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Citation for published version:
Atkinson, N, Laeitao, N, Orr, DJ, Meyer, MT, Carmo-Silva, E, Griffiths, H, Smith, AM & McCormick, A 2017, ‘Rubisco small subunits from the unicellular green alga Chlamydomonas complement Rubisco-deficient mutants of Arabidopsis’ New Phytologist. DOI: 10.1111/nph.14414

Digital Object Identifier (DOI):
10.1111/nph.14414

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
New Phytologist

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Rubisco small subunits from the unicellular green alga *Chlamydomonas* complement Rubisco-deficient mutants of Arabidopsis

Nicky Atkinson¹, Nuno Leitão², Douglas J. Orr³, Moritz T. Meyer⁴, Elizabete Carmo-Silva³, Howard Griffiths⁴, Alison M. Smith² and Alistair J. McCormick¹,²

¹SynthSys & Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Edinburgh, EH9 3BF, UK; ²Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK; ³Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK; ⁴Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, UK

Author for correspondence:
Alistair J. McCormick
Tel: +44 (0)1316505316
Email: alistair.mccormick@ed.ac.uk

Received: 31 July 2016
Accepted: 24 November 2016

New Phytologist (2017) doi: 10.1111/nph.14414

Key words: Arabidopsis thaliana, carbon concentrating mechanism (CCM), Chlamydomonas reinhardtii, chloroplast, photosynthesis, pyrenoid, Rubisco, tobacco.

Summary

- Introducing components of algal carbon concentrating mechanisms (CCMs) into higher plant chloroplasts could increasephotosynthetic productivity. A key component is the Rubisco-containing pyrenoid that is needed to minimise CO₂ retro-diffusion for CCM operating efficiency.
- Rubisco in Arabidopsis was re-engineered to incorporate sequence elements that are thought to be essential for recruitment of Rubisco to the pyrenoid, namely the algal Rubisco small subunit (SSU, encoded by rbcS) or only the surface-exposed algal SSU α-helices.
- Leaves of Arabidopsis rbcS mutants expressing ‘pyrenoid-competent’ chimeric Arabidopsis SSUs containing the SSU α-helices from *Chlamydomonas reinhardtii* can form hybrid Rubisco complexes with catalytic properties similar to those of native Rubisco, suggesting that the α-helices are catalytically neutral.
- The growth and photosynthetic performance of complemented Arabidopsis rbcS mutants producing near wild-type levels of the hybrid Rubisco were similar to those of wild-type controls. Arabidopsis rbcS mutants expressing a *Chlamydomonas* SSU differed from wild-type plants with respect to Rubisco catalysis, photosynthesis and growth. This confirms a role for the SSU in influencing Rubisco catalytic properties.

Introduction

Rubisco (EC 4.1.1.39) catalyses net CO₂ assimilation in all photosynthetic organisms. Despite this central role, Rubisco is an inefficient enzyme that limits photosynthetic productivity, particularly in plants with the C₃ photosynthetic pathway. Rubisco has a slow carboxylation rate (kcat) and a relatively low affinity for CO₂, with a Kₘ for CO₂ at ambient O₂ (KₘCO₂) close to the CO₂ concentration in a C₃ leaf mesophyll cell (Galmés et al., 2014).

Rubisco also catalyses d-ribulose-1,5-bisphosphate (RuBP) oxygenation, resulting in the energetically expensive photorespiratory pathway where previously fixed CO₂ is lost (Sharkey, 1988). These features necessitate a large investment in the enzyme (up to 50% of leaf soluble protein) to support adequate rates of CO₂ assimilation (Parry et al., 2013). Increasing the operating efficiency of Rubisco and reducing photorespiration are important approaches for improving yields in C₃ crop plants (Whitney et al., 2011; Parry et al., 2013; Carmo-Silva et al., 2015; Long et al., 2015; Ort et al., 2015).

The operating efficiency of Rubisco in C₃ plants could be enhanced by elevating the CO₂ concentration in the chloroplast by means of carbon concentrating mechanisms (CCMs). Possibilities include using components of biochemical CCMs (as in C₄ and CAM photosynthesis) and/or the biophysical inorganic carbon accumulation mechanisms from cyanobacteria and eukaryotic algae (von Caemmerer et al., 2012; Price et al., 2013; Meyer et al., 2016). In algal CCMs, bicarbonate transporters and localisation of Rubisco and carbonic anhydrase within the chloroplast, and in most instances within the pyrenoid (a microcompartment commonly present in chloroplasts of microalgae), result in saturating CO₂ concentrations around Rubisco (Morita et al., 1998; Giordano et al., 2005; Wang et al., 2015). Modelling approaches suggest that algal CCMs with a pyrenoid are likely to be more effective in maintaining elevated CO₂ concentrations around Rubisco than those without (Badger et al., 1998). Modelling also reveals that the confinement of Rubisco to a microcompartment would be required for effective operation of a biophysical CCM in a higher plant (Price et al., 2013; McGrath & Long, 2014). Recent work has shown that algal CCM components, including carbonic anhydrases and bicarbonate transporters, can be expressed in...
appropriate subcellular locations in angiosperms (Atkinson et al., 2016). To achieve a functional algal CCM in an angiosperm it will also be necessary to introduce a Rubisco capable of assembling into a pyrenoid-like structure.

Pyrenoid formation in the model green alga *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*) depends on the amino acid sequences of the small subunit of Rubisco (SSU, encoded by the *rbcS* nuclear gene family) and, more specifically, on two surface-exposed α-helices, which differ markedly between *Chlamydomonas* and higher plants (Meyer et al., 2012). In *Chlamydomonas*, *rbcS* deletion mutants can be rescued with a SSU variant from angiosperms (Arabidopsis, spinach or sunflower) without compromising in vitro Rubisco catalysis (Genkov et al., 2010). However, these hybrid Rubisco no longer assembled into a pyrenoid. Accordingly, lines expressing in vitro SSU variant from angiosperms (Arabidopsis, spinach or sunflower) and higher plants (Meyer et al., 2010). However, these hybrid Rubisco no longer assembled into a pyrenoid. Accordingly, lines expressing the hybrid Rubisco lacked a functional CCM, resulting in growth only at high CO₂. Pyrenoid formation and CCM function were restored by expression of a chimeric SSU, where a higher plant SSU was modified with the algal SSU α-helices (Meyer et al., 2012). Thus, assembling a pyrenoid-like microcompartment in chloroplasts would probably require the incorporation of *Chlamydomonas*-like α-helical sequence into the native angiosperm SSU, in addition to other proteins involved in pyrenoid formation such as the Rubisco-associated protein EPYC1 (Mackinder et al., 2016).

Here we examine how the incorporation of SSUs with α-helices from *Chlamydomonas* SSUs influences the biogenesis and catalysis of Rubisco in Arabidopsis leaves. The Rubisco large subunits (LSUs, encoded by *rbcL*) harbour the catalytic sites and are highly conserved between algae and angiosperms (Arabidopsis and *Chlamydomonas* LSUs are 88% identical at the level of amino acid sequences). By contrast, the SSU isoforms of Arabidopsis and *Chlamydomonas* are only 40–43% identical, even though their tertiary structures are extremely similar, including the positions of the α-helices (Spreitzer, 2003). Although located on the distal ends of the octameric LSU core of Rubisco and distant from the catalytic sites, the amino acid sequence of the SSUs can affect the catalytic properties of the enzyme (Genkov & Spreitzer, 2009).

