Targeted knockdown of ribulose-1, 5-bisphosphate carboxylase-oxygenase in rice mesophyll cells

Chirag Maheshwari a, Robert A. Coe a, Shanta Karki a, Sarah Covshoff b, Ronald Tapia a, Aruna Tyagi c, Julian M. Hibberd b, Robert T. Furbank d, William Paul Quick a, e, Hsiang-Chun Lin b, e, *

a C4Rice Centre, International Rice Research Institute (IRRI), Los Baños, Philippines
b ARC Centre of Excellence for Translational Photosynthesis, Research School of Biology, The Australian National University, Acton, 2601, Australia
c Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, United Kingdom
d Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, United Kingdom

* Corresponding author.
E-mail address: h.lin@irri.org (H.-C. Lin).

A R T I C L E  I N F O
Keywords: Oryza sativa Antisense Rubisco RBCS C4 rice

A B S T R A C T
We generated antisense constructs targeting two of the five Rubisco small subunit genes (OsRBCS2 and 4) which account for between 30–40 % of the RBCS transcript abundance in leaf blades. The constructs were driven by a maize phosphoenolpyruvate carboxylase (PEPC) promoter known to have enriched expression in mesophyll cells (MCs). In the resulting lines leaf, Rubisco protein content was reduced by between 30–50 % and CO2 assimilation rate was limited under photorespiratory and non-photorespiratory conditions. A relationship between Rubisco protein content and CO2 assimilation rate was found. This was associated with a significant reduction in dry biomass accumulation and grain yield of between 37–70%. In addition to serving as a resource for reducing Rubisco accumulation in a cell-preferential manner, these lines allow us to characterize gene function and isoform specific suppression on photosynthesis and growth. Our results suggest that the knockdown of multiple genes is required to completely reduce Rubisco accumulation in MCs.

1. Introduction

In C4 photosynthesis the first step of CO2 fixation is carried out by ribulose-1, 5-bisphosphate carboxylase-oxygenase (Rubisco, EC 4.1.1.39) in the chloroplast of mesophyll cells (MCs). Photosynthetic rate is limited by the activity of Rubisco because of its extremely low catalytic turnover rate and competing oxygenation reaction, which leads to the formation of toxic metabolites which must be broken-down by a series of reactions in a process known as photorespiration (von Caemmerer et al., 2012) was established with this goal in mind. To investigate

Abbreviation: A, net rate of CO2 assimilation; ACi, photosynthesis CO2 response curves; BSC, bundle sheath cell; Ca, ambient CO2 concentration; CE, carboxylation efficiency; Chl, chlorophyll; GI, intercellular CO2 concentration; rbcL, large subunit of Rubisco; MC, mesophyll cell; PPDF, photosynthetic photon flux density; R4, dark respiration rate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; RBCS, small subunit of Rubisco.

https://doi.org/10.1016/j.jplph.2021.153395
Received 28 October 2020; Received in revised form 6 February 2021; Accepted 21 February 2021
Available online 23 February 2021
0176-1617/© 2021 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
the feasibility of such an endeavor a toolkit of transgenic resources is being assembled (Kajala et al., 2011; Ermakova et al., 2019). Among these, there are lines in which parts of the photorespiratory cycle have been selectively downregulated to test the hypothesis of whether this primes a plant for C₂ photosynthesis (Lin et al., 2016). Here we report on the downregulation and translocation of Rubisco from MCs to BSCs and the effect on rice photosynthesis and growth.

In rice, the Rubisco holoenzyme is made up of eight large Rubisco subunits (rbcLs) encoded by a single gene on the chloroplast DNA and eight small Rubisco subunits (RBCSs) encoded by a five multigene family on the nucleus DNA (Dean et al., 1989; Rodermel, 1999; Sasanuma, 2000). OsRBCS5 is located on chromosome 2 but is not expressed in the leaf blade (Morita et al., 2014; Suzuki et al., 2007, 2009). The remaining four genes (OsRBCS2-OsRBCS5) are in a tandem array on chromosome 12 and are highly expressed in photosynthetic active tissues such as leaf blades (Suzuki et al., 2009). The deduced amino acid sequence without the transit peptide for targeting the chloroplast are completely identical, leading to the assumption that there are no functional differences within the transit peptide for targeting the chloroplast are completely identical, leading to the assumption that there are no functional differences within the RCCS gene family within a species. It has previously been shown that suppression of a RCCS gene leads to a reduction in the holoenzyme (Hudson et al., 1992; Makino et al., 1997; Rodermel et al., 1988) with the expression of \( \text{rbcL} \) regulated at its transcript level in response to the availability of RCCS protein (Makino et al., 1997; Suzuki and Makino, 2012). Although RCCS protein has no catalytic function for \( \text{CO}_2 \) fixation, it is important for maximal activity and structural stability for the Rubisco holoenzyme (Andersson and Backlund, 2008).

