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Citation for published version:

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

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
New Phytologist

Publisher Rights Statement:
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<th>New Phytologist</th>
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<td>Date Submitted by the Author:</td>
<td>n/a</td>
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| Key Words:      | carbon concentrating mechanism, Arabidopsis thaliana, Chlamydomonas reinhardtii, tobacco, pyrenoid, chloroplast, photosynthesis, Rubisco |
Rubisco small subunits from the unicellular green alga Chlamydomonas complement Rubisco-deficient mutants of Arabidopsis

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Running title: Expression of algal Rubisco small subunits in Arabidopsis
Summary word count: 165
Word count for main text: 5785
Word count for each main section: Introduction, 839; Materials and Methods, 1274; Results, 1786; Discussion, 1815; Acknowledgements, 71
Number of Figures: 6 (Fig. 4 in colour)
Number of Tables: 3
Supporting Information: Notes S1; Fig. S1-3; Tables S1-6

Keywords: Arabidopsis thaliana, carbon concentrating mechanism, Chlamydomonas reinhardtii, chloroplast, photosynthesis, pyrenoid, Rubisco, tobacco.
Summary

- Introducing components of algal carbon concentrating mechanisms (CCMs) into higher plant chloroplasts could increase photosynthetic productivity. A key component is the Rubisco-containing pyrenoid that is needed to minimise CO$_2$ retro-diffusion for CCM operating efficiency.

- Rubisco in Arabidopsis was re-engineered to incorporate sequence elements which are thought to be essential for recruitment of Rubisco to the pyrenoid, viz the algal Rubisco small subunit (SSU, encoded by rbcS) or only the surface-exposed 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 the 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

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) catalyses net CO$_2$ assimilation in all photosynthetic organisms. Despite this central role, Rubisco is an inefficient enzyme that limits photosynthetic productivity, particularly in plants with the C$_3$ photosynthetic pathway. Rubisco has a slow carboxylation rate ($k_{\text{cat}}^c$) and a relatively low affinity for CO$_2$, with a $K_m$ for CO$_2$ at ambient O$_2$ ($K_c^\text{air}$) close to the CO$_2$ concentration in a C$_3$ leaf mesophyll cell (Galmés et al., 2014). Rubisco also catalyses D-ribose-1,5-bisphosphate (RuBP) oxygenation, resulting in the energetically-expensive photorespiratory pathway where previously-fixed CO$_2$ 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 CO$_2$ assimilation (Parry et al., 2013). Increasing the operating efficiency of Rubisco and reducing photorespiration are important approaches for improving yields in C$_3$ crop plants.
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 levels 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 (Chlamydomonas throughout) 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 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 likely 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 SSU influences the biogenesis and catalysis of Rubisco in Arabidopsis leaves. The Rubisco large subunits (LSUs, encoded by \textit{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 sequence). In 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. \textit{rbcS1A} on chromosome 1 accounts for ~50% of SSU transcript, the remainder being contributed by the \textit{rbcS1B}, \textit{rbcS2B} and \textit{rbcS3B} genes located contiguously on chromosome 5 (Yoon et al., 2001). An Arabidopsis double mutant lacking expression of \textit{rbcS1A} and with strongly reduced expression of \textit{rbcS3B} (the 1a3b mutant) has a low Rubisco content (30% of wild-type plants) and slow growth (Izumi et al., 2012). In this study the 1a3b mutant was complemented with either the Arabidopsis \textit{rbcS1A} (control), an \textit{rbcS1A} variant encoding the Chlamydomonas α-helix sequences or the native \textit{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 1a3b 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.

\section*{Materials and Methods}
\subsection*{Plant material and growth conditions}
Arabidopsis (\textit{Arabidopsis thaliana}, Col-0) seeds were sown on compost, stratified for 3 d at 4°C and grown at 20°C, ambient CO$_2$, 70% relative humidity and 150 μmol photons m$^{-2}$ s$^{-1}$ in 12 h light, 12 h 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 (\textit{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
rbcS1a rbcS2b mutant (double mutant 1a2b) was generated by crossing T-DNA insertion
lines GABI_608F01 (At1g67090) and GABI_324A03 (At5g38420). The 1a3b 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). PCR reactions
were performed as in McCormick & Kruger (2015) using gene-specific primers (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 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 (RHIP1) (Czechowski et al., 2005)] were used for
normalisation. Calculations of relative expression ratios were performed according to Pfaffl