In Arabidopsis the SSUs are encoded by four genes, *rbcS1A* on chromosome 1 accounts for ~50% of SSU transcript, the remainder being contributed by the *rbcS1B*, *rbcS2B* and *rbcS3B* genes located contiguously on chromosome 5 (Yoon et al., 2001). An Arabidopsis double mutant lacking expression of *rbcS1A* and with strongly reduced expression of *rbcS3B* (the *l*a*b* mutant) has a low Rubisco content (30% of wild-type plants) and slow growth (Izumi et al., 2012). In this study the *l*a*b* mutant was complemented with either the Arabidopsis *rbcS1A* (control), an *rbcS1A* variant encoding the *Chlamydomonas* α-helix sequences or the native *rbcS2* gene from *Chlamydomonas*. We compared the Rubisco content, catalytic properties, leaf photosynthesis and growth of multiple lines for each genotype produced. Our results show that the *l*a*b* mutant is a valuable background for attempts to assemble an algal CCM in an angiosperm chloroplast, and for wider examination of the contribution made by SSU genetic diversity to Rubisco properties.

### Materials and Methods

#### Plant material and growth conditions

*Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh. Col-0) seeds were sown on compost, stratified for 3 d at 4°C and grown at 20°C, ambient CO₂, 70% relative humidity and 150 μmol photons m⁻² s⁻¹ in 12:12 h light:dark. For comparisons of different genotypes, plants were grown from seeds of the same age and storage history, harvested from plants grown in the same environmental conditions. Tobacco (*Nicotiana benthamiana* L.) was cultivated in a glasshouse (minimum 20°C, natural light supplemented to give light periods of at least 12 h). An Arabidopsis *rbc3a1a rbc2a2b* mutant (double mutant *l*a*b*) was generated by crossing T-DNA insertion lines GABI_608F01 (At1g67090) and GABI_32A403 (At5g38420). The *l*a*b* mutant (GABI_608F01 (At1g67090); SALK_117835 (At5g38410)) was provided by Hiroyuki Ishida, Department of Applied Plant Science, Tohoku University, Japan.

#### DNA and RNA extraction, PCR and RT-qPCR

Genomic DNA was extracted from rosettes according to Li & Chory (1998). PCRs were performed as in McCormick & Kruger (2015) using gene-specific primers (Supporting Information Table S1). Insertion copy numbers were obtained by quantification of 35S promoter copies (performed by iDNA Genetics, www.idnagenetics.com). mRNA was isolated from the sixth and seventh leaves of 28-d-old rosettes and complementary DNA was synthesized with oligo(dT) primers. Reverse transcription quantitative PCR (RT-qPCR) was carried out as in Andriotis et al. (2010). Primers to test for expression of SSU genes were designed to amplify the unique 3′ region of the transcripts (Table S1). Amplification efficiency was determined with a calibration curve for each primer set. Three reference genes (At4g05320 (UBQ10), At1g13320 (PP2A) and At4g26410 (RHLP1) (Czechowski et al., 2005)) were used for normalisation. Calculations of relative expression ratios were performed according to Pfaffl (2001).

#### Expression of *rbcS* genes in *N. benthamiana* and Arabidopsis *l*a*b* mutants

The α-helices of *rbcS1A* (At1g67090) were replaced with those from the *Chlamydomonas* *rbcS* family (Fig. 1) using overlapping PCR with Phusion® High-Fidelity DNA polymerase (as per the manufacturer’s instructions; New England BioLabs). The promoter region (2 kb) upstream of *rbcS1A* was fused to the complete cDNA sequences of native or modified SSUs. The *rbcS1A* chloroplast transit peptide (TP) sequence was fused to the mature *Chlamydomonas* *rbcS2* (Cre02.g120150) (Goldschmidt-Clermont & Rahire, 1986) CDS before promoter addition. Promoter–cDNA fusions were cloned into Gateway Entry vectors (pCR®8GW/TOPO®TA Cloning® Kit; Thermo Fisher Scientific), then into the binary destination vector pGBW4 (Nakagawa et al., 2009) or pB7WG (Karimi et al., 2002) (Notes S1). Stop codons were removed to allow in-frame C-terminal
fusion to a sequence encoding green fluorescent protein (GFP) in pGWB4. Binary vectors were transformed into *Agrobacterium tumefaciens* (AGL1) for transient gene expression in tobacco (Schob et al., 1997) or stable insertion in Arabidopsis plants by floral dipping (Clough & Bent, 1998). Homozygous insertion lines were identified in the T3 generation by seedling segregation ratios on Murashige & Skoog (MS) medium (half-strength) plates containing phosphinothricin (BASTA®, final concentration 10 ng ml⁻¹) as a selectable marker. Lines used for subsequent analysis were checked for the presence of T-DNA insertions at the *rbcS1A* and *rbcS3B* loci.

Protein quantification and Rubisco content

For determination of leaf protein and Rubisco contents on an area basis, soluble protein was extracted from 2 cm² of snap frozen leaf material from 32-d-old plants (sixth and seventh leaf) in 500 μl of 50 mM Tricine-NaOH (pH 8.0), 10 mM EDTA, 1% (w/v) PVP 40, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 10 μM leupeptin. Following centrifugation at 2380 g for 5 min at 4°C, soluble protein was quantified using a Bradford-based assay (Bio-Rad) against BSA standards (Thermo Fisher Scientific). Rubisco content was determined in an aliquot of the extract via ¹⁴C-CABP (carboxy-D-arabinitol 1,5-bisphosphate) binding following incubation with 10 mM NaHCO₃, 20 mM MgCl₂ and the addition of 3 μl 12 mM ¹⁴C-CABP (37 MBq mmol⁻¹) for 25 min at room temperature (Whitney et al., 1999).

Subunit ratios were estimated by immunoblotting. Extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–12% (w/v) polyacrylamide gel (Bolt® Bis-Tris Plus Gel; Thermo Fisher Scientific), transferred to polyvinylidene fluoride (PVDF) membrane then probed with rabbit serum raised against wheat Rubisco at 1 : 10 000 dilution (Howe et al., 1982) followed by Li-Cor IRDye® 800CW goat anti-rabbit IgG (Li-Cor Inc.) at 1 : 10 000 dilution, then viewed on an Li-Cor Odyssey CLx Imager. The contributions of LSU and SSUs were estimated from a five-point standard curve of a wild-type sample of known Rubisco content (0.1–2.4 μg Rubisco).

Fig. 1 Gene expression cassettes for native and heterologous Rubisco small subunits. *rbcS1A* from *Arabidopsis thaliana* (1AAt) (a), *rbcS1A* with α-helices from the *Chlamydomonas reinhardtii rbcS* family (1AAtMOD) (b), and mature *rbcS2* from *Chlamydomonas* (S2Cr) (c) were expressed using the *rbcS1A* promoter (not drawn to scale) and 35S terminator. For S2Cr, the chloroplast transit peptide (TP) of *Chlamydomonas* rbcS2 (45 amino acids) was replaced with the rbcS1A TP (55 amino acids) from Arabidopsis to facilitate localisation of the mature rbcS2 to the chloroplast. (d) Alignments of the mature SSU peptides generated in this study. Numbering is relative to the *Chlamydomonas* rbcS2 sequence. Residues that comprise the two α-helices A and B are underlined, and those different from rbcS1A are in bold. For comparison with 1AAtMOD, the modified spinach SSU generated by Meyer et al. (2012) is included.

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Rubisco catalytic properties

Whole 45-d-old rosettes (20–30 cm²) were rapidly frozen in liquid nitrogen (N₂) and Rubisco was extracted as described by Prins et al. (2016), then activated for 45 min on ice before assays were conducted at 25°C. Catalytic properties of Rubisco from wild-type and transgenic lines were determined from 14CO₂ consumption, essentially as described by Prins et al. (2016) with alterations as per Orr et al. (2016), using 40 μl of extract. Six CO₂ concentrations were used with O₂ concentrations of 0 and 21%.