To reduce Rubisco protein accumulation, we generated antisense constructs targeting two of the five \( \text{RCCS} \) genes (OsRBCS2 and 4) expressed in photosynthetic tissue. Together these account for between 30–40 % of the \( \text{RCCS} \) transcript abundance in leaf blades (Suzuki et al., 2009), allowing a substantial reduction in Rubisco protein accumulation to be achieved without a lethal phenotype developing. Lines must produce viable seed to be crossed with lines expressing \( \text{C}_4 \) enzyme genes facilitating gradual replacement of \( \text{C}_4 \) with \( \text{C}_2 \) photosynthesis (Kajala et al., 2011). The antisense genes against OsRBCS2 and 4 are driven by a maize phosphoenolpyruvate carboxylase (PEPC) promoter known to have enriched expression in MCs (Matsuoaka et al., 1994). In addition to serving as a resource for reducing Rubisco accumulation in a cell-specific manner, these lines allow us to characterize gene function and isoform specific suppression on photosynthesis and growth.

2. Materials and methods

2.1. Generation of OsRBCS2 and OsRBCS4 knockdown transgenic rice lines

To individually reduce OsRBCS2 (Os12g17600) and OsRBCS4 (Os12g19470) expression in rice MCs, the full coding sequences (CDS) of LOC_Os12g17600.1 and LOC_Os12g19470.2 were cloned separately in the antisense direction via RT-PCR using the following primers: \( 5′-\text{CACCTTAGGTGCCACCGACTCT} \) and \( 5′-\text{ATGGGCCCCTCCTGGTAT} \) for OsRBCS2, \( 5′-\text{CACCTTAGGTGCCACCGACTCT} \) and \( 5′-\text{ATGGGCTCCCTGGGATGC} \) for OsRBCS4. The CAC at the 5′ end of the forward primer allowed directional cloning into pENTR/D-TOPO (ThermoFisher Scientific, USA) and subsequent Gateway cloning into the pSC110 expression vector. The pSC110 vector was created by Gibson Assembly and contained the B73 ZmPEPC promoter to drive gene expression in rice MCs (Gibson et al., 2009; Osborn et al., 2017; Matsuoaka et al., 1994). Both antisense OsRBCS2 and OsRBCS4 vectors were verified by sequencing and transformed into rice (Oryza sativa indica cv IR64) using Agrobacterium-mediated transformation following the method described by Yin et al. (2019).

2.2. Plant growth conditions

Plants were grown at the International Rice Research Institute (IRRI), Los Baños, Philippines, 14°9′53.58″N, 121°15′32.19″E. Seeds were sown and germinated in 100 mL roottrainers (http://roottrainers.co.uk/) after one-week plants were transplanted into 7-liter pots filled with soil from the IRRI upland farm. Plants were grown in a containment transgenic screen house with a day/night temperature of 35 ± 3 °C/28 ± 3 °C and relative humidity of 70–90 %. Maximum light intensity was 2000 μmol photons m\(^{-2}\) s\(^{-1}\) on a clear sunny day.

2.3. PCR screening

Transgenic plants were subjected to genomic PCR screening to confirm the presence of OsRBCS antisense DNA sequence. PCR was carried out by using the KAPA 3 G plant PCR kit (Kapa Biosoym, USA; https://www.sigmaaldrich.com/life-science/roche-biochemica-l-reagents/kapa-genomics-reagents.html) with the gene-specific primers (5′-AGGACTGCCCATCTCATAAT and 5′-TGGATGTCGTCGTATCATC) for OsRBCS2, 5′-CATGTGACCAAATGCATC and 5′-AGCTGACAAAT GCGGAA for OsRBCS4). Plasmid DNA containing antisense OsRBCS2 and OsRBCS4 were used as positive controls and non-transgenic rice and water as negative controls. PCR conditions were as follows: pre-denaturation for 5 min at 95 °C, 30 cycles of the polymerization reaction consisting of a denaturation step for 20 s at 95 °C, an annealing step for 15 s at 60 °C, and an extension step for 1 min at 72 °C, and a final extension step for 5 min at 72 °C.

