psrl and MluI restriction sites at the 5′ and 3′ end of F2, respectively. The Se-rbcL gene was amplified from pGEM-Teasy-Se-rbcL with F1OLOBrlcF and 4ERBcRev primers adding an overlap to the 3′ end of F1 at the 5′ end of Se-rbcL and four restriction sites, MauBI, NotI, Psrl and MluI, at the 3′ end of Se-rbcL. This amplified Se-rbcL gene was designed to replace the tobacco rbcL in frame and allow the synthetic expansion of the operon. F1for and F2rev primers were used to amplify F1 from its pCR8 vector and the resulting product was then joined with the Se-rbcL amplicon by the overlap extension PCR procedure. The F1-Se-rbcL segment was then digested with Apal and MluI restriction enzymes and ligated into pGEM-Teasy-Se-rbcL template treated with the same two enzymes to obtain the pGEM-F1-rbcL vector. F2 was digested out of its pCR8 vector with Psrl and MluI enzymes and ligated into the similarly digested pGEM-F1-rbcL vector to yield the pGEM-PF1-rbcL-SMO-F2 vector. The selectable marker openor (SMO) containing loxp-Ppsba-adA-Tpsrl6-loxp was amplified from a previously reported chloroplast transformation vector, pTetCBgC28, with SMOfor and SMOrev primers, digested with Psrl and ligated in forward orientation to the Psrl-digested pGEM-F1-rbcL-F2 vector to obtain the pGEM-PF1-rbcL-SMO-F2 vector.

The rbcL terminator (TrcL) was amplified from the tobacco DNA with TrbcLfor and TrbcLrev primers, digested with MauBI and Bsp120I enzymes and ligated between the MauBI and NotI sites of the pGEM-F1-rbcL-SMO-F2 vector to obtain the pCT-rbcL, which is ready to replace the tobacco rbcL with Se-rbcL and the SMO by the chloroplast transformation procedure. The Se-rbcL operon driven by the native rbcL promoter in pCT-rbcL was then expanded at the MauBI site with Se-rbcL, Se-rbcX and Se-cmcm35s as follows.

Three terminators from the Arabidopsis thaliana (At) chloroplast genome, TpetD(At), TpsbA(At) and Trps16(At), were amplified with their respective primer pairs, TpetDAtfor-TpetDAtrev, TpsbAAtfor-TpsbAAtrev and Trps16Atfor-Trps16Atrev, adding an overlap to the intercistronic expression element (IEE) at the 3′ end and two restriction sites, MauBI and NotI at the 5′ end of each terminator. Each terminator was extended at the 3′ end by IEE-s.d or IEE-SD18 fragment present in the four intergenic regions, IG1, IG2, IG3, and IG4 in Fig. 1a. The Se-rbcX and Se-cmcm35s genes were amplified from the genomic DNA extracted from Se7942 using the primer pairs rbcXfor-rbcXrev and M35sfor-M35srev respectively, adding an overlap to the IEE-s.d fragment at the 5′ end and an MauBI site at the 3′ end of each gene. Similarly, Se-rbcS was amplified from pGEM-Teasy-Se-rbcL using the primer pair rbcSfor-rbcSrev. Then, Ig1-rbcS, Ig2-rbcS, Ig3-rbcS and G4-rccm35s fragments were similarly generated by joining each intergenic fragment with the corresponding vector using the overlap extension PCR procedure. The MauBI-digested Ig2-rbcX and Ig4-cmcm35s modules were each inserted into the MauBI site of the pCT-rbcL to obtain pCT-rbcL-rbcX and pCT-rbcL-cmcm35s respectively. Then the MauBI-digested Ig1-rbcS and Ig3-rbcS modules were each inserted into the MauBI site of pCT-rbcL-rbcX and pCT-rbcL-cmcm35s to obtain pCT-rbcL-rbcX and pCT-rbcL-cmcm35s vectors, respectively, which were used in the following chloroplast transformation procedure to replace the native rbcL gene with the cyanobacterial genes.