Rubisco specificity factor was determined on Rubisco purified from each genotype from c. 300 cm² rosette tissue using the method described by Prins et al. (2016), with the omission of the final Sephacryl S-200 step, which was found to be unnecessary for obtaining a clean extract (Orr et al., 2016). Rubisco CO₂/O₂ specificity (S_C/O) was determined using the method of Parry et al. (1989). At least 10 measurements were made on the Rubisco purified from each genotype. Values were normalised based on measurements made in the same experiment on purified wheat (Triticum aestivum) Rubisco, which has an established S_C/O of 100 (Parry et al., 1989).

Chlorophyll quantification

Leaf discs (c. 10 mg fresh weight) were frozen in liquid N₂, powdered, and then mixed with 100 volumes of ice-cold 80% (v/v) acetone, 10 mM Tris–HCl. Following centrifugation at 17 200 g for 10 min, chlorophyll was quantified according to Porra et al. (1989).

Measurement of photosynthetic parameters

Gas exchange and chlorophyll fluorescence were determined using a Li-Cor LI-6400 portable infra-red gas analyser with a 6400-40 leaf chamber on either the sixth or the seventh leaf of 35- to 45-d-old nonflowering rosettes grown in large pots to generate leaf area sufficient for gas exchange measurements (Flexas et al., 2007). For all gas exchange experiments, leaf temperature and chamber relative humidity were 20°C and c. 70%, respectively. Gas exchange data were corrected for CO₂ diffusion from the measuring chamber as in Bellasio et al. (2016). Light response curves for net photosynthetic CO₂ assimilation (A) were generated at ambient CO₂ (400 μmol mol⁻¹). A nonrectangular hyperbola was fitted to the light response (Marshall & Biscoe, 1980; Thornley, 1998). The response of A to varying substomatal CO₂ concentration (C) was measured at 1500 μmol photons m⁻² s⁻¹. To calculate the maximum rate of Rubisco carboxylation (V_c,max) and the maximum photosynthetic electron transport rate (J_max), the A/C data were fitted to the C₃ photosynthesis model as in Ether & Livingston (2004) using the catalytic parameters K_c and affinity for O₂ (K_o) values for wild-type Arabidopsis Rubisco at 20°C as reported in Walker et al. (2013). For estimates of the ratio of Rubisco oxygenase to carboxylase activity (V_o/V_c), leaves were measured under photorespiratory (ambient oxygen (O₂), 21% (v/v)) or low-photorespiratory (low O₂, 2% (v/v)) conditions (Bellasio et al., 2014).

Maximum quantum yield of photosystem II (PSII) (F_v/F_m) was measured using a Hansatech Handy PEA continuous excitation chlorophyll fluorimeter (Hansatech Instruments) (Maxwell & Johnson, 2000). Nonphotochemical quenching (NPQ) analyses were performed using a Hansatech FMS1 pulse-modulated chlorophyll fluorimeter. Rapid light response curves were generated by measuring the fluorescence response to a saturating pulse (applied every 30 s) under increasing levels of actinic light (0–1500 μmol photons m⁻² s⁻¹). Quenching parameters, including NPQ₁ and NPQ₆, were derived as in Griffiths & Maxwell (1999).

Confocal laser scanning microscopy

Leaves were imaged with a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems) as in Atkinson et al. (2016).

Results

The 1a3b mutant of Arabidopsis provided a suitable genotype for examining the influence of heterologous SSUs on leaf photosynthesis and growth. Some aspects of the 1a3b mutant phenotype may reflect loss of distinct Rubisco isoforms (i.e. forms with different SSU compositions), as well as loss of total Rubisco activity. As a first step to evaluate this possibility, a second mutant, lacking expression of rbcS1A and a different minor SSU, rbcS2B (the 1a2b mutant) was included in some of the analyses. Quantification of T-DNA copy numbers indicated that neither double mutant contained T-DNA insertions other than those at their respective rbcS loci.

Design and targeting of native and heterologous SSUs

Binary vectors were generated to express either the full-length native Arabidopsis rbcS1A (1Aₐₐ), the mature Chlamydomonas rbcS2 N-terminally fused to the chloroplast TP sequence from Arabidopsis rbcS1A (S₂Cₐ), or the full-length Arabidopsis rbcS1A modified to contain α-helices matching the amino acid sequence as those of the Chlamydomonas SSU family (1Aₐ₌,MOD) (Fig. 1; Notes S1). Chlamydomonas and Arabidopsis SSU α-helices have the same number of amino acids, but differ in terms of chemical composition. Expression of the introduced proteins was driven by the promoter of Arabidopsis rbcS1A, which has the highest expression level of the Arabidopsis rbcS genes (Izumi et al., 2012).

To check the subcellular locations of introduced SSUs, they were initially generated as C-terminal fusions to GFP and transiently expressed in leaves of N. benthamiana. Fluorescence microscopy revealed that all three fusion proteins were located in the chloroplast stroma (Fig. S1). Untagged SSUs were then stably expressed in the Arabidopsis 1a3b mutant.
Expression levels, leaf protein and Rubisco content of native and heterologous SSU isoforms

In wild-type plants, rbcS1A transcripts were the most abundant (43% of the rbcS pool), followed by rbcS3B (28%), rbcS2B (21%) and rbcS1B (8%) (Fig. 2; Table S2). The 1a3b mutant had no detectable transcript for rbcS1A and much reduced levels of transcript for rbcS3B (c. 10% of wild-type levels). Both rbcS1A and rbcS2B transcripts were below the level of detection in the 1a2b mutant. In the 1a3b mutant, transcript levels for the two undisrupted rbcS genes, rbcS1B and rbcS2B, were 50% and 170% of those in wild-type plants, respectively. In the 1a2b mutant, rbcS1B and rbcS3B transcript levels were 120% and 140% of those in wild-type plants, respectively. For both mutants, transcript levels for rbcL (ATCG00490) and for the overall rbcS pool were 50% of those in wild-type plants.

For each of the three transgenic genotypes expressing native or heterologous SSUs in the 1a3b mutant background, at least six independent lines segregated in the T2 generation. Transgenic plants were screened for faster growth rates and maximum quantum yield of PSII (measured by dark-adapted leaf fluorescence; $F_v/F_m$) compared to the 1a3b mutant (Fig. S2). For further analysis, homozygous T3 lines for each genotype were selected from the three best-performing T2 segregating lines.

For each line of each transgenic genotype, transcript levels for the inserted transgene were comparable to those of the native rbcS1A gene in wild-type plants (Fig. 2; Table S2). Levels of transcript of the undisrupted native Rubisco genes were altered in these lines relative to wild-type plants. For rbcL, transcript levels were higher in transgenic than in 1a3b mutant plants, and at least one independent line for each construct they were as high as in wild-type plants. As in 1a3b mutants, transcript levels for rbcS2B in transgenic plants were generally higher than those in wild-type plants (Fig. 2; Table S2).

The leaf Rubisco content in the 1a3b and 1a2b mutants was reduced by 70 and 50%, respectively, relative to wild-type plants (Fig. 3a). Total soluble protein content in leaves of the mutants was also c. 60% of wild-type values in both cases. This reduction was larger than could be accounted for by the reduction in Rubisco content alone (Fig. 3b; Table S3).

Complementation of the 1a3b mutant restored total Rubisco to 75% of wild-type levels for 1AAt and 1AAtMOD lines, and to 65% of wild-type levels for S2Cr lines. Immunoblotting revealed that the heterologous SSUs 1AAtMOD and S2Cr had different mobilities on SDS-PAGE gels from the native SSUs (Fig. 3c).