2.4. DNA blot analysis

Large-scale genomic DNA was extracted from leaves at the mid-tillering stage. DNA blot analysis was carried out as described by Lin et al. (2016) except genomic DNA was digested with BglII restriction endonuclease (New England Biolabs, USA; https://international.neb.com/).

2.5. Quantitative RT-PCR

Total RNA was extracted and analyzed as described in Lin et al. (2016) except for the primer pairs used. The mRNA level of each OsRBCS gene was quantified on a fold change basis in comparison with wild-type plants. 3′- untranslated regions (UTRs) of the OsRBCS gene family have different nucleotide sequences and so were utilized for amplification. The Elongation factor 1-alpha gene (OsEF1α; Os03g0177500) was used as an internal standard. Primers pairs were: 5′-CTTCGGCAAGTCA-GGATTG and 5′-AGCCGGCGGCTAAATATCA for OsRBCS2; 5′-TTCCAA GGGTCAAGTCCCAC and 5′-ATAGGCGAAGTCCACG for OsRBCS3; 5′-GTGGCCGTATGGGATTATTA and 5′-TCAGCCGCAACG for OsRBCS4; 5′-GGTTGGGGCGATGAAAGG and 5′-ACTGGGGGAAAGG GAAACCAAT for OsRBCS5; 5′-ACAGTGGTGTTGTGTGACG and 5′-GGGCT CTTGACCCAGTACAG for OsEF1α.

2.6. Soluble leaf protein

Leaf samples for soluble protein were harvested between 09:00 and 11:00 h from the fourth fully expanded leaf. Proteins were extracted and fractionated as described previously Lin et al. (2016). Samples were loaded based on an equal leaf area (0.396 mm² for rbcL and RBCS). After electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride membrane and probed with antisera against Rubisco protein (provided by Richard Leegood, Sheffield University, UK) at a dilution of 1:200. A peroxidase-conjugated secondary antibody was used at a dilution of 1:200 and immunoreactive bands were visualized with ECL Western Blotting Detection Reagents (GE Healthcare, USA; https://www.ge lifesciences.com). Rubisco protein content was quantified from SDS-page gels using ALPHA-Ease FC software (Alpha Innotech, USA). Measurements were made from one leaf per plant and four plants per line. Values are expressed as the percentage protein abundance compared to the wild-type.
2.7. Leaf chlorophyll content, plant growth analysis, and destructive harvesting

Leaf chlorophyll content was measured with a SPAD 502 Chlorophyll Meter (SPAD, Konica Minolta; https://www.konicaminolta.com) at the mid-tillering stage on the upper fully expanded leaves. Values given are the average ± SE of three measurements from six plants per line. Plant height was measured from the soil surface to the tip of the youngest fully expanded leaf. The total tiller number was counted prior to harvesting for destructive measurements. All above-ground biomass (leaves, stems, and sheaths) were harvested, weighed, and placed in paper bags, and oven dried at 70 °C until a constant dry biomass weight was achieved. Values of plant height, total tiller number, and dry biomass are presented as the average ± SE of ten plants per line.

2.8. Gas exchange measurement

Leaf gas exchange measurements were made at IRRI using a LI-6400XT portable photosynthesis system (LI-COR Biosciences, USA; https://www.licor.com) as described in Lin et al. (2016). Measurements were made from the three youngest fully expanded leaves for each plant during the tillering stage, 60–65 days post-germination.

2.9. Immunolocalization

Leaf samples were harvested between 09:00 h and 11:00 h from 9-week-old plants. The middle portion of the seventh fully expanded leaf was dissected and fixed for four hours in a solution containing 4% paraformaldehyde, 0.2% glutaraldehyde, and 25 mM sodium phosphate buffer pH 7.2. After fixation, sections were rinsed four times in 25 mM phosphate buffer over a period of 60 min. Thin leaf sections were cut using a razor blade and blocked in TBST buffer (0.1% Tween 20, 20 mM Tris, 154 mM NaCl) containing 3% milk for two hours at room temperature. Sections were probed with antisera against Rubisco protein diluted 1:100 in blocking solution and incubated overnight at 4 °C. The remaining steps were performed at room temperature. Sections were washed six times with blocking solution and then incubated for two hours with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, USA; https://www.thermofisher.com/ph/en/home/brands/invitrogen.html) at 37 °C in the dark. Sections were washed six times with blocking solution, post-stained with 0.05% calcofluor white for five min, and washed with distilled water. Sections were mounted on microscope slides in 50% glycerol and examined on a BX61 Disk Scanning microscope (Olympus, USA; https://www.olympus-global.com) with fluorescence function under DAPI, RFP and GFP filters.