Expression of rbcS genes in Nicotiana benthamiana and Arabidopsis 1a3b 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 manufacturer’s instructions, www.neb.com). 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) cDNA prior to promoter addition.
Promoter-cDNA fusions were cloned into Gateway Entry vectors (pCR®8/GW/TOPO®-TA
Cloning® Kit, www.lifetechnologies.com), then into the binary destination vector pGWB4
(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 GFP in pGWB4. Binary
vectors were transformed into Agrobacterium tumefaciens (AGL1) for transient gene
expression in tobacco (Schöb 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 µl⁻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 PMSF and 10 µM Leupeptin. Following centrifugation at 2,380 g for 5 min at 4°C, soluble protein was quantified using a Bradford-based assay (www.biorad.com) against BSA standards (www.thermofisher.com). Rubisco content was determined in an aliquot of the extract via \(^{14}\text{C}-\text{CABP}\) (carboxy-D-arabinitol 1,5-bisphosphate) binding following incubation with 10 mM NaHCO₃, 20 mM MgCl₂ and the addition of 3 µL 12mM \(^{14}\text{C}-\text{CABP}\) (37 MBq mmol⁻¹) for 25 min at RT (Whitney et al., 1999).

Subunit ratios were estimated by immunoblotting. Extracts were subjected to SDS-PAGE on a 4-12% (w/v) polyacrylamide gel (Bolt® Bis-Tris Plus Gel, www.lifetechnologies.com), transferred to 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 (www.licor.com) at 1:10,000 dilution, then viewed on an LI-COR Odyssey CLx Imager. The contribution 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).

**Rubisco catalytic properties**

Whole 45-d-old rosettes (20-30 cm²) were rapidly frozen in liquid N₂ and Rubisco 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 \(^{14}\text{CO}_2\) 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 ca. 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 (Sₚ/O) was determined using the method of Parry et al. (1989). At least ten 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 Sₚ/O of 100 (Parry et al., 1989).

Chlorophyll quantification

Leaf discs (ca. 10 mg fresh weight) were frozen in liquid N₂, powdered, then mixed with 100 vol 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 seventh leaf of 35- to 45-d-old non-flowering 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 ca. 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 non-rectangular hyperbola was fitted to the light response (Marshall & Biscoe, 1980; Thornley, 1998). The response of A to varying substomatal CO₂ concentration (Cᵢ) was measured at 1,500 µmol photons m⁻² s⁻¹. To calculate the maximum rate of Rubisco carboxylation (Vₐₘₐₓ) and the maximum photosynthetic electron transport rate (Jₘₐₓ), the A/Cᵢ data were fitted to the C₃ photosynthesis model as in Ethier & Livingston (2004) using the catalytic parameters Kₐir and affinity for O₂ (Kₒ) 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 (Vₒ/Vₐ), 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 (www.hansatech-instruments.com) (Maxwell & Johnson, 2000). Non-photochemical 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 to 1,500 µmol photons m$^{-2}$ s$^{-1}$). Quenching parameters, including NPQ$_v$ and NPQ$_f$, were derived as in Griffiths & Maxwell (1999).

Confocal laser scanning microscopy

Leaves were imaged with a Leica TCS SP2 laser scanning confocal microscope (www.leica-microsystems.com) 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 (1A$_{At}$), the mature Chlamydomonas rbcS2 N-terminally fused to the chloroplast TP sequence from Arabidopsis rbcS1A (S2$_{Cr}$), or the full-length Arabidopsis rbcS1A modified to contain $\alpha$-helices matching the amino acid sequence as those of the Chlamydomonas SSU family (1A$_{At}$MOD) (Fig. 1, Notes S1). Chlamydomonas and Arabidopsis SSU $\alpha$-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* (~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; *Fv*/*Fm*) 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 in 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 about 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 levels 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. In 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 i) wild-type plants, ii) the parental 1a3b mutant, and iii) representative non-transgenic 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 wild-type for two out of the three lines. For 1A\textsubscript{At}MOD and S2\textsubscript{Cr} 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).