This enabled quantification of the relative contributions of the LSU, the native SSUs and the heterologous SSUs to total Rubisco content (Fig. 3a). There were no significant differences in the ratio of LSU to SSU protein between any of the lines tested (Table S3). The 1AAtMOD and S2Cr transgenic lines retained the same amount of native SSU (i.e. products of the rbcS1B and rbcS2B genes) as the 1a3b mutant. Heterologous SSU levels were 2.4-fold higher than native SSU levels in 1AAtMOD. By contrast, heterologous SSU levels were 1.4-fold lower than native SSU levels in S2Cr lines.

Rubisco activity in mutant and transgenic plants

The in vitro catalytic properties of Rubisco from wild-type plants (Table 1) were in good agreement with those of Galmés et al. (2014). The catalytic properties of Rubisco from 1a3b and 1a2b mutants were comparable to values for Rubisco from wild-type plants. Rubisco from 1AAt lines had the same catalytic properties as Rubisco from wild-type plants. This was also true for 1AAtMOD lines, despite the modification to the Rubisco SSU in these plants. However, $k_{cat}$ and $S_{C/O}$ values were significantly lower for Rubisco from S2Cr lines than for Rubisco from wild-type plants.

Growth phenotypes

Growth of transgenic lines was compared with that of (1) wild-type plants, (2) the parental 1a3b mutant and (3) representative nontransgenic 1a3b mutant lines selected as out-segregants from the T2 populations (Fig. 4). Fresh and dry weights of the out-segregant mutant lines were the same as those of the parental 1a3b mutant at 28 d (Fig. 4c; Table S4). Out-segregant lines had lower rates of rosette expansion than the parental 1a3b mutant (Fig. 4b), but this did not affect interpretation of the effects of the transgenes on growth.

As reported previously, 1a3b mutants had very low growth rates (Izumi et al., 2012). All three transgenic genotypes had greater rates of rosette expansion than 1a3b lines, with 1AAt and 1AAtMOD having higher expansion rates than S2Cr (Fig. 4b). The dry weight of 1AAt rosettes at 28 d was on average 84% of that of wild-type plants, and was not significantly different from the wild-type for two of the three lines. For 1AAtMOD and S2Cr lines, dry weight was on average 75 and 56%, respectively, of that of wild-type plants. There was no significant difference in the ratio of dry weight to fresh weight between wild-type plants and transgenic lines. All three transgenic genotypes had higher leaf area to weight ratios (rosette area per unit fresh or dry weight) than 1a3b mutants, and were not significantly different in this respect from wild-type plants (Table S4).

Fig. 2 Transcript abundances of the Rubisco gene family in rbcS mutants and transgenic lines of Arabidopsis thaliana. Abundances of rbcS1A (AT1g67090), rbcS1B (At5g38430), rbcS2B (At5g38420), rbcS3B (At5g38410) and rbcL (At5g00490) transcripts were quantified relative to wild-type levels (set at 100) from 28-d-old rosettes using RT-qPCR with gene-specific primers (Supporting Information Table S1). For wild-type, 1a3b and 1a2b lines, the values ± SE of measurements made on three individual 28-d-old rosettes. For transgenic lines, values are the means ± SE of measurements made on nine rosettes, three from each of the three lines. Full expression data are shown in Table S2. HET, heterologous rbcS.

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CO2 at 0% O2; for Rubisco specificity, which is the mean calculated using the Michaelis–Menten model as described in Prins et al. (2016). The table shows mean ± SD values for three biological replicates, except for Rubisco specificity, which is the mean ± SD of the numbers of technical replicates shown in parentheses. All values were measured at 25°C. $K_c$, $K_m$ for CO2 at 0% O2; $K_c^\text{air}$, $K_m$ for CO2 at 21% O2; $k_{\text{cat}}^c$, turnover number (mol carboxylation product mol$^{-1}$ active site s$^{-1}$); $k_{\text{cat}}^c/K_c^\text{air}$, Rubisco carboxylation efficiency at 21% O2; $S_{\text{C/O}}$, Rubisco specificity factor.

Table 1 Catalytic parameters of Rubisco in rbcs mutants and transgenic lines of Arabidopsis thaliana

|            | Wild-type | 1a3b   | 1a2b   | 1AAt   | 1AAtMOD | S2Cr |
|------------|-----------|--------|--------|--------|---------|------|
| $k_{\text{cat}}^c$ (s$^{-1}$) | 4.1 ± 0.1 | 4.2 ± 0.1 | 4.1 ± 0.2 | 4.0 ± 0.1 | 4.1 ± 0.1 | 3.6 ± 0.1* |
| $K_c$ (µM)  | 10.7 ± 0.7| 9.5 ± 0.7| 9.4 ± 1.1| 10.4 ± 1.1| 11.5 ± 0.9 | 9.6 ± 1.0 |
| $K_c^\text{air}$ (µM) | 15.8 ± 1.0 | 14.3 ± 0.5 | 15.4 ± 1.5 | 16.9 ± 1.8 | 17.1 ± 1.0 | 16.4 ± 1.2 |
| $k_{\text{cat}}^c/K_c^\text{air}$ | 0.25 ± 0.01 | 0.3 ± 0.02 | 0.27 ± 0.02 | 0.25 ± 0.03 | 0.24 ± 0.02 | 0.22 ± 0.03 |
| $S_{\text{C/O}}$ | 92.5 ± 1.0 (27) | 96.3 ± 1.7 (11) | 93.4 ± 1.7 (10) | 91.8 ± 1.0 (17) | 92.7 ± 0.8 (18) | 87.8 ± 0.9* (14) |

Rubisco specificity was determined from at least 10 replicate measurements for the enzyme purified from each line. Other catalytic parameters are calculated using the Michaelis–Menten model as described in Prins et al. (2016). The table shows mean ± SD values for three biological replicates, except for Rubisco specificity, which is the mean ± SD of the numbers of technical replicates shown in parentheses. All values were measured at 25°C. $K_c$, $K_m$ for CO2 at 0% O2; $K_c^\text{air}$, $K_m$ for CO2 at 21% O2; $k_{\text{cat}}^c$, turnover number (mol carboxylation product mol$^{-1}$ active site s$^{-1}$); $k_{\text{cat}}^c/K_c^\text{air}$, Rubisco carboxylation efficiency at 21% O2; $S_{\text{C/O}}$, Rubisco specificity factor.

*Significant difference ($p < 0.05$) as determined by ANOVA followed by Tukey’s HSD tests.

Rosette expansion rates and fresh and dry weights in the 1a2b mutant were greater than in the 1a3b mutant, but lower than those of wild-type and transgenic lines (Fig. 4c). The 1a2b mutant had a lower ratio of fresh to dry weight than the 1a3b mutant (Table S4). Although the specific leaf areas (rosette area per unit dry weight) of 1a2b and 1a3b mutants were comparable, rosette area per unit fresh weight was significantly higher in 1a2b than in 1a3b mutants.

Photosynthetic characteristics

At ambient CO2 and saturating light, all three transgenic genotypes had much higher rates of CO2 assimilation ($A_{\text{max}}$) than the 1a3b mutant ($A_{\text{max}}$ photosynthetically active radiation (PAR) curves, Fig. 5a). $A_{\text{max}}$ was similar to that of wild-type plants in 1AAt and 1AAtMOD lines but lower in S2Cr lines (Table 2). $A_{\text{max}}$ was higher in the 1a2b than in the 1a3b mutant, and was comparable in 1a2b and S2Cr lines. The apparent quantum efficiency (Φ) for all three transgenic lines was higher than in the 1a3b mutant and comparable with the wild-type value. Light compensation point and respiration rate in the dark ($R_d$) were the same in all lines.

