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 d, Robert T. Furbank e, William Paul Quick a, b, c, Hsiang-Chun Lin a, *,

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

Abstract

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
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 photosynthetic cycle have been selectively downregulated to test the hypothesis of whether this primes a plant for C4 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, 2001). OsRBCS1 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 RBCS gene family within a species. It has previously been shown that suppression of a RBCS gene leads to a reduction in the holoenzyme (Hudson et al., 1992; Makino et al., 1997; Rodermel et al., 1988) with the expression of rbcL regulated at its transcript level in response to the availability of RBCS protein (Makino et al., 1997; Suzuki and Makino, 2012). Although RBCS protein has no catalytic function for CO2 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 RBCS genes (OsRBCS2 and 4) expressed in photosynthetic tissue. Together these account for between 30–40 % of the RBCS 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 C4 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 Biosystem, USA; https://www.sigmaaldrich.com/life-science/roche-biochemica-l-reagents/kapa-genomics-reagents.html) with the gene-specific primers (5′-AGGACTGGCCATCTCCFATT and 5′-TGATGGTCCTCGGATATCATC for OsRBCS2; 5′-CATGGACCAACGCGATTCA and 5′-AGGCTCGAAATGGGGAAAA for OsRBCS4). Plasmid DNA containing antisense OsRBCS2 and OsRBC4 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 BgII 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′-CTTGGGCAACTCTGAGCAATG and 5′-AGGCGGGCGGTAAATCTA for OsRBCS2; 5′-TTCCCAAGGGTCAAGTCTCAC and 5′-ATAGGCGGAGGAGGAGGAGG for OsRBCS3; 5′-GAGCCCGGATGGCAAGGATTA and 5′-TCAGCTCCACCGAGGAGG for OsRBCS4; 5′-GGTGTCGGCGGATGAAG and 5′-ACTGGGGAAAAAAGGGGAAACAT for OsRBCS5; 5′-CCACTGTTGTTTGTAGG and 5′-GGCGCCTTACKAGTCAG 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.gelifesciences.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 ps) 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 (rbc2-165 and rbc2-266) and two OsRBCS4 knockdown events (rbc4-022 and rbc4-053) 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 rbc2-165 and rbc2-266) and 40 T2 generation OsRBCS4 knockdown plants (from two events rbc4-022 and rbc4-053) (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 immunoblot analysis showed a reduction in the accumulation of RBCS in both OsRBCS2 and OsRBCS4 knockdown lines and no detectable RBCS protein in event rbc2-266 and rbc4-53 (Fig. 2B). There are coordinated reductions of RBCS and rbcL in both rbc2 and rbc4 lines and rbcL protein were reduced about 28 % in rbc2-266, 41 % in rbc2-266, 50 % in rbc4-022 and 39 % rbc4-53 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. rbc2-266 and rbc4-022 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

| Table 1 | Rubisco large subunit protein content. |
|---------|--------------------------------------|
| Percentage abundance to WT |
| rbc2-165 | 72 ± 0.20 | * |
| rbc2-266 | 59 ± 0.19 | * |
| rbc4-022 | 50 ± 0.25 | * |
| rbc4-053 | 61 ± 0.30 | * |

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.
discernable relationship between CO₂ assimilation rates less than half those of wild-type plants. There was no highest intercellular CO₂ curvature and convergence in under photorespiratory conditions, except for a slight decrease in curvature 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 rbcs2 and rbcs4 lines at all intercellular CO₂ concentrations compared to wild-type rice, although photosynthesis was not saturated in any of the lines. Rates of CO₂ assimilation were much lower in rbcs4-022 and rbcs2-226 lines compared to the other lines for the same gene; this was related to percentage reduction in Rubisco protein as rbcs4-22 and rbcs2-226 had lowest Rubisco content among four rbcs 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 rbcs2-165 and wild-type response at the highest intercellular CO₂ 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. CO₂ compensation points (r) were significantly higher in event rbcs2-266 and rbcs4-022 compared with wild-type plants under both conditions (Table 2). In response to changes in irradiance, CO₂ 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 CO₂ assimilation rates less than half those of wild-type plants. There was no discernable relationship between CO₂ assimilation rate and Rubisco protein reduction (Fig. S4), although there were line specific differences, with notably higher rates in rbcs4-053 compared to other antisense lines. Quantum efficiency (Φ) and respiration rates (Rd) were statistically significantly lower in all rbcs lines compared with wild-type plants (Table 2).