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 CO\textsubscript{2} and saturating light, all three transgenic genotypes had much higher rates of CO\textsubscript{2} assimilation (A\textsubscript{max}) than the 1a3b mutant (A/PAR curves, Fig. 5A). A\textsubscript{max} was similar to that of wild-type plants in 1A\textsubscript{At} and 1A\textsubscript{At}MOD lines but lower in S2\textsubscript{Cr} lines (Table 2). A\textsubscript{max} was higher in the 1a2b than in the 1a3b mutant, and was comparable in 1a2b and S2\textsubscript{Cr} 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\textsubscript{d}) were the same in all lines.

There were substantial differences between the 1a3b mutant and the transgenic genotypes in the response of CO\textsubscript{2} assimilation to changing external CO\textsubscript{2} concentrations under saturating light (A/C\textsubscript{i} curves, Fig. 5B). Several photosynthetic parameters can be derived from A/C\textsubscript{i} curves (Table 2). The maximum rate of Rubisco carboxylation (V\textsubscript{c,max}) and maximum photosynthetic electron transport rate (J\textsubscript{max}) were not significantly different between wild-type, 1A\textsubscript{At} and 1A\textsubscript{At}MOD plants, but were lower in S2\textsubscript{Cr} plants than in wild-type plants. The initial linear slope of the A/C\textsubscript{i} 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 V\textsubscript{c,max}, J\textsubscript{max}, the sub-
stomatal CO₂ compensation point ($I$) and the initial slope of the $A/C_i$ curve were different from those of the $1a3b$ mutant, but similar to values for the S2$_{Ct}$ lines.

Gas exchange rates and chlorophyll fluorescence measurements under photorespiratory [ambient O₂ (21%)] and non-photorespiratory [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 ($V_o/V_c$) (Bellasio et al., 2014).

The transgenic genotypes had higher $GA$ and $J_{NADPH}$ values than the $1a3b$ mutant. Values for $1A_{At}$ and $1A_{At}$MOD lines were similar to those of wild-type plants, but values for S2$_{Ct}$ lines were lower. $GA$ and $J_{NADPH}$ in the $1a2b$ mutant were higher than in the $1a3b$ mutant, and comparable with values for the S2$_{Ct}$ 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). $1a3b$ mutants had higher levels of non-photochemical quenching (NPQ) than wild-type plants, but NPQ in transgenic genotypes was comparable with that of wild-type plants (Fig. 6). In contrast, the NPQ value for the $1a2b$ mutant was lower than that of wild-type plants. NPQ has two components: fast relaxing quenching ($q_E$: NPQ$_{fast}$) associated with photoprotection, and slow relaxing quenching ($q_I$: 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). $q_I$ was lower in both transgenic genotypes that in wild-type plants, but $q_E$ was elevated in the $1a3b$ mutant and reduced in the $1a2b$ mutant. The $q_E$:$q_I$ 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.

*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: (i) 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); (ii) 1AAt plants, with about 78% of wild-type Rubisco content and with Rubisco containing mainly rbcS1A and rbcS2B; (iii) the 1a2b mutant, with 45% of wild-type Rubisco content and with Rubisco containing mainly rbcS3B, and (iv) the 1a3b mutant, with 30% of wild-type Rubisco content and with Rubisco containing rbcS2B. The catalytic properties $k_{cat} c$, $K_c^{air}$ and $S_{C/O}$ of Rubisco at 25°C were similar in these four genotypes (Table 1), thus they are largely independent of the native SSU composition of Rubisco in Arabidopsis.

Nearly all of 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 CO$_2$ (Fig. 5), $\Gamma$ and $J_{max}$ (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 CO$_2$ 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; Lauerer et al., 1993; Fichtner et al., 1993; Stitt & Schulze, 1994), and Arabidopsis plants with strong suppression of expression of all four SSU genes (Zhan et al., 2014).

Some features of the four genotypes did not vary consistently with Rubisco content. For example, chlorophyll content, $F_v/F_m$ and $\Phi$ were strongly affected only in the genotype with the lowest levels of Rubisco, 1a3b (Table 2, Table S5). Other parameters including leaf
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soluble protein content and specific leaf area were affected only in genotypes with less than 50% of wild-type Rubisco levels (Fig. 3, Table S4). 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 around 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 CO₂ assimilation, 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, since 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 C₄ plants [i.e. higher \( k_{cat} \), but also higher \( K_c \) (lower CO₂ 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 are typically very similar. In Chlamydomonas, for example, the two SSUs differ by only four amino acid residues (all outside the α-helices) and appear to be functionally equivalent (Rodermelm et al., 1996; Genkov et al., 2010). In Arabidopsis, the mature rbcS1A differs from rbcS1B, rbcS2B and rbcS3B by only eight amino acids, six of which are conserved between the three B-class SSUs (Fig. S3). Two of these are located in the first α-helix.