Generation of transplastomic tobacco plants. We used the Biolistic PDS-1000/ He Particle Delivery System (Bio-Rad Laboratories) and a tissue-culture leaf selection method. Two-week-old tobacco (Nicotiana tabacum cv. Samsun) seedlings germinated in sterile MS agar medium were boredamed with 0.6 μm gold particles carrying the appropriate chloroplast transformation vector. Two days later, the leaves were cut in half and put on RMOP agar plates containing 200 μl of 70% ethanol and air-dried before it was dissolved in 100 μl of double-distilled water. After quality and concentration of the DNA samples were determined by a Nanodrop method, 1 μg of each DNA sample was digested by NdeI and Nhel restriction enzymes, and the digested fragments were separated on a 1% agarose gel. The DNA pieces in the gel were depurinated, denatured and then transfected and cross-linked to a nylon membrane according to the manufacturer’s protocols. The DNA samples on the membrane blot were hybridized with the DIG-labelled probe, which was then detected with anti-digoxigenin alkaline phosphatase antibody using CDP-star chemiluminescent substrate (Roche) according to the manufacturer’s specifications.

Analyses of the transcripts by RT–PCR. Total RNA was extracted from each leaf tissue sample with a standard TRizol procedure. The leaf tissues frozen with liquid nitrogen were ground in 800 μl of trizol and incubated at 22°C for 5 min. After the insoluble pieces were removed by centrifugation, 160 μl of chloroform was added to the supernatant, mixed vigorously for 15 s and incubated at 22°C for 3 min. The two aqueous phases were separated in a centrifuged at 4°C for 15 min and the upper layer transferred to a new tube was mixed with 500 μl of isopropanol. The sample was incubated at 22°C for 10 min and centrifuged at 4°C for 10 min. The pellet was resuspended in 800 μl of 75% ethanol and centrifuged again at 4°C for 10 min. The pellet was air-dried and resuspended in 50 μl of molecular biology grade water. The RNA samples were treated with DNase using Ambion DNA-free kit (Life Technologies) and the cDNA for each gene was generated with its corresponding reverse primer using Sensiscript Reverse Transcription kit (Qiagen) according to the manufacturer’s protocols. The cDNA samples were amplified with the PCR master mix (Bioline) and analysed in a 1% agarose gel.

SDS page, immunoblot and determination of CcmM35/Rubisco content. The crude leaf homogenates used in the carboxylase activity measurements were separated by SDS–PAGE using 4–20% polyacrylamide gradient gels (Thermo Scientific, UK). For each sample, the same amount of protein, as determined by Bradford assay, was loaded onto the gel. After electrophoresis, the resolved proteins were transferred to a nitrocellulose membrane (Hybond-C Extra from GE Healthcare Life Sciences) using a western blot apparatus. The nitrocellulose membranes were immunoblotted using one of four primary polyclonal antibodies raised against: cyanobacterial (Se PCC6301) Rubisco; tobacco Rubisco; the small subunit of tobacco Rubisco; and CcmM from Se PCC7942. The primary polyclonal antibody to detect CcmM35 was generated in rabbit with His-tagged CmM35 protein purified from E.coli (Cambridge Research Biochemicals, UK) and used at a dilution of 1:500 in the immunoblots and from 1:500 to 1:2,000 for immunogold labelling, and was highly specific for CcmM (Fig. 2a). The secondary antibodies were visualized by means of a secondary goat anti-rabbit peroxidase-conjugated antibody (Sigma). The red and relative amount of Synechococcus CcmM35 in the same leaves were determined using immunoblots with antibodies against CcmM and cyanobacterial Rubisco. The amounts of Rubisco and CcmM35 present in crude leaf homogenates were estimated by comparison with authentic protein standards (purified CcmM35 and cyanobacterial Rubisco). Amounts of CcmM35 and cyanobacterial Rubisco (μmol m−2) were the mean ± standard deviation for duplicate determinations. The band intensities were obtained using Image software (NIH, USA) and the standard curves using Microsoft Excel.