There were substantial differences between the 1a3b mutant and the transgenic genotypes in the response of CO2 assimilation to changing external CO2 concentrations under saturating light ($A/C_i$ curves, Fig. 5b). Several photosynthetic parameters can be derived from $A/C_i$ curves (Table 2). The maximum rate of Rubisco carboxylation ($V_{\text{c,max}}$) and maximum photosynthetic electron transport rate ($J_{\text{max}}$) were not significantly different between wild-type, 1AAt and 1AAtMOD plants, but were lower...
in S2Cr plants than in wild-type plants. The initial linear slope of the A/Ci curve (a measure of the carboxylation efficiency and activation state of Rubisco) was lower for transgenic genotypes than for wild-type plants due to reduced Rubisco content in the transgenic lines (Fig. 3a). In the 1a2b mutant, Vc,max, Jmax, the substomatal CO2 compensation point (Γ) and the initial slope of the A/Ci curve were different from those of the 1a3b mutant, but similar to values for the S2Cr lines.

Gas exchange rates and chlorophyll fluorescence measurements under photorespiratory (ambient O2 (21%)) and nonphotorespiratory (low O2 (2%)) conditions were used to derive information about photorespiration (Table 3). Gross CO2 assimilation rates (GA, CO2 assimilation in the absence of respiration) and NADPH production (estimated from the photosynthetic electron transport rate, $J_{NADPH}$) can together be used to estimate the ratio of Rubisco oxygenase to carboxylase activity ($V_o/V_c$) (Bellaio et al., 2014).

The transgenic genotypes had higher GA and $J_{NADPH}$ values than the 1a3b mutant. Values for 1AaI and 1AaI.MOD lines were similar to those of wild-type plants, but values for S2Cr lines were lower. GA and $J_{NADPH}$ in the 1a2b mutant were higher than in the 1a3b mutant, and comparable with values for the S2Cr lines. There were no significant differences in $V_o/V_c$ values between any of the lines, indicating that relative photorespiratory rates were similar across genotypes under the conditions used.

Chlorophyll content and dark-adapted $F_v/F_m$ values in the transgenic lines and the 1a2b mutant were higher than in the 1a3b mutant, and were not significantly different from those of wild-type plants (Table S5). The 1a3b mutants had higher levels of NPQ than wild-type plants, but NPQ in transgenic genotypes was comparable with that of wild-type plants (Fig. 6). By contrast, the NPQ value for the 1a2b mutant was lower than that of wild-type plants. NPQ has two components: fast relaxing quenching ($qE$: NPQ$_{fast}$) associated with photoprotection, and slow relaxing quenching ($qI$: NPQ$_{slow}$) associated with chronic photoinhibition (Walters & Horton, 1991). To calculate the contribution of these components in the mutant lines, NPQ was tracked following a period of high light (600 µmol photons m$^{-2}$ s$^{-1}$ for 1 h) and subsequent recovery in darkness (1 h). $qI$ was lower in both transgenic genotypes that in wild-type plants, but $qE$ was elevated in the 1a3b mutant and reduced in the 1a2b mutant. The $qE/qI$ ratio was higher in the 1a3b mutant but lower in the 1a2b mutant than in wild-type plants (Table S6).

**Discussion**

Our results illustrate the impact of varying Rubisco content and native SSU composition on plant performance in Arabidopsis. Furthermore, we have shown that heterologous, pyrenoid competent SSUs assemble with the native LSU to produce a functional hybrid Rubisco with catalytic properties similar to the native Rubisco. This is a significant step towards the introduction of a functional algal CCM into higher plants.

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Fig. 4 Growth analysis of rbcs mutants and transgenic lines of Arabidopsis thaliana. (a) Representative examples of 28-d-old rosettes (T3) for mutants and transgenic genotypes. (b) Rosette expansion of homozygous transgenic and 1a3b out-segregant plants compared to that of wild-type and 1a3b mutant plants. (c) Fresh and dry weights were compared after 28 d. For wild-type (WT), 1a3b and 1a2b values are the means ± SE of measurements made on 10 individual rosettes. For transgenic lines values are means ± SE of measurements made on 30 rosettes, 10 from each of the three lines. See Supporting Information Table S4 for full dataset. seg, segregating T3 wild-type.
Differences in native SSU composition of Rubisco have only minor implications for plant performance in Arabidopsis

The data presented here suggest that the four native SSUs in Arabidopsis are largely equivalent in the properties they convey to the Rubisco enzyme under the growth conditions tested. Four genotypes provided data that lead to this conclusion: (1) wild-type plants, with the highest Rubisco content and with Rubisco containing almost exclusively rbcS1A, rbcS2B and rbcS3B SSUs (because of its very low transcript levels it is assumed that rbcS1B makes a very minor contribution to the SSU population); (2) 1AAt plants, with c. 78% of wild-type Rubisco content and with Rubisco containing mainly rbcS1A and rbcS2B; (3) the 1a2b mutant, with 45% of wild-type Rubisco content and with Rubisco containing mainly rbcS3B; and (4) the 1a3b mutant, with 30% of wild-type Rubisco content and with Rubisco containing rbcS2B. The catalytic properties of Rubisco at 25°C were similar in these four genotypes (Table 1), and thus they are largely independent of the native SSU composition of Rubisco in Arabidopsis.

Nearly all the phenotypic differences between the four genotypes with different native SSU compositions can be explained by the differences in total Rubisco content alone. Across these four genotypes, parameters including leaf protein content (Fig. 3; Table S3), the response of photosynthesis to light and to CO₂ (Fig. 5), Γ and Jmax (Table 2), and the rates of biomass accumulation and rosette expansion (Fig. 4; Table S4) responded to decreasing Rubisco activity in the manner expected for a single enzyme exercising a moderate degree of control over CO₂ assimilation (Stitt & Schulze, 1994). Additionally, the responses were broadly in line with those observed for tobacco plants with varying amounts of Rubisco activity of probably constant SSU composition (Quick et al., 1991; Fichtner et al., 1993; Lauer et al., 2017)

Table 2 Variables derived from photosynthetic response curves, based on gas exchange analysis of 35- to 45-d-old Arabidopsis thaliana plants

|                | Wild-type | 1a3b    | 1a2b    | 1AAt    | 1AAtMOD | S2Ct   |
|----------------|-----------|---------|---------|---------|---------|--------|
| A_{amb} (μmol CO₂ m⁻² s⁻¹) | 5.7 ± 0.1 a | 3.4 ± 0.3 c | 4.7 ± 0.1 b | 5.3 ± 0.2 abc | 5.1 ± 0.2 ab | 5.0 ± 0.1 abc |
| A_{max} (μmol CO₂ m⁻² s⁻¹) | 13.5 ± 0.5 a | 6.8 ± 0.5 c | 10.4 ± 0.2 b | 12.8 ± 0.6 ab | 12.3 ± 0.7 ab | 10.9 ± 0.4 abc |
| g_s (mol CO₂ m⁻² s⁻¹) | 0.34 ± 0.06 a | 0.42 ± 0.06 b | 0.33 ± 0.02 a | 0.3 ± 0.02 a | 0.36 ± 0.03 a | 0.41 ± 0.03 a |
| Φ (mmol CO₂ mol⁻¹ photons) | 55.9 ± 1.9 a | 42.2 ± 2.3 b | 55.3 ± 1.4 a | 53.5 ± 3.3 a | 51.5 ± 1.8 a | 53.6 ± 0.4 a |
| LCP (μmol CO₂ m⁻² s⁻¹) | 16.6 ± 1.3 a | 18.8 ± 0.7 a | 22.7 ± 0.8 a | 18.0 ± 2.6 a | 17.0 ± 1.5 a | 20.9 ± 1.6 a |
| V_{c,max} (μmol CO₂ m⁻² s⁻¹) | 31.4 ± 1.4 a | 14.6 ± 0.4 d | 20.9 ± 0.4 c | 27.1 ± 1.3 b | 26.1 ± 1.1 b | 22.2 ± 0.6 bc |
| J_{max} (mol e⁻ m⁻² s⁻¹) | 73.3 ± 2.8 a | 32.8 ± 1.3 d | 53.7 ± 0.9 b | 66.6 ± 3.0 ab | 63.7 ± 2.1 ab | 56.3 ± 1.6 bc |
| Γ (μmol CO₂ mol⁻¹) | 39.4 ± 2.8 a | 60.0 ± 3.9 a | 42.8 ± 1.6 b | 39.1 ± 0.9 b | 41.4 ± 0.5 b | 43.2 ± 0.8 b |
| R_o (μmol CO₂ m⁻² s⁻¹) | 1.9 ± 0.2 a | 1.8 ± 0.1 a | 2.0 ± 0.1 b | 1.8 ± 0.1 a | 1.9 ± 0.1 a | 1.8 ± 0.1 a |
| Initial slope (A/C_i) | 0.055 ± 0.003 a | 0.024 ± 0.007 d | 0.034 ± 0.006 c | 0.045 ± 0.002 b | 0.044 ± 0.002 b | 0.036 ± 0.001 bc |