2.10. Leaf anatomy analysis

Leaf width was measured at the middle portion of the fully expanded penultimate leaf. Between 2 to 4 mm² of the middle portion of the penultimate leaf was cut and fixed in formaldehyde-acetic acid-alcohol (FAA) solution under vacuum (20 psig) at room temperature for at least 12 h. Leaf sections were rinsed twice in water for 60 min. Leaf sections were dehydrated using an ethanol series, incubated twice in 70%
ethanol for 30 min; once in 80 % ethanol for 30 min; once in 90 % ethanol for 30 min, and finally three times in 100 % ethanol for 30 min. Leaf sections were infiltrated in 10 % ethanol and varying concentrations of Spurr’s resin solution (10%–100%). Sections were incubated for at least 60 min at each concentration solution. Leaf sections were then finally infiltrated twice for 4 h with 100 % Spurr’s resin solution. Sections were placed in fresh 100 % Spurr’s resin solution in molds. Samples were polymerized overnight at 70 °C. Embedded leaf sections were cut into 10–15 μm thick sections on a microtome (MT2-B, DuPont-Instruments-Sorvall, USA) and then dried. Sections were stained with 0.05 % toluidine blue O stain in 0.1 % sodium carbonate, pH 11.1 for 5 min then rinsed four times with distilled water, each for 5 min. Sections were dried and mounted on slides using Permount™ (Fischer Scientific, USA; https://www.fishersci.com/us/en/home.html). Transverse images were acquired using a BX51 Olympus microscope (Olympus, USA; https://www.olympus-global.com/ ) at 4X and 40X magnification. Mesophyll cell number was counted by calculating the numbers of mesophyll cells in between minor veins. Leaf thickness, interveinal distance (IVD), vein number (major and minor), mesophyll cell length was measured as described in Chatterjee et al. (2016).

2.11. Statistics

Statistical analyses were performed using Statistical Tool for Agricultural Research (STAR) software (International Rice Research Institute, Philippines) using a one-way analysis of variance (ANOVA) or a Student’s t-test with a P-value of < 0.05.

3. Results

3.1. OsRBCS2 and OsRBCS4 specific knockdown lines

A total of 190 T3 plants were PCR positive for the antisense-OsRBCS2 construct and 30 T0 plants for the antisense-OsRBCS4 construct. Plants with reduced Rubisco protein accumulation relative to the wild-type rice were selected for DNA blot analysis in order to confirm T-DNA integration. Two OsRBCS2 knockdown events (rbcs2-165 and rbcs2-266) and two OsRBCS4 knockdown events (rbcs4-022 and rbcs4-053) with the lowest Rubisco accumulation were selected from T1 progeny. These were advanced generating a total of 40 PCR positive T2 generation OsRBCS2 knockdown plants (from two events rbcs2-165 and rbcs2-266)

<table>
<thead>
<tr>
<th>(A)</th>
<th>rbc2-165</th>
<th>rbc2-266</th>
<th>rbc2-022</th>
<th>rbc2-053</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbcL</td>
<td>WT 1 2 3 4</td>
<td>WT 1 2 3 4</td>
<td>WT 1 2 3 4</td>
<td>WT 1 2 3 4</td>
</tr>
<tr>
<td>RBCS</td>
<td>WT 1 2 3 4</td>
<td>WT 1 2 3 4</td>
<td>WT 1 2 3 4</td>
<td>WT 1 2 3 4</td>
</tr>
</tbody>
</table>

Fig. 2. Soluble leaf protein. (A) SDS-PAGE (B) Immunoblots with antibodies against rbcL and RBCS. Wild-type (WT) and plants of rbc2s and rbc4s knockdown lines (numbers). Total extracted proteins were separated by 12 % SDS-gel. Samples were loaded based on equal leaf area of 3.96 mm².