Purification of cyanobacterial Rubisco and CcmM35 proteins. Synechococcus Rubisco was expressed in E. coli BL21 (DE3) cells using the vector pA929 as previously described which was expressed by autoinduction in buffer containing 0.1 M Bicine-NaOH pH 8.0, 20 mM MgCl2, 50 mM NaHCO3, 100 mM PMSF and bacterial protease inhibitor cocktail (Sigma). All steps in the purification were conducted at 0°C. The harvested cells were sonicated and cell debris removed by centrifugation (17,400g, 20 min, 4°C). PEG–4000 and MgCl2 were added to the supernatant, giving final concentrations of 20% (w/v) and 20 mM, respectively. After 30 min at 0°C, the precipitated Rubisco was sedimented by centrifugation (17,400g, 20 min, 4°C) and the pellet resuspended in 25 mM triethanolamine (pH 7.8, HCI), 5 mM MgCl2, 0.5 mM EDTA, 1 mM e-aminocaproic acid, 1 mM benzamidine, 12.5% (v/v) glycerol, 2 mM DTT and 5 mM NaHCO3. This resuspended fraction was subjected to anion-exchange chromatography using a 5 ml HiTrap Q column (GE-Healthcare) pre-equilibrated with the same buffer. Rubisco was eluted with a 0–600 mM NaCl gradient in the same buffer. Fractions containing the most Rubisco activity (as judged by RuBP-dependent 14CO2 assimilation) were further purified and desalted by size-exclusion chromatography using a 20 × 2.6 cm diameter
column of Sephacryl S-200 HR (GE-Healthcare) pre-equilibrated and developed with (50 mM Bicine-NaOH pH 8, 20 mM MgCl₂, 0.2 mM EDTA, 2 mM DTT). The resulting protein peak was concentrated by ultrafiltration using 20 ml capacity /150 kDa cut-off centrifugal concentrations (Thermo Pierce). The PCR-amplified ccmM35 gene from Se PCC7942 was cloned into pCR8/GW/TOPO TA vector (Life Technologies) and subsequently transferred to the Gateway pDEST17 E. coli expression vector (Life Technologies), which utilizes the T7 promoter to express the inserted gene and incorporates a 6×His tag at the N terminus of the translated protein. The expression vector was transformed into Rosetta (DE3) competent cells, and the protein expression was induced with 0.5 mM IPTG at OD₆₀₀nm of 0.5. The cells in 0.5 litre LB culture were harvested after 4 h of growth at 37°C and 250 r.p.m. The cells were resuspended in about 10 ml of ice cold 50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole at pH 8.0 and broken with sonication. The cell debris were removed by centrifugation and the supernatant was mixed with 2 ml of Ni-NTA resin, which was then washed with 15 ml of the cell suspension buffer in a gravity-flow column and the bound protein was eluted with the buffer containing 200 mM imidazole. The purity of CcmM35 was assessed with SDS–PAGE, and its expression was determined by the Bradford method.

For the immuno-gold labelling, gold grids carrying ultrathin sections (60–90 nm) of leaf tissue embedded in HM20 were treated using different rabbit primary antibodies against: cyanobacterial Rubisco from Se PCC6301; tobacco Rubisco; and CcmM35 (produced by Cambridge Research Biochemicals). A secondary goat anti-rabbit antibody conjugated with 10 nm gold particles (Abcam, UK) was used for the labelling.

Images were obtained using a transmission electron microscope (Jeol 2011 F) operating at 200 kV, equipped with a Gatan Ultrascan CCD camera and a Gatan Dual Vision CCD camera.

Plant material and growing conditions. Both transgenic and wild-type Nicotiana tabacum var. Samsun NN were grown in the same controlled environment chamber with 16 h of fluorescent light (43%) and 8 h dark, at 24°C during the day and 22°C during the night. The relative humidity was 70% during the day and 80% during the night. The atmospheric CO₂ concentration was kept constant at 9,000 p.p.m. (air containing 0.9% v/v CO₂).

Quantification of protein, Rubisco, and chlorophyll. Total soluble protein in the leaf homogenates was determined by the standard Bradford method. Rubisco active site concentration in the crude homogenate was determined using the [¹⁴C]-CABP binding assay or by quantifying LSU band intensity by immunoblotting. Each approach gave very similar results. Chlorophyll concentration was determined spectrophotometrically using unfractionated leaf homogenates.

Carboxylase activity measurements. Leaf discs (1 cm²) were cut and promptly homogenized using an ice-cold pestle and mortar, in the presence of 500 µl of ice-cold extraction buffer (50 mM EPSS-NaOH pH 8.0; 10 mM MgCl₂; 1 mM EDTA; 1 mM EGTA; 50 mM 2-mercaptoethanol; 20 mM DTT; 20 mM NaHCO₃; 2 mM NaHPO₄; Sigma plant protease inhibitor cocktail (diluted 1:100); 1 mM PMSF; 2 mM benzamidine; 5 mM e-aminocaproic acid). Rubisco carboxylase activity was measured immediately in 300 µl of assay buffer containing 100 mM EPSS-NaOH pH 8.0, 20 mM MgCl₂, 0.8 mM RuBP and 10 mM, 20 mM or 50 mM NaH¹⁴CO₃ (18.5 kBq per mol) at room temperature (22°C). The assay was initiated by the addition of 20 µl of the leaf homogenate, and was quenched after 2, 4, 6 or 10 min, by the addition of 100 µl of 10 M formic acid. The samples were oven dried and the acid stable ¹⁴C determined by liquid scintillation counting, following residue rehydration (400 µl H₂O) and the addition of 3.6 ml liquid scintillation cocktail (Ultima Gold, PerkinElmer, UK).