For measurements of net photosynthetic CO₂ assimilation (A)/photosynthetically active radiation (PAR), relative humidity was maintained at 68 ± 4% and ambient CO₂ levels at 400 μmol mol⁻¹. For measurements of A/sub-stomatal CO₂ concentration (C_i), relative humidity was maintained at 73 ± 1% under a constant illumination of 1500 μmol photons m⁻² s⁻¹. All measurements were performed at 20°C. Values are the mean ± SE of measurements made on four leaves, each from a different plant (as shown in Fig. 5) followed by letters indicating significant differences (P < 0.05) as determined by ANOVA followed by Tukey’s HSD tests. Values followed by the same letter are not significantly different. A_{amb}, net photosynthesis measured at ambient CO₂ and growth chamber light levels; A_{max}, light-saturated CO₂ assimilation rate at ambient CO₂; g_s, stomatal conductance to CO₂ (at ambient CO₂); Φ, apparent quantum efficiency; LCP, light compensation point; V_{c,max}, maximum rate of Rubisco carboxylation; J_{max}, maximum electron transport rate; Γ, CO₂ compensation point (C_i = A); R_o, respiration in the dark.

Fig. 5 Photosynthesis response curves of rbcS mutants and transgenic lines of Arabidopsis thaliana. Measurements were made on the sixth or seventh leaf of 35- to 45-d-old nonflowering rosettes. (a) A/PAR curves show the response of CO₂ assimilation rates to different light levels at ambient CO₂ levels of 400 μmol mol⁻¹. (b) A/C_i curves showing the response of net CO₂ assimilation to different sub-stomatal concentrations of CO₂ (C_i) under saturating light (1500 μmol photons m⁻² s⁻¹). For wild-type, 1a3b and 1a2b values are the means ± SE of measurements made on individual leaves from four different rosettes. For transgenic lines values are means ± SE of measurements made on 12 rosettes, four from each of the three lines.
Some features of the four genotypes did not vary consistently with Rubisco content. For example, chlorophyll content, $F_{\text{m}}/F_{\text{m}}$, and $\Phi$ were strongly affected only in the genotype with the lowest levels of Rubisco, 1a3b (Tables 2, S5). Other parameters including leaf soluble protein content and specific leaf area were affected only in genotypes with less than 50% of wild-type levels of Rubisco, 1a3b and 1a2b (Tables 2, S5). Our data in these respects are reminiscent of those obtained for tobacco under limiting light in which Rubisco activity was varied by expression of antisense RNA that targeted all of the SSUs (Quick et al., 1991; Fichtner et al., 1993; Lauerer et al., 1993; Stitt & Schulze, 1994). Reductions of 40% or less in Rubisco activity in tobacco plants under limiting light (as in our experiments) had relatively little effect on the rate of photosynthesis and few pleiotropic consequences. Greater reductions progressively affected photosynthesis and downstream processes, different processes being affected at different levels of Rubisco reduction (Quick et al., 1991; Stitt & Schulze, 1994). Future experiments will investigate which phenotypic differences between the lines are exaggerated when plants are grown in saturating light.

For processes associated with photoprotection, qualitatively different phenotypes were observed in the 1a3b and 1a2b mutants. NPQ was elevated in the 1a3b mutant. NPQ was also elevated in tobacco and rice with reduced levels of Rubisco (Quick et al., 1991; Lauerer et al., 1993; Ruuska et al., 2000; Ushio et al., 2003; von Caemmerer et al., 2004): this effect may result from reduced ATP consumption for CO$_2$ assimilation, and hence a higher ΔpH across the thylakoid membrane. Lumen acidification promotes activity of the energy-dissipating xanthophyll cycle (Ruuska et al., 2000; Johnson et al., 2009; Zaks et al., 2012). By contrast, with 1a3b plants and other plant species with reduced Rubisco, NPQ was reduced in 1a2b mutants. In particular, 1a2b plants had a much reduced rate of relaxation of NPQ immediately following the onset of darkness (the qE or fast component of NPQ). The exact mechanism underlying qE is not known (e.g. Johnson et al., 2009; Zaks et al., 2012). However, as mature rbcS2B and rbcS3B have identical amino acid sequences, the difference in NPQ between the 1a2b and 1a3b mutants is likely to stem from the pleiotropic effects of the different degrees of reduction of Rubisco activity in the two mutants, rather than from the different SSU compositions of their Rubiscos.

It is clear from previous work that SSUs can influence Rubisco catalysis. For example, overexpression of specific native or heterologous SSU proteins altered the catalysis of Rubisco in rice leaves, resulting in properties that are more like those of C$_4$ plants (i.e. higher $k_{\text{cat}}$, but also higher $K_c$ (lower CO$_2$ affinity) than for native rice Rubisco) (Ishikawa et al., 2011; Morita et al., 2014). Over-expression in Arabidopsis of a pea SSU, differing from Arabidopsis SSUs by 40 amino acids, resulted in Rubisco with slightly reduced carboxylase activity and capacity for activation (Getzoff et al., 1998). Similarly, Rubisco properties were changed by introduction of a sorghum SSU into rice (Ishikawa et al., 2011). However, except in the case of the rice SSU above, little is known about the functional importance of sequence variation between SSUs within a species. SSU isoforms in a single species