Chlamydomonas-like SSUs generate a functional hybrid Rubisco in Arabidopsis

Introduction of either a Chlamydomonas SSU (S2Cr) 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 S2Cr or 1AAtMOD increased Rubisco content in the 1a3b mutant without significantly enhancing levels of the remaining native SSUs, 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 ~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 S2Cr plants had lower $k_{cat}$ and $S_{C/O}$ values than those of wild-type and 1AAtMOD Rubisco, even though the S2Cr SSU pool contained only ~40% Chlamydomonas SSU. S2Cr lines generally performed less well than 1AAtMOD lines. Neither S2Cr nor 1AAtMOD lines
are likely to be Rubisco-limited because they both have ~70% of the Rubisco content of wild-type plants (Quick et al., 1991). Differences in photosynthesis and growth between S2_Cr and 1A_At_MOD lines are thus likely to result largely from SSU-dependent differences in Rubisco catalytic properties. In Chlamydomonas, expression of a higher plant SSU can impart improved catalysis and S_C/O (Genkov et al., 2010). Data shown here demonstrates that the reverse is also true, an algal SSU can negatively affect catalytic properties of the hybrid Rubisco in a higher plant. Since the 1A_At_MOD and S2_Cr 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_C/O (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 S2_Cr 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 1A_At_MOD, 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 1A_{MOD} and S2_{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 AMS and AJM respectively, and a Ph.D. studentship from the John Innes Foundation to NL). ECS 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
AJM and NA planned and designed the research and wrote the manuscript. AMS, DO, MM, HG, ECS assisted in experimental design, data analysis and writing of the manuscript. AJM, NA and NL performed the research, data analysis, collection, and assisted with data interpretation and writing.

References


conformational changes in the crystal structure of ribulose-1,5-bisphosphate carboxylase/oxygenase. Biochemistry 44: 9851-9861.


Figure Legends

Figure 1. Gene expression cassettes for native and heterologous Rubisco small subunits. \textit{rbcS1A} from Arabidopsis (1A\textsubscript{At}) (A), \textit{rbcS1A} with \(\alpha\)-helices from the \textit{Chlamydomonas reinhardtii} \textit{rbcS} family (1A\textsubscript{At}MOD) (B), and mature \textit{rbcS2} from \textit{Chlamydomonas reinhardtii} (S2\textsubscript{Cr}) (C) were expressed using the \textit{rbcS1A} promoter (not drawn to scale) and \textit{35S} terminator. For S2\textsubscript{Cr}, the chloroplast transit peptide (TP) of \textit{Chlamydomonas} \textit{rbcS2} (45 aa) was replaced with the \textit{rbcS1A} TP (55 aa) from Arabidopsis to facilitate localisation of the mature \textit{rbcS2} to the chloroplast. Alignments of the mature SSU peptides generated in this study are shown (D). Numbering is relative to the \textit{Chlamydomonas} \textit{rbcS2} sequence. Residues that comprise the two \(\alpha\)-helixes A and B are underlined, those different from \textit{rbcS1A} are in bold. For comparison with 1A\textsubscript{At}MOD, the modified spinach SSU generated by Meyer \textit{et al.} (2012) is included.

Figure 2. Transcript abundances of the Rubisco gene family in \textit{rbcs} mutants and transgenic lines. Abundances of \textit{rbcS1A} (At1g67090), \textit{rbcS1B} (At5g38430), \textit{rbcS2B} (At5g38420), \textit{rbcS3B} (At5g38410) and \textit{rbcL} (Atcg00490) transcripts were quantified relative to wild-type levels (set at 100) from 28-d-old rosettes using RT-qPCR with gene-specific primers (Table S1). For wild-type, \textit{1a3b} and \textit{1a2b} values are the means ± standard error (SE) of measurements made on three individual 28-d-old rosettes. For transgenic lines values are means ± SE of measurements made on nine rosettes, three from each of the three lines. Full expression data are shown in Table S2. Abbreviation: \textit{HET}; heterologous \textit{rbcS}.