3.3. Phenotypic perturbations associated with reduced Rubisco

Reduction of Rubisco protein in rbcs2 and rbcs4 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 rbcs2-165, there was a corresponding statistically significant reduction in dry biomass, with rbcs4 lines exhibiting the largest decrease relative to the wild-type plants. There were no statistically significant differences in tiller number but rbcs4 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 rbcs4 lines and higher chlorophyll contents in rbcs2-266. The difference in phenotype perturbations between lines follows protein abundance with rbcs2-165 exhibiting the lowest Rubisco protein reduction and smallest phenotypic perturbations and rbcs4-022 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 rbcs knockdown lines (Table 4), with rbcs2-165 having the widest IVD and rbcs4-022 line the lowest. Mesophyll cell number was significantly reduced in all the rbcs knockdown lines. The mesophyll cell length was increased in all rbcs knockdown lines but was
CO₂ compensation point (r), carboxylation efficiency (CE), respiration rate (Rₚ) and quantum efficiency (Φ) of wild type and rbcs2 and rbcs4 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 Rₚ 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 leaves 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.

Table 3
Leaf chlorophyll content, plant height, tiller number, dry biomass and grain yield of wild-type and rbcs knockdown lines.

| Chl (SPAD value) | Plant height (cm) | Tiller number | Dry biomass (g) | Grain yield (g) |
|-----------------|------------------|---------------|-----------------|-----------------|
| Wild-type       | 43.14 ± 0.73ab   | 105 ± 1.4ab   | 26.1 ± 4.5bc    | 58.16 ± 10.93ab |
| rbcs2-165       | 42.30 ± 0.71bc   | 105.6 ± 1.9a  | 29.2 ± 3.9a     | 57.36 ± 6.81a   |
| rbcs2-266       | 44.04 ± 0.93a    | 103.6 ± 1.2a  | 27.5 ± 4.4ab    | 44.32 ± 5.12b   |
| rbcs4-022       | 41.84 ± 1.20a    | 101.4 ± 1.8a  | 22.6 ± 4.9a     | 32.60 ± 8.76c   |
| rbcs4-053       | 42.00 ± 0.97a    | 99.4 ± 3.0b   | 23.4 ± 4.8b     | 33.19 ± 8.23c   |

Chlorophyll contents (SPAD value) are the average ± SE of three leaves from ten individual plants. Plant height, tiller number, dry biomass and grain yield are the average ± SE of ten individual plants; 77 days post-germination. Biomass is the total dry weight of leaf, stem and sheath tissue. Different letters within groups indicate statistical significance based on a one-way ANOVA (p < 0.05) with a Least Significant Difference (LSD) test for post-hoc pairwise comparison.

Table 4
Leaf width, leaf thickness, interveinal distance (IVD), vein number and mesophyll cell characteristics of wild-type and rbcs knockdown lines.

| Leaf width (cm) | Leaf thickness (μm) | IVD (μm) | No. of major vein | No. of minor vein | No. of Mesophyll cell | Mesophyll cell length (μm) |
|----------------|---------------------|----------|------------------|------------------|-----------------------|---------------------------|
| Wild-type      | 1.4 ± 0.08a        | 177.45 ± 7.86a | 88.82 ± 5.44a    | 240.94 ± 11.72ab | 10.4 ± 0.9a        | 8.22 ± 0.4a               | 23.82 ± 0.20b             |
| rbcs2-165      | 1.36 ± 0.06a       | 175.10 ± 11.42a | 87.93 ± 4.63a    | 245.80 ± 9.27a   | 11.6 ± 0.5a         | 8.00 ± 0.5a               | 26.68 ± 1.50a             |
| rbcs2-266      | 1.34 ± 0.05a       | 167.05 ± 8.86a | 87.26 ± 3.12a    | 230.11 ± 4.74bc  | 11.6 ± 0.5a         | 42.3 ± 3.7a               | 25.35 ± 0.67bc            |
| rbcs4-022      | 1.16 ± 0.07ab      | 174.35 ± 7.98ab | 85.82 ± 1.76a    | 216.38 ± 7.11c   | 10.6 ± 0.7a         | 43.2 ± 1.0a               | 25.16 ± 0.84ab            |
| rbcs4-053      | 1.20 ± 0.09b       | 159.95 ± 9.56b | 82.70 ± 3.71b    | 226.89 ± 7.52bc  | 10.6 ± 0.6b         | 40.8 ± 2.6a               | 7.53 ± 0.28bc             |

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. Interveninal 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.

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 rbcs2 knockdown lines. In rbcs2 knockdown 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 rbcs2 knockdown lines, the rbcs4 knockdown 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
suggest 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 from MCs to BSCs.

The targeted antisense-rbcs plants would also be required for understanding the requirement for Rubisco synthesis was decreased in Rubisco knockdown rice from MCs to BSCs. This provides a useful foundation for installing C₄ photosynthesis to increase the yield of rice-rational and feasibility. Curr. Opin. Plant Biol. 11 (2), 228–231. https://doi.org/10.1016/j.pbi.2007.11.002.