For Rubisco inhibition using the tight binding Rubisco inhibitor, 2-carboxy-D-arabinitol-1,5-bisphosphate (CABP), leaf homogenates were incubated on ice for 15 min in the presence of 50 µM CABP. Residual carboxylase activity (if any) was then measured as described above.

28. Gray, B. N., Yang, H., Ahner, B. A. & Hanson, M. R. An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. Plant Mol. Biol. 76, 345–355 (2011).
29. Bainbridge, G. et al. Engineering Rubisco to change its catalytic properties. J. Exp. Bot. 46, 1269–1276 (1995).
30. Wintermans, J. F. & de Mots, A. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim. Biophys. Acta 109, 448–453 (1965).
Extended Data Figure 1 | Rubisco and CcmM35 content of SeLSM35 tobacco leaves. The stated concentrations of purified Se Rubisco (a) and CcmM35 (b) proteins were used as standards. a, Immunoblot using an antibody against cyanobacterial LSU (top) and the standard curve used to estimate the amount of cyanobacterial Rubisco in samples S1–S3 extracted from SeLSM35 tobacco leaves (bottom). b, Immunoblot using an antibody against CcmM (top) and the standard curve used to estimate the amount of CcmM35 in samples S4–S6 extracted from SeLSM35 tobacco leaves (bottom). The band intensities in the two standard curves were obtained with ImageJ software and the standard curves with Microsoft Excel. c, The absolute and relative amounts (mean ± standard deviation) of CcmM35 and cyanobacterial Rubisco in SeLSM35 tobacco line from two separate measurements. Each Rubisco holoenzyme is assumed to be composed of 8 LSU and an unknown quantity of SSU.
Extended Data Figure 2 | Electron micrographs of ultrathin sections of leaf mesophyll cells from the chloroplast transformant SelSM35. Large compartments containing cyanobacterial Rubisco and CcmM35 in the chloroplast stroma are indicated by black arrows. Leaf tissues were prepared by high pressure freeze fixation (HPF) in combination with immunogold labelling using an antibody against CcmM. A secondary antibody conjugated with 10-nm gold particles was used for the labelling. Scale bars, 500 nm.
Extended Data Figure 3 | Rubisco-specific $^{14}$CO$_2$ fixation by crude leaf homogenates from tobacco lines expressing cyanobacterial Rubisco (SeLSX and SeLSM35) and wild-type tobacco (WT). a, Carboxylase activity assayed with (+) and without (−) RuBP. b, Carboxylase activity assayed with (+) and without (−) the inhibitor CABP. The rates of carboxylase activity (mols fixed per mol act sites per s) are the means ± standard deviation derived from the 2, 4 and 10 min data obtained in assays at 125 μM CO$_2$ (corresponding to 10 mM NaH$^{14}$CO$_3$, at pH 8.0).
Extended Data Table 1 | Rubisco, total soluble protein and chlorophyll content of the wild-type and transformed homoplastomic tobacco leaves of similar size, development and canopy position

| Sample    | Rubisco (g/m²) | Total soluble protein (g/m²) | Chlorophyll a & b (g/m²) |
|-----------|----------------|-----------------------------|--------------------------|
| Wild-type | 0.91 ± 0.09    | 3.74 ± 0.06                 | 0.32 ± 0.02              |
| SeLSX     | 0.11 ± 0.01    | 1.85 ± 0.02                 | 0.21 ± 0.01              |
| SeLSM35   | 0.16 ± 0.01    | 1.46 ± 0.09                 | 0.18 ± 0.01              |