Figure 3. Rubisco and protein contents in \textit{rbcs} mutants and transgenic lines. Rubisco (A) and total protein contents (B) are shown for 32-d-old plants. Rubisco content was determined via \(^{14}\text{C}-\text{CABP} \) binding, and subunit ratios were estimated by immunoblotting. For wild-type, \textit{1a3b} and \textit{1a2b} values are the means ± SE of measurements made on three individual rosettes. For transgenic lines values are means ± SE of measurements made on nine rosettes, three from each of the three lines. Representative immunoblots for wild-type plants and transgenic lines, probed with a serum containing polyclonal antibodies against Rubisco (C). Standard curves (0.1-2.4 µg Rubisco) are shown for wild-type LSU (55 kD) and SSUs (14.8 kD), followed by protein amounts in different lines. Native LSU, SSU and heterologous SSUs (15.5 kD and 14 kD, respectively) are indicated by dark grey, light grey and white arrows, respectively. Quantitation of soluble protein and Rubisco is shown in Table S3.
Figure 4. Growth analysis of rbcs mutants and transgenic lines. Representative examples of 28-d-old rosettes (T₃) are shown for mutants and transgenic genotypes (A). Rosette expansion of homozygous transgenic and la3b out-segregant plants was compared to that of wild-type and la3b mutant plants (B). Fresh and dry weights were compared after 28 d (C). For wild-type, la3b and la2b values are the means ± SE of measurements made on ten individual rosettes. For transgenic lines values are means ± SE of measurements made on thirty rosettes, ten from each of the three lines. See Table S4 for full dataset. Abbreviation: seg; segregating T₃ wild-type.

Figure 5. Photosynthesis response curves of rbcs mutants and transgenic lines. Measurements were made on the sixth or seventh leaf of 35-45-d-old non-flowering rosettes. A/PAR curves show the response of CO₂ assimilation rates to different light levels (PAR, photosynthetic active radiation) at ambient CO₂ levels of 400 µmol mol⁻¹ (A). A/Cᵢ curves show the response of net CO₂ assimilation to different sub-stomatal concentrations of CO₂ (Cᵢ) under saturating light (1,500 µmol photons m⁻² s⁻¹) (B). For wild-type, la3b and la2b 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 twelve rosettes, four from each of the three lines.

Figure 6. Non-photochemical quenching response to light in leaves of rbcs mutants and transgenic lines. All plants were 28-d-old. For wild-type, la3b and la2b 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 twelve plants, four from each of the three lines.
Table 1. Catalytic parameters of Rubisco in *rbcs* mutants and transgenic lines. Rubisco specificity was determined from at least ten 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. Abbreviations: $K_c$, $K_m$ for CO$_2$ at 0% O$_2$; $K_c^\text{air}$, $K_m$ for CO$_2$ at 21% O$_2$; $k_{\text{cat}}$, turnover number (mol carboxylation product mol$^{-1}$ active site s$^{-1}$); $k_{\text{cat}}/K_c^\text{air}$, Rubisco carboxylation efficiency at 21% O$_2$; $S_{C/O}$, Rubisco specificity factor.

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

* indicates significant difference (P< 0.05) as determined by ANOVA followed by Tukey’s HSD tests.
Table 2. Variables derived from photosynthetic response curves, based on gas exchange analysis of 35- to 45-d-old plants. For measurements of \( A/\text{PAR} \), relative humidity was maintained at 68 ± 4% and ambient CO\(_2\) levels at 400 µmol mol\(^{-1}\). For measurements of \( A/C_i \), relative humidity was maintained at 73 ± 1% under a constant illumination of 1,500 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. Abbreviations: \( A_{\text{amb}} \), net photosynthesis measured at ambient CO\(_2\) and growth chamber light levels; \( A_{\text{max}} \), light saturated CO\(_2\) assimilation rate at ambient CO\(_2\); \( g_s \), stomatal conductance to CO\(_2\) (at ambient CO\(_2\)); \( \Phi \), apparent quantum efficiency; LCP, light compensation point; \( V_{c,\text{max}} \), maximum rate of Rubisco carboxylation; \( J_{\text{max}} \), maximum electron transport rate; \( \Gamma \), CO\(_2\) compensation point (\( C_i-A \)); \( R_d \), respiration in the dark.