Table 3 Estimates of in vivo Rubisco oxygenase and carboxylase activities made from measurements of gas exchange and chlorophyll fluorescence under ambient (21%) or low (2%) O$_2$

|                      | Wild-type | 1a3b | 1a2b | 1AM | 1AM/MOD | S2S
|----------------------|-----------|------|------|-----|--------|----
| $G_{\text{A}}$ ($\mu$mol m$^{-2}$ s$^{-1}$) | 9.48 ± 0.56$^a$ | 4.58 ± 0.43$^c$ | 5.69 ± 0.53$^c$ | 8.73 ± 0.09$^{ab}$ | 8.57 ± 0.64$^{ab}$ | 6.72 ± 0.49$^{bc}$
| $G_{\text{AM}}$ ($\mu$mol m$^{-2}$ s$^{-1}$) | 6.17 ± 0.36$^a$ | 2.98 ± 0.25$^d$ | 3.67 ± 0.36$^{cd}$ | 5.6 ± 0.15$^{ab}$ | 5.49 ± 0.42$^{ab}$ | 4.36 ± 0.38$^{bc}$
| $I_{\text{NADPH}}$ ($\mu$mol m$^{-2}$ s$^{-1}$) | 18.9 ± 1.1$^a$ | 9.1 ± 0.8$^c$ | 11.4 ± 1.1$^c$ | 17.5 ± 0.2$^b$ | 17.1 ± 1.3$^b$ | 13.4 ± 0.9$^g$.
| $I_{\text{NADPH}}$ ($\mu$mol m$^{-2}$ s$^{-1}$) | 19.9 ± 0.93$^a$ | 9.2 ± 0.7$^c$ | 11.9 ± 0.9$^{bc}$ | 18.4 ± 0.2$^a$ | 18.2 ± 1.4$^a$ | 14.1 ± 1$^b$
| $V_c$ (µmol m$^{-2}$ s$^{-1}$) | 2.21 ± 0.13$^a$ | 1.06 ± 0.11$^b$ | 1.34 ± 0.12$^b$ | 2.09 ± 0.06$^a$ | 2.06 ± 0.17$^a$ | 1.57 ± 0.07$^a$
| $V_c/V_c$ | 0.304 ± 0.002$^a$ | 0.302 ± 0.013$^a$ | 0.307 ± 0.008$^a$ | 0.313 ± 0.015$^a$ | 0.316 ± 0.012$^a$ | 0.307 ± 0.011$^a$

Fig. 6 Nonphotochemical quenching response to light in leaves of rbcS mutants and transgenic lines of Arabidopsis thaliana. All plants were 28 d old. For wild-type, 1a3b and 1a2b values are the means ± SE of measurements made on individual leaves from four different rosettes. For transgenic lines values are means ± SE of measurements on leaves from 12 plants, four from each of the three lines.
Chlamydomonas-like SSUs generate a functional hybrid
Rubisco in Arabidopsis

Introduction of either a Chlamydomonas SSU (S2c) or a modified version of rbcS1A (1AAtMOD) into the Arabidopsis 1a3b mutant substantially complemented several aspects of the 1a3b phenotype. In a previous study, a Chlamydomonas SSU introduced into pea chloroplasts was not processed to the mature, active form, probably due to differences in chloroplast import machinery between Chlamydomonas and higher plants (Su & Boschetti, 1994). In this study, replacing the Chlamydomonas SSU TP with the rbcS1A TP directed the mature protein to the chloroplast stroma (Fig. S1). Expression of S2c or 1AAtMOD increased Rubisco content in the 1a3b mutant without significantly enhancing levels of the remaining native SSUs, and thus both introduced proteins promoted expression of the native LSU and assembled into catalytically active hybrid Rubiscos. These results are consistent with the idea that rbcL transcription and LSU synthesis adjust according to the availability of SSU (Wollman et al., 1999; Wostrikoff & Stern, 2007; Wostrikoff et al., 2012; Zhan et al., 2014).

Photosynthesis was restored almost to wild-type levels in 1AAtMOD (Fig. 5). Furthermore, the catalytic characteristics of Rubisco in 1AAtMOD plants, where c. 70% of the SSU pool was heterologous, were comparable to those of 1AAt and wild-type plants (Table 1). This suggests that the SSU α-helix regions alone do not affect Rubisco biogenesis or catalysis, and that Rubisco in higher plants can be made compatible with the requirements of the algal CCM without affecting enzyme performance.

Rubisco in S2c plants had lower kCa and S values than those of wild-type and 1AAtMOD Rubisco, even though the S2c SSU pool contained only c.40% Chlamydomonas SSU. S2c lines generally performed less well than 1AAtMOD lines. Neither S2c nor 1AAtMOD lines are likely to be Rubisco-limited because they both have c. 70% of the SSU content of wild-type plants (Quick et al., 1991). Differences in photosynthesis and growth between S2c and 1AAtMOD lines are thus likely to result largely from SSU-dependent differences in Rubisco catalytic properties. In Chlamydomonas, expression of a higher plant LSU can impart improved catalysis and S values (Genkov et al., 2010). The data shown here demonstrate that the reverse is also true: an algal LSU can negatively affect catalytic properties of the hybrid Rubisco in a higher plant. Since the 1AAtMOD and S2c SSUs have the same α-helices, differences in catalytic properties of the hybrid enzyme must arise from sequence differences in regions of the SSU outside of these helices.

The Chlamydomonas SSU protein differs in several respects from the Arabidopsis SSUs, including the presence of additional amino acid residues at the C-terminus and in the loop between β-strands A and B (Spreitzer, 2003). The latter forms the entrance of the solvent channel and may be important for carboxylation rates and S (Karkehabadi et al., 1995; Esquivel et al., 2013). Hybrid Rubisco enzymes with SSUs that diverge significantly in amino acid sequence from the native SSU frequently have altered stability and properties, and a lower capacity for assembly with the native LSU. The poor complementation of Arabidopsis Rubisco in S2c warrants further study to expand upon existing knowledge in this area, including the functional capacity of the chaperone Rubisco activase when presented with hybrid Rubiscos.

rbcS mutants of Arabidopsis are a useful platform for Rubisco analyses and the assembly of an algal CCM

This study shows that Arabidopsis mutants lacking SSU isoforms are a useful platform for attempts to assemble a functional algal CCM in higher plants. Introduction of 1AAtMOD, containing α-helices believed to be necessary for pyrenoid assembly, had no apparent effect on Rubisco function and assembly, and plant performance was generally close to wild-type levels under our growth conditions.

For aggregation of Rubisco into a pyrenoid, additional algal CCM components will be required. Cryo-electron tomography of Chlamydomonas pyrenoids showed that Rubisco proteins are not randomly arranged, and periodicity is consistent with hexagonal close packing, with a space of 2–4.5 nm between each protein depending on their relative orientations (Engel et al., 2015). Other factors, such as linker proteins, are probably needed. Recently, a multiple repeat linker-protein, EPYC1 (formerly known as LCI5), has been identified in Chlamydomonas that is associated with Rubisco during aggregation within the pyrenoid (Mackinder et al., 2016). The 1AAtMOD and S2c Arabidopsis lines are ideal backgrounds in which to test candidates for these other factors as they emerge, to clarify the nature of SSU-associated interactions, and to integrate other essential algal CCM components (Atkinson et al., 2016).

Acknowledgements

This work was funded by the UK Biotechnology and Biological Sciences Research Council (Institute Strategic Programme Grant BB/J004561/1 to the John Innes Centre, grants BB/I024453/1 and BB/M006468/1 to A.M.S. and A.J.M., respectively, and a PhD studentship from the John Innes Foundation to N.L.). E.C-S. acknowledges financial support from the Lancaster Environment Centre. We thank Hiroyuki Ishida (Tohoku University) for seeds of the 1a3b mutant and the reviewers for their helpful comments.

Author contributions

A.J.M. and N.A. planned and designed the research and wrote the manuscript. A.M.S., D.J.O., M.T.M., H.G. and E.C-S. assisted in experimental design, data analysis and writing of the
manuscript. A.J.M., N.A. and N.L. performed the research, data analysis, collection, and assisted with data interpretation and writing.

References

Andriotis VM, Pike MJ, Bunnewell S, Hills MJ, Smith AM. 2010. The plastidial glucose-6-phosphate/phosphate antiporter GPT1 is essential for morphogenesis in Arabidopsis embryos. *Plant Journal* 64: 128–139.

Atkinson N, Feike D, Mackinder IC, Meyer MT, Griffiths H, Jonikas MC, Smith AM, McCormick AJ. 2016. Introducing an algal carbon-concentrating mechanism into higher plants: location and incorporation of key components. *Plant Biotechnology Journal* 14: 1302–1315.