References

Andersson, L., Backlund, A., 2008. Structure and function of rubisco. Plant Physiol. Biochem. 46 (3), 275–291. https://doi.org/10.1016/j.phyto.2008.01.001.

Chatterjee, J., D ionora, J., Elmidio-Mabiliangan, A., Wanchana, S., Thakur, V., Rondigyosphere, A., Quick, W.F., 2016. The evolutionary basis of naturally diverse rice leaves anatomy. PLoS One 11 (10), e0164532. https://doi.org/10.1371/journal.pone.0164532.

Dean, C., Favreau, M., Bedbrook, J., Dummuir, P., 1989. Sequences S 5′-translation start regulate expression of petunia rbcS genes. Plant Cell 1 (2), 209–215. https://doi.org/10.1105/tpc.1.2.209.

Ermakova, M., Danila, F.R., Furbank, R.T., von Caemmerer, S., 2019. On the road to C4 rice: advances and perspectives. Plant J. 101 (4), 490–950. https://doi.org/10.1111/tpj.14565.

Evans, J.R., von Caemmerer, S., Setchell, B.A., Hudson, G.S., 1994. The relationship between CO₂ transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of rubisco. Aust. J. Plant Physiol. 21 (4), 475–495. https://doi.org/10.1071/PP9940475.

Ghanoum, O., Evans, J.R., von Caemmerer, S., 2011. Nitrogen and water use efficiency in C₃ plants. In: Raghavendra, A.S., Sage, R.F. (Eds.), C₄ Photosynthesis and Related CO₂ Concentrating Mechanisms, Advances in Photosynthesis. Springer Verlag, Berlin, pp. 129–146. https://doi.org/10.1007/978-94-007-5407-0_6.

Gibson, D.G., Young, L., Chiang, R.Y., Venter, J.C., Hutchison III, C.A., Smith, H.O., 1994. The promoters of plant growth and N allocation in transgenic rice plants? Plant Physiol. 114 (2), 483–494. https://doi.org/10.1104/pp.114.2.483.

Hibberd, J.M., Sheehy, J.E., Langdale, J.A., 2008. Using C₄ photosynthesis to increase plant growth and N allocation in transgenic rice plants. Proc. Natl. Acad. Sci. U. S. A. 105 (24), 8215–8220. https://doi.org/10.1073/pnas.0800222105.

Hudson, G.S., Evans, J.R., von Caemmerer, S., Arvidsson, B.Y., Andrews, T.J., 1992. Reduction of ribulose-1, 5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. Plant Physiol. 98 (1), 294–302. https://doi.org/10.1104/pp.98.1.294.

Kajala, K., Covshoff, S., Karki, S., Woodfield, H., Tolley, B.J., Dionora, M.J.A., Mogul, R. T., Mabiliangan, A.E., Danila, F.R., Hibberd, J.M., Quick, W.P., 2011. Strategies for engineering a two-celled C₄ photosynthetic pathway into rice. J. Exp. Bot. 62 (9), 3001–3010. https://doi.org/10.1093/jxb/err022.

Kanno, K., Suzuki, Y., Makino, A., 2017. A small decrease in Rubisco content by individual suppression of rbcS genes leads to improvement of photosynthesis and greater biomass production in rice under conditions of elevated CO₂. Plant Cell Physiol. 58 (3), 635–642. https://doi.org/10.1093/pcp/pcx018.

Lin, H., Karki, S., Coe, R.A., Bagha, S., Khoshkran, R., Babaladha, C.P., Quick, W.P., 2016. Targeted knockdown of GDCH in rice leads to a photosynthetic-deficient phenotype useful as a building block for C₄ rice. Plant Cell Physiol. 57 (5), 919–932. https://doi.org/10.1093/pcp/pcw032.

Makino, A., Shimada, T., Takumi, S., Kaneko, K., Matsuzuka, M., Shimamoto, K., Yamamoto, N., 1997. Does decrease in ribulose-1, 5-bisphosphate carboxylase by antisense RbcS lead to a higher N-use efficiency of photosynthesis under conditions of saturating CO₂ and light in rice plants? Plant Physiol. 114 (2), 483–491. https://doi.org/10.1104/pp.114.2.483.

Makino, A., Nakano, H., Ma, T., Shimada, T., Yamamoto, N., 2000. Photosynthesis, plant growth and N allocation in transgenic rice plants with decreased Rubisco under CO₂ enrichment. J. Exp. Bot. 51 (suppl. 1), 383–389. https://doi.org/10.1093/jxb/er51.suppl.1.383.

Matsuzuka, M., Koyuzuka, J., Shimamoto, K., Kano-Murakami, Y., 1994. The promoters of two carboxylases in a C₄ plant (maize) direct cell