<table>
<thead>
<tr>
<th>Variable (µmol CO(_2) m(^{-2}) s(^{-1}))</th>
<th>wild-type</th>
<th>1a3b</th>
<th>1a2b</th>
<th>1A(_{\text{ml}})</th>
<th>1A(_{\text{mMOD}})</th>
<th>S2(_{\text{Cr}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A_{\text{amb}} )</td>
<td>5.7 ± 0.1(^a)</td>
<td>3.4 ± 0.3(^c)</td>
<td>4.7 ± 0.1(^b)</td>
<td>5.3 ± 0.2(^{ab})</td>
<td>5.1 ± 0.2(^{ab})</td>
<td>5.0 ± 0.1(^{ab})</td>
</tr>
<tr>
<td>( A_{\text{max}} )</td>
<td>13.5 ± 0.5(^b)</td>
<td>6.8 ± 0.5(^c)</td>
<td>10.4 ± 0.2(^a)</td>
<td>12.8 ± 0.6(^{ab})</td>
<td>12.3 ± 0.7(^{ab})</td>
<td>10.9 ± 0.4(^{bc})</td>
</tr>
<tr>
<td>( g_s ) (mol CO(_2) m(^{-2}) s(^{-1}))</td>
<td>0.34 ± 0.06(^a)</td>
<td>0.42 ± 0.06(^a)</td>
<td>0.33 ± 0.02(^a)</td>
<td>0.3 ± 0.02(^a)</td>
<td>0.36 ± 0.03(^a)</td>
<td>0.41 ± 0.03(^a)</td>
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<tr>
<td>( \Phi ) (mmol CO(_2) mol(^{-1}) photons)</td>
<td>55.9 ± 1.9(^a)</td>
<td>42.2 ± 2.3(^b)</td>
<td>55.3 ± 1.4(^a)</td>
<td>53.5 ± 3.3(^a)</td>
<td>51.5 ± 1.8(^a)</td>
<td>53.6 ± 0.4(^a)</td>
</tr>
<tr>
<td>LCP (µmol CO(_2) m(^{-2}) s(^{-1}))</td>
<td>16.6 ± 1.3(^a)</td>
<td>18.8 ± 0.7(^a)</td>
<td>22.7 ± 0.8(^a)</td>
<td>18.0 ± 2.6(^a)</td>
<td>17.0 ± 1.5(^a)</td>
<td>20.9 ± 1.6(^a)</td>
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<tr>
<td>( V_{c,\text{max}} ) (µmol CO(_2) m(^{-2}) s(^{-1}))</td>
<td>31.4 ± 1.4(^a)</td>
<td>14.6 ± 0.4(^c)</td>
<td>20.9 ± 0.4(^c)</td>
<td>27.1 ± 1.3(^{ab})</td>
<td>26.1 ± 1.1(^{ab})</td>
<td>22.2 ± 0.6(^{bc})</td>
</tr>
<tr>
<td>( J_{\text{max}} ) (mmol e(^{-}) m(^{-2}) s(^{-1}))</td>
<td>73.3 ± 2.8(^a)</td>
<td>32.8 ± 1.3(^d)</td>
<td>53.7 ± 0.9(^c)</td>
<td>66.6 ± 3.0(^{ab})</td>
<td>63.7 ± 2.1(^{ab})</td>
<td>56.3 ± 1.6(^{bc})</td>
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<tr>
<td>( \Gamma ) (µmol CO(_2) mol(^{-1}))</td>
<td>39.4 ± 2.2(^b)</td>
<td>60.0 ± 3.9(^a)</td>
<td>42.8 ± 1.6(^b)</td>
<td>39.1 ± 0.9(^b)</td>
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<td>( R_d ) (µmol CO(_2) m(^{-2}) s(^{-1}))</td>
<td>1.9 ± 0.2(^a)</td>
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<td>1.8 ± 0.1(^a)</td>
<td>1.9 ± 0.1(^a)</td>
<td>1.8 ± 0.1(^a)</td>
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<td>Initial slope (( A/C_i ))</td>
<td>0.055 ± 0.003(^a)</td>
<td>0.024 ± 0.007(^d)</td>
<td>0.034 ± 0.006(^c)</td>
<td>0.045 ± 0.002(^b)</td>
<td>0.044 ± 0.002(^b)</td>
<td>0.036 ± 0.001(^{bc})</td>
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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$_2$. Plants (35 to 40-d-old) were measured under 300 µmol photons m$^{-2}$ s$^{-1}$, and ambient CO$_2$ of 300 µmol mol$^{-1}$ as in Bellasio *et al.* (2014). For wild-type, *1a3b* and *1a2b* values are the means ± SE of measurements made on individual leaves from five different rosettes. For transgenic lines values are means ± SE of measurements made on fifteen rosettes, five from each of the three lines. Values are followed by letters indicating significant difference (P <0.05), as determined by ANOVA followed by Tukey’s HSD tests. Values followed by the same letter are not significantly different. Abbreviations: $GA_{low}$, gross photosynthetic rate ($A+R_d$) under 2% O$_2$ (2%); $GA_{amb}$, gross photosynthetic rate under 21% O$_2$; $J_{NADPH_{low}}$, NADPH produced for photosynthesis (derived from electron transport rate) under 2% O$_2$; $J_{NADPH_{amb}}$, NADPH produced for photosynthesis under 21% O$_2$; $V_O$, Rubisco oxygenation rate; $V_C$, Rubisco carboxylation rate.