Badger MR, Andrews TJ, Whitney SM, Ludwig M, Yellowlees DC, Leggett W, Price GD. 1998. The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO₂-concentrating mechanisms in algae. *Canadian Journal of Botany* 76: 1052–1071.

Bellasio C, Beefling DJ, Griffiths H. 2016. An Excel tool for deriving key photosynthetic parameters from combined gas exchange and chlorophyll fluorescence: theory and practice. *Plant, Cell & Environment* 59: 1180–1197.

Bellasio C, Burgess SJ, Griffiths H, Hibberd JM. 2014. A high throughput gas exchange screen for determining rates of photorespiration or regulation of C₄ activity. *Journal of Experimental Botany* 65: 3769–3779.

von Caemmerer S, Lawson T, Oxorough K, Baker NR, Andrews TJ, Raines CA. 2004. stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. *Journal of Experimental Botany* 55: 1157–1166.

von Caemmerer S, Quick WP, Furbank RT. 2012. The development of C₄ rice: current progress and future challenges. *Science* 336: 1671–1672.

Carmo-Silva E, Scales JC, Madgwick PJ, Parry MA. 2015. Optimizing Rubisco and its regulation for greater resource use efficiency. *Plant, Cell & Environment* 38: 1817–1832.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant Journal* 16: 735–743.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology* 139: 5–17.

Engel BD, Schaffer M, Kuhn Cuellar L, Whitney SM, Ludwig M, Yellowlees DC, Leggett W, Price GD. 1998. The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO₂-concentrating mechanisms in algae. *Canadian Journal of Botany* 76: 1052–1071.

Ethier GJ, Livingston NJ. 2004. On the need to incorporate sensitivity to CO₂ transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. *Plant, Cell & Environment* 27: 137–153.

Fichtner K, Quick WP, Schulze ED, Mooney HA, Rodermel SR, Bogorad L, Stitt M. 1993. Decreased ribulose-1,5-bisphosphate carboxylase/oxygenase in transgenic tobacco transformed with ‘antisense’ rbcs. V. Relationship between photosynthetic rate, storage strategy, biomass allocation and vegetative plant growth at three different nitrogen supplies. *Planta* 190: 332–345.

Flexas J, Ortuño MF, Ribas-Carbo M, Díaz-Enpejo A, Florez-Sarasa ID, Medrano H. 2007. Mesophyll conductance to CO₂ in Arabidopsis thaliana. *New Phytologist* 175: 501–511.

Galjers K, Kapralov MV, Andralojc PJ, Conesa MA, Keys AJ, Parry MAJ, Flexas J. 2014. Expanding knowledge of the Rubisco kinetics variability in plant species: environmental and evolutionary trends. *Plant, Cell & Environment* 37: 1989–2001.

Genkov T, Meyer M, Griffiths H, Spreiter RJ. 2010. Functional hybrid Rubisco enzymes with plant small subunits and algal large subunits: engineered rbcsDNA for expression in Chlamydomonas. *Journal of Biological Chemistry* 285: 19833–19841.

Genkov T, Spreiter RJ. 2009. Highly conserved small subunit residues influence Rubisco large subunit catalysis. *Journal of Biological Chemistry* 284: 30105–30112.

Getzoff TP, Zhu GH, Bohnert HJ, Jensen RG. 1998. Chimeric Arabidopsis thaliana ribulose-1,5-bisphosphate carboxylase/oxygenase containing a pea small subunit protein is compromised in carboxylation. *Plant Physiology* 116: 695–702.

Giordano M, Beardall J, Raven JA. 2005. CO₂ concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *Annual Review of Plant Biology* 56: 99–131.

Goldschmidt-Clermont M, Rahire M. 1986. Sequence, evolution and differential expression of the two genes encoding variant small subunits of ribulose bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. *Journal of Molecular Biology* 191: 421–432.

Griffiths H, Maxwell K. 1999. In memory of C. S. Pittendrigh: does exposure in forest canopies relate to photoprotective strategies in ephiphytic bromeliads? *Functional Ecology* 13: 15–23.

Howe CJ, Aufrert AD, Doherty A, Bowman CM, Dyer TA, Gray JC. 1982. Location and nucleotide sequence of the gene for the proton-translocating subunit of wheat chloroplast ATP synthase. *Proceedings of the National Academy of Sciences, USA* 79: 6903–6907.

Ishikawa C, Hatanaka T, Misso S, Miyake C, Fukayama H. 2011. Functional incorporation of sorghum small subunit increases the catalytic turnover rate of Rubisco in transgenic rice. *Plant Physiology* 156: 1603–1611.

Izumi M, Tsunoda H, Suzuki Y, Makino A, Ishida H. 2012. RBCS1A and RBCS3B, two major members within the Arabidopsis RBCS multigene family, function to yield sufficient Rubisco content for leaf photosynthetic capacity. *Journal of Experimental Botany* 63: 2159–2170.

Johnson MP, Perez-Bueno ML, Zia A, Horton P, Ruban AV. 2009. The zeaxanthin-independent and zeaxanthin-dependent qE components of nonphotochemical quenching involve common conformational changes within the photosystem II antenna in Arabidopsis. *Plant Physiology* 149: 1061–1075.

Karimi M, Inze D, Depicker A. 2002. GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science* 7: 193–195.

Karkehabadi S, Peddi SR, Anwaruzzaman M, Taylor TC, Cederlund A, Genkov T, Andersson I, Spreiter RJ. 1995. Chimeric small subunits influence catalysis without causing global conformational changes in the crystal structure of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Biochemistry* 44: 9851–9861.

Lauerer M, Safric D, Quick WP, Labate C, Fichtner K, Schulze ED, Rodermel SR, Bogorad L, Stitt M. 1993. Decreased ribulose-1,5-bisphosphate carboxylase/oxygenase in transgenic tobacco transformed with ‘antisense’ rbcs. VI. Effect on photosynthesis in plants grown at different irradiance. *Planta* 190: 332–345.

Li J, Chory J. 1998. Preparation of DNA from Arabidopsis. *Methods in Molecular Biology* 82: 55–60.

Long SP, Marshall-Colon A, Zhu XG. 2015. Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. *Cell* 161: 56–66.

Mackinder LCM, Meyer MT, Mettler-Altman T, Chen VK, Mitchell MC, Caspari O, Rosenzweig ESF, Pallesen L, Reeves G, Itakura A et al. 2016. A repeat protein links Rubisco to form the eukaryotic carbon-concentrating organelle. *Proceedings of the National Academy of Sciences, USA* 113: 5958–5963.

Marshall B, Biscoe PV. 1980. A model for C₃ leaves describing the dependence of net photosynthesis on irradiance. *Journal of Experimental Botany* 31: 29–39.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence – a practical guide. *Journal of Experimental Botany* 51: 659–668.

McCormick AJ, Kruger NJ. 2015. Lack of fructose 2,6-bisphosphate compromises photosynthesis and growth in Arabidopsis in fluctuating environments. *Plant Journal* 81: 670–683.

 McGrath JM, Long SP. 2014. Can the cyanobacterial carbon-concentrating mechanism increase photosynthesis in crop species? A theoretical analysis. *Plant Physiology* 164: 2247–2261.

Meyer MT, McCormick AJ, Griffiths H. 2016. Will an algal CO₂-concentrating mechanism work in higher plants? *Current Opinion in Plant Biology* 31: 181–188.
**Table S5** Chlorophyll characteristics and maximum quantum yield of PSII ($F_v/F_m$) for *rbcS* mutants and transgenic lines

**Table S6** Photosynthetic nonphotochemical quenching capacity for *rbcS* mutants

**Notes S1** Expression vectors for Rubisco small subunit (*rbcS*) cassettes

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