Table 1

<table>
<thead>
<tr>
<th>Rubisco large subunit protein content.</th>
<th>Percentage abundance to WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbc2-165</td>
<td>72 ± 0.20 *</td>
</tr>
<tr>
<td>rbc2-266</td>
<td>59 ± 0.19 *</td>
</tr>
<tr>
<td>rbc4-022</td>
<td>50 ± 0.25 *</td>
</tr>
<tr>
<td>rbc4-053</td>
<td>61 ± 0.30 *</td>
</tr>
</tbody>
</table>

Measurements were made from one leaf per plant and four plants per line. Values are expressed as the percentage protein abundance compared to the wild-type (WT). Values are the average ± SE. Asterisk denotes statistically significant differences with WT, Student t-test, p-value ≤ 0.05.

and 40 T2 generation OsRBCS4 knockdown plants (from two events rbcs4-022 and rbcs4-053) (Fig. S1). DNA blot analysis showed that these transgenic lines carried between two and four copies of the antisense constructs (Fig. S2). This led to a reduction in the accumulation of OsRBCS2 transcripts of between 15 and 24-fold (Fig. 1A) and OsRBCS4 transcripts of between 6 and 12-fold in the respective knockdown lines (Fig. 1C). Reduction of either isoform was also associated with a slight increase in the accumulation of OsRBCS3 and OsRBCS5 transcripts (Fig. 1B & D). These results indicate that the OsRBCS2 and OsRBCS4 genes were selectively suppressed by the antisense approach. Correspondingly the immunoblot analysis showed a reduction in the accumulation of RBCS in both OsRBCS2 and OsRBCS4 knockdown lines and no detectable RBCS protein in event rbcs2-266 and rbcs4-53 (Fig. 2B). There are coordinated reductions of RBCS and rbcL in both rbcs2 and rbcs4 lines and rbcL protein were reduced about 28 % in rbcs2-165, 41 % in rbcs2-266, 50 % in rbcs4-022 and 39 % rbcs4-53 compared to wild-type plants (Fig. 2A; Table 1). Immunolocalization confirmed reduction of Rubisco protein preferentially in the MCs (Fig. S3). Reduction in Rubisco content was correlated with the number of antisense inserts. rbcs2-266 and rbcs4-022 carried four and three antisense inserts, respectively, and their Rubisco protein contents were lower compared to the events carrying fewer antisense inserts.

3.2. Photosynthetic perturbations associated with reduced Rubisco

The response of the net rate of CO2 assimilation (A) to intercellular CO2 concentration (Ci) was measured under photorespiratory (21 % O2; Fig. 3A) and non-photorespiratory conditions (2% O2; Fig. 3B) at high
discernable relationship between CO$_2$ assimilation rates less than half those of wild-type plants. There was no highest intercellular CO$_2$ curvation and convergence in responses under non-photorespiratory conditions were similar to those lowest Rubisco content among four percentage reduction in Rubisco protein as compared to the other lines for the same gene; this was related to per assimilation were much lower in irradiance (2000 μmol photons m$^{-2}$ s$^{-1}$) where RuBP-saturated rates of Rubisco are limiting to photosynthesis. Under photorespiratory conditions, A was significantly lower in both rbsc2 and rbsc4 lines at all intercellular CO$_2$ concentrations compared to wild-type rice, although photosynthesis was not saturated in any of the lines. Rates of CO$_2$ assimilation were much lower in rbsc4-022 and rbsc2-226 lines compared to the other lines for the same gene; this was related to percentage reduction in Rubisco protein as rbsc2-22 and rbsc2-226 had lowest Rubisco content among four rbsc knockdown lines (Fig. 1). The responses under non-photorespiratory conditions were similar to those under photorespiratory conditions, except for a slight decrease in curvature and convergence in rbsc2-165 and wild-type response at the highest intercellular CO$_2$ concentrations (Fig. 3B). These results suggest that photosynthesis was limited by Rubisco under almost all conditions. Consistent with this, carboxylation efficiency (CE) was significantly reduced under both conditions, but most markedly under photorespiratory conditions (Table 2), indicative of Rubisco limited capacity in the leaf. CO$_2$ compensation points (r) were significantly higher in event rbsc2-266 and rbsc4-022 compared with wild-type plants under both conditions (Table 2). In response to changes in irradiance, CO$_2$ assimilation in wild-type plants was not saturated at 2000 μmol photon m$^{-2}$ s$^{-1}$ (Fig. 3C). In the knockdown lines, photosynthesis was marginally increased above PPFD 1000 μmol photon m$^{-2}$ s$^{-1}$ with CO$_2$ assimilation rates less than half those of wild-type plants. There was no discernable relationship between CO$_2$ assimilation rate and Rubisco protein reduction (Fig. S4), although there were line specific differences, with notably higher rates in rbsc4-053 compared to other antisense lines. Quantum efficiency (Φ) and respiration rates (Rd) were statistically significantly lower in all rbsc lines compared with wild-type plants (Table 2).