The wild-type plants were grown in air and the transformants in air supplemented with 0.9% (v/v) CO₂. Fresh 4 cm² leaf samples were homogenized in (1 ml) ice-cold extraction buffer. The crude homogenate was used for determination of chlorophyll and Rubisco content. The total soluble protein was determined by the Bradford method following extract clarification (13,200g, 5 min, 4°C). Values are means ± standard deviation from 3 different leaves per sample.
Extended Data Table 2 | Oligonucleotides used in the construction of chloroplast transformation vectors, DNA blot analyses of the tobacco chloroplast rbcL locus and RT–PCR analyses of the tobacco chloroplast rbcL gene and transgenes introduced in the transplastomic lines

| Primers     | Nucleotide sequences                                      |
|-------------|-----------------------------------------------------------|
| F1OlrbcLfor | CATGAGTTGTAGGAGAGGATTTATGCTTAAAACCCAAAGTGCTG            |
| 4EREbcLrev  | ATACCGCGTTCTGAGGCGACGCCCGCCGGCGCGGGTTAAAAATCTTACCTTGCTCAA |
| F1for       | GCCCCCACTACTGACCTGAACTACC                                |
| F1for2      | AGTCTGGGCCCCAATAATGATT                                  |
| F1rev       | AATTCCTTACCACAATCCTG                                    |
| F2for       | ATGGCTGCAAGATGAGGTCAGACCATATGAAACAGATACATTAGCAGATAAATTAG |
| F2rev       | TCCAACGGGTGTGAATAATACCAACATTTAGCGACACTAGAATTGG          |
| SM0for      | CTATGGCTCTCTTTTTTCTGCAG                                 |
| SM0rev      | ATGGCTGCAAGATGAGGTCAGACCATATGAAACAGATACATTAGCAGATAAATTAG |
| TrbcLfor    | AGATCGGCGCGGAAAGCTAGACATTAGCAGATAAATTAG                 |
| TrbcLrev    | AGATCGGCGCGGAAAGCTAGACATTAGCAGATAAATTAG                 |
| IEESDrev    | GTATATCTCTTCTTGTAGATCTTGTGTATACCATTTCCGTTGTAATAATGTGTC  |
| IEESD18rev  | CCCATATGTATATCTCTTCTCTCCCATATGATATATCTCTCTTCTGAGATCTGTGAC |
| SD18rev2    | CATGGGTATATCTCTTCTCTCCCATATGATATATCTCTCTTCTCCCATATGTA   |
| TpetDAfor   | AGATGGGCCCCACGCCGTCGCACGCGTTTCAATTTATATCAATATATGAAATACAG |
| TpetDArev   | CCCATCCGTTGTATAAATATGATCTTAACCCTATTTTAATTTAATTTAATTTAG  |
| TpbaAAtfor  | AGATCGGCGCGGACGCCGCTGCACTTGTTAGCTGTTAGCTGAGATCTGAC     |
| TpbaAArev   | CCATTCCGTTGTAATAATAGCTTATATATGATACCTCTTAATAATTTGCTC     |
| Trps16AAtfor| AGATCGGCGCGGACGCCGCTGCACTTGTTAGCTGTTAGCTGAGATCTGAC     |
| Trps16AArev | CCATTCCGTTGTAATAATAGCTTATATATGATACCTCTTAATAATTTGCTC     |
| rbc5for     | GTACAAGCTCTCAGAAAGGAGATATACCTCGATGATGAAATACCGAAGAGAG   |
| rbc5rev     | AGATCGGCGCGGACGCCGCTGCACTTGTTAGCTGTTAGCTGAGATCTGAC     |
| rbcXfor     | GTACAAGCTCTCAGAAAGGAGATATACCTCGATGATGAAATACCGAAGAGAG   |
| rbcXrev:    | AGATCGGCGCGGACGCCGCTGCACTTGTTAGCTGTTAGCTGAGATCTGAC     |
| M3for       | GTCAACAGATCTCTCCGAAGGAGATATACCTCGATGAGCCTTTAAACCGCAAGG |
| M3rev       | AGATCGGCGCGGACGCCGCTGCACTTGTTAGCTGTTAGCTGAGATCTGAC     |
| addAfor     | ATGGCTGCTGAAGCGGTATTAG                                  |
| addArev:    | TTTTGGCACAATCTACCTATGTGATCTCG                          |
| SBpr3for    | ACCATGCAATTTGGACGGATCATTAG                              |
| SBpr3rev    | TGATATCTCTTCTTGTATATATAGCAGACACTAA                     |
| N-rbc5for   | AGTCAACGAAACGAGAAGACTAA                                 |
| N-rbc5rev   | TTACTATCCAAAGCTCCACTG                                  |

The restriction sites are underlined.