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>1a3b</th>
<th>1a2b</th>
<th>1A$_{At}$</th>
<th>1A$_{At}$MOD</th>
<th>S2$_{Cr}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$GA_{low}$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>9.48 ± 0.56$^a$</td>
<td>4.58 ± 0.4$^e$</td>
<td>5.69 ± 0.53$^a$</td>
<td>8.73 ± 0.09$^{ab}$</td>
<td>8.57 ± 0.64$^{ab}$</td>
<td>6.72 ± 0.49$^{de}$</td>
</tr>
<tr>
<td>$GA_{amb}$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>6.17 ± 0.36$^a$</td>
<td>2.98 ± 0.25$^a$</td>
<td>3.67 ± 0.36$^{ab}$</td>
<td>5.6 ± 0.15$^{ab}$</td>
<td>5.49 ± 0.42$^{ab}$</td>
<td>4.36 ± 0.38$^{bc}$</td>
</tr>
<tr>
<td>$J_{NADPH_{low}}$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>18.9 ± 1.1$^a$</td>
<td>9.1 ± 0.8$^c$</td>
<td>11.4 ± 1.1$^c$</td>
<td>17.5 ± 0.2$^{ab}$</td>
<td>17.1 ± 1.3$^{ab}$</td>
<td>13.4 ± 0.9$^{bc}$</td>
</tr>
<tr>
<td>$J_{NADPH_{amb}}$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>19.9 ± 0.9$^a$</td>
<td>9.2 ± 0.7$^c$</td>
<td>11.9 ± 0.9$^{bc}$</td>
<td>18.4 ± 0.2$^a$</td>
<td>18.2 ± 1.4$^a$</td>
<td>14.1 ± 1$^b$</td>
</tr>
<tr>
<td>$V_O$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>2.21 ± 0.13$^a$</td>
<td>1.06 ± 0.11$^b$</td>
<td>1.34 ± 0.12$^b$</td>
<td>2.09 ± 0.06$^a$</td>
<td>2.06 ± 0.17$^a$</td>
<td>1.57 ± 0.07$^{abc}$</td>
</tr>
<tr>
<td>$V_C$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>7.27 ± 0.43$^a$</td>
<td>3.52 ± 0.29$^d$</td>
<td>4.35 ± 0.41$^c$</td>
<td>6.64 ± 0.12$^{ab}$</td>
<td>6.52 ± 0.49$^{ab}$</td>
<td>5.15 ± 0.42$^{bc}$</td>
</tr>
<tr>
<td>$V_O/V_C$</td>
<td>0.304 ± 0.002$^a$</td>
<td>0.302 ± 0.013$^a$</td>
<td>0.307 ± 0.008$^a$</td>
<td>0.313 ± 0.015$^a$</td>
<td>0.316 ± 0.012$^a$</td>
<td>0.307 ± 0.011$^a$</td>
</tr>
</tbody>
</table>
Figure 1

200x157mm (300 x 300 DPI)
Figure 2
Fig. 2
99x41mm (300 x 300 DPI)
Figure 3
Fig. 3
139x89mm (300 x 300 DPI)
Figure 4
Fig. 4
189x109mm (300 x 300 DPI)
Figure 5
Fig. 5
249x283mm (300 x 300 DPI)
Figure 6
Fig. 6
129x70mm (300 x 300 DPI)