3.3. Phenotypic perturbations associated with reduced Rubisco

Reduction of Rubisco protein in rbsc2 and rbsc4 knockdown lines led to a statistically significant reduction in grain yield of between 37–70% (Table 3), with considerable variation observed between individual lines. In all lines except rbsc2-165, there was a corresponding statistically significant reduction in dry biomass, with rbsc4 lines exhibiting the largest decrease relative to the wild-type plants. There were no statistically significant differences in tiller number but rbsc4 lines were slightly shorter than wild-type plants (Table 3). There were no consistent differences in leaf chlorophyll contents, with small but statistically significant reductions in rbsc4 lines and higher chlorophyll contents in rbsc2-266. The difference in phenotype perturbations between lines follows protein abundance with rbsc2-165 exhibiting the lowest Rubisco protein reduction and smallest phenotypic perturbations and rbsc4-022 the largest Rubisco protein reduction and most significant phenotypic perturbations (Fig. S5).

3.4. Effect of reduced Rubisco on leaf anatomy

There were statistically significant differences in interveinal distance (IVD) between individual rbsc knockdown lines (Table 4), with rbsc2-165 having the widest IVD and rbsc4-022 line the lowest. Mesophyll cell number was significantly reduced in all the rbsc knockdown lines. The mesophyll cell length was increased in all rbsc knockdown lines but was
only statistically significant in rbc2-165 (Table 4). No difference in the number and thickness of major or minor veins were found. Leaves of both rbc2-4 knockout lines were significantly narrower than either the wild-type or rbc2-2 lines, which correspond with reduced IVD and MC number.

4. Discussion

To mimic the downregulation of part of the Calvin–Benson cycle in MCs that is required for C₄ photosynthesis, we have successfully targeted two OsRBCS genes, OsRBCS2 and OsRBCS4. Genetic redundancy within the OsRBCS gene family does not completely compensate for a reduction in the expression of a single multigene family member (Kanno et al., 2017; Ogawa et al., 2012). Constructs designed to independently reduce expression of OsRBCS2 and OsRBCS4 in the MCs led to a significant reduction in gene transcript for the target gene with modest increases in the other multigene family members (Fig. 1). These results are consistent with previous studies showing that the expressions of each OsRBCS genes were regulated independently from other OsRBCS genes (Kanno et al., 2017; Ogawa et al., 2012). In leaf blades of wild-type plants, OsRBCS2 transcripts accumulate to similar levels as those of OsRBCS4 (Suzuki et al., 2009). However, the effect of suppression of OsRBCS2 antisense was stronger with a larger reduction in OsRBCS2 transcript accumulation in rbc2 knockout lines. In rbc2 knockout lines, Rubisco protein accumulation was 30–40 % lower, which is consistent with reports from previous studies where Rubisco content was reduced by up to 45 % in OsRBCS2 cDNA antisense rice lines (Makino et al., 1997, 2000). Compared to the rbc2 knockout lines, the rbc2-4 knockout lines had a smaller reduction in OsRBCS4 transcript accumulation, but had a larger reduction in Rubisco protein accumulation. These results indicate that the transcript expressions of the OsRBCS genes were not followed by proportional changes in the Rubisco content, suggesting that the OsRBCS genes are independently controlled and a post-transcriptional regulation is involved in Rubisco protein accumulation (Whitney et al., 2011; Ogawa et al., 2012). Although we did not generate lines targeting all the OsRBCS multigene family, these results

**Table 2**

Comparison of photosynthesis parameters.

<table>
<thead>
<tr>
<th>r µmol CO₂ m⁻² s⁻¹</th>
<th>rCE µmol CO₂ m⁻² s⁻¹ µmol CO₂ mol⁻¹</th>
<th>rR µmol CO₂ m⁻² s⁻¹</th>
<th>Φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 % O₂</td>
<td>51.69 ± 1.59</td>
<td>0.126 ± 0.02a</td>
<td>0.53 ± 0.05a</td>
</tr>
<tr>
<td>rbc2-165</td>
<td>51.92 ± 0.15</td>
<td>0.067 ± 0.05b</td>
<td>0.41 ± 0.03b</td>
</tr>
<tr>
<td>rbc2-266</td>
<td>66.72 ± 1.45</td>
<td>0.046 ± 0.00</td>
<td>0.39 ± 0.07b</td>
</tr>
<tr>
<td>rbc2-022</td>
<td>57.77 ± 1.58</td>
<td>0.05 ± 0.01b</td>
<td>0.35 ± 0.02b</td>
</tr>
<tr>
<td>rbc2-053</td>
<td>49.85 ± 2.71</td>
<td>0.06 ± 0.01b</td>
<td>0.48 ± 0.05b</td>
</tr>
<tr>
<td>2 % O₂</td>
<td>13.32 ± 0.95</td>
<td>0.166 ± 0.04b</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 3**

Leaf chlorophyll content, plant height, tiller number, dry biomass and grain yield of wild-type and rbc2 knockout lines.

<table>
<thead>
<tr>
<th>Chl (SPAD value)</th>
<th>Plant height cm</th>
<th>Tiller number</th>
<th>Dry biomass g</th>
<th>Grain yield g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>43.14 ± 0.75c</td>
<td>105 ± 1.4a</td>
<td>26.1 ± 4.5bc</td>
<td>58.16 ± 10.93a</td>
</tr>
<tr>
<td>rbc2-165</td>
<td>42.30 ± 0.71bc</td>
<td>105.6 ± 1.9a</td>
<td>29.2 ± 3.9a</td>
<td>57.36 ± 6.81a</td>
</tr>
<tr>
<td>rbc2-266</td>
<td>44.04 ± 0.93a</td>
<td>103.6 ± 1.2a</td>
<td>27.5 ± 4.4ab</td>
<td>44.32 ± 5.12b</td>
</tr>
<tr>
<td>rbc2-022</td>
<td>41.84 ± 1.20a</td>
<td>101.4 ± 1.8b</td>
<td>22.6 ± 4.9bc</td>
<td>32.60 ± 8.76c</td>
</tr>
<tr>
<td>rbc2-053</td>
<td>42.00 ± 0.97b</td>
<td>99.4 ± 3.0b</td>
<td>23.4 ± 4.8a</td>
<td>33.19 ± 8.23c</td>
</tr>
</tbody>
</table>

**Table 4**

Leaf width, leaf thickness, interveinal distance (IVD), vein number and mesophyll cell characteristics of wild-type and rbc2 knockout lines.

<table>
<thead>
<tr>
<th>Leaf width (cm)</th>
<th>Leaf thickness (µm)</th>
<th>IVD (µm)</th>
<th>No. of major vein</th>
<th>No. of minor vein</th>
<th>No. of Mesophyll cell</th>
<th>Mesophyll cell length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.4 ± 0.08a</td>
<td>177.45 ± 7.86a</td>
<td>88.82 ± 5.44a</td>
<td>240.94 ± 11.75ab</td>
<td>10.4 ± 0.9a</td>
<td>40.2 ± 1.4a</td>
</tr>
<tr>
<td>rbc2-165</td>
<td>1.36 ± 0.06a</td>
<td>175.10 ± 11.42a</td>
<td>87.93 ± 4.63a</td>
<td>245.80 ± 9.27a</td>
<td>11.6 ± 0.5a</td>
<td>40.8 ± 2.5a</td>
</tr>
<tr>
<td>rbc2-266</td>
<td>1.34 ± 0.05a</td>
<td>167.05 ± 8.66a</td>
<td>87.26 ± 3.12a</td>
<td>230.11 ± 4.74bc</td>
<td>11.6 ± 0.5a</td>
<td>42.4 ± 3.7a</td>
</tr>
<tr>
<td>rbc2-022</td>
<td>1.16 ± 0.07b</td>
<td>174.35 ± 7.98a</td>
<td>85.82 ± 1.76a</td>
<td>216.38 ± 7.11a</td>
<td>10.6 ± 0.7a</td>
<td>43.2 ± 1.0a</td>
</tr>
<tr>
<td>rbc2-053</td>
<td>1.2 ± 0.09b</td>
<td>159.95 ± 9.56a</td>
<td>82.70 ± 3.71a</td>
<td>226.89 ± 7.52ab</td>
<td>10.6 ± 0.6b</td>
<td>40.6 ± 2.6a</td>
</tr>
</tbody>
</table>

Leaf width is the average ± SE of two fully expanded penultimate leaves from ten individual plants. Leaf thickness of major veins is the average ± SE of four major veins of one fully expanded penultimate leaf from five individual plants. Leaf thickness of minor veins is the average ± SE of 10–12 minor veins of one fully expanded penultimate leaf from five individual plants. Interveinal distance (IVD) is the average ± SE of 5–6 transverse sections at both the left- and right-hand side of one fully expanded penultimate leaf from five individual plants. Major and minor vein numbers are the average ± SE of five individual plants. Mesophyll cell number and Mesophyll cell length (µm) are the average ± SE of 10–12 different sections of one fully expanded penultimate leaf from five individual plants. Different letters indicate statistical significance based on a one-way ANOVA (p < 0.05) with a Least Significant Difference (LSD) test for post-hoc pairwise comparison.

CO₂ compensation point (r), carboxylation efficiency (CE), respiration rate (Rₗ) and quantum efficiency (Φ) of wild type and rbc2 and rbc4 antisense lines. Measurements for CE and r were made at a photosynthetic photon flux density (PPFD) of 2000 µmol photons m⁻² s⁻¹, leaf temperature 30 °C and either 21 % or 2 % O₂. Measurements of Rₗ were made after 30 min in the dark. Measurements of Φ were made at a CO₂ concentration of 400 µmol CO₂ mol⁻¹ and a leaf temperature of 30 °C. Values represent the mean ± SE of three days from three individual plants. Different letters within groups indicate statistical significance from a one-way ANOVA (p < 0.05) and a least significant difference test (LSD) for post-hoc pairwise comparison.
suggested that targeting multiple genes is required to completely reduce Rubisco accumulation in MCs. In addition, insufficient chloroplast number and volume in the BSCs of rice is also a limitation for Rubisco translocation from MCs to BSCs. However, the genes regulating the activation of BS organelles in rice are still uncertain (Wang et al., 2017; Ermakova et al., 2019).

Reducing Rubisco protein content by 30 % or more in the antisense rice plants limits CO2 assimilation rates under photorespiratory conditions (21 % O2). A relationship between the reduction in Rubisco protein content and CO2 assimilation rate was observed, which is consistent with previous studies (Makino et al., 1997; Suzuki et al., 2012). On the other hand, other studies also reported that a relatively small decrease in Rubisco content (65–90 % of wild-type Rubisco) can lead to an increase in CO2 assimilation rate of between 5–15 % at elevated CO2 due to the excessive amount of Rubisco for photosynthesis in rice (Kanno et al., 2017; Makino et al., 1997, 2000). However here both rbcS2 and rbcS4 knockdown lines did not have a higher CO2 assimilation rate than wild-type plants in elevated CO2 conditions. The differences between results in this study and previous studies may due to the reduction of OsRBCS2 and OsRBCS4 being preferentially targeted to the MCs leading to a more severe reduction in Rubisco content in the MCs. On a protein quantity basis, the reduction of rbcS2 lines had a more significant impact on CO2 assimilation rate than rbcS4 lines given the percentage reduction in Rubisco protein. In addition, other studies reported that a reduction of Rubisco content slowed down the Calvin cycle, thus impacting the amounts of photosynthetic and related primary metabolites in Rubisco transgenic rice plants (Suzuki et al., 2012; Sudo et al., 2014). The demand for Rubisco synthesis was decreased in Rubisco knockdown rice line and that tended to increase the amounts of amino acids (Suzuki et al., 2012). Further metabolome analysis in the preferentially MC targeted antisense-rbcS plants would also be required for understanding how plants deal with the downregulation and translocation of Rubisco from MCs to BSCs.

A reduction in Rubisco protein content and CO2 assimilation was associated with a significant reduction in dry biomass accumulation and grain yield. The magnitude of the reduction was proportional to the reduction in Rubisco protein content (Fig. S5). Previous studies have shown that tobacco plants with more than 50 % reduced Rubisco had changes in plant growth such as a decrease in plant weight, root weight, and changes in leaf geometry (Quick et al., 1991). The rbcS knockdown lines had no difference in number of major and minor veins or leaf thickness compared to wild-type plants (Table 4). Evans et al. (1994) reported a small reduction (92 % of wild-type) in leaf thickness in the rbcS antisense tobacco lines when Rubisco was removed by more than 50 %. We also observed that the numbers of MCs in between minor veins were reduced and the mesophyll cell length was slightly increased in rbcS knockdown lines (Table 4). This suggests that there may be anatomical changes to the leaf associated with altering Rubisco protein content.

Our results show that preferentially MC targeted antisense-rbcS plants can be used to reduce Rubisco accumulation in rice to levels that induce a phenotype without lethality. The resulting knockdown plants have reduced CO2 assimilation rates with moderate effects on plant growth. This provides a useful foundation for installing C4 photosynthesis in rice.

Funding

This work was funded by C4 Rice Project grants from the Bill & Melinda Gates Foundation to IRRI (Grant ID#51586).

CRediT authorship contribution statement


Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

We wish to thank Efren Bagunu, Florencio Montecillo, Juvy Reyes, and Irma Canicosa for their help with plant transformation, husbandry, and physiological measurements at IRRI C4 Rice Centre.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2021.153395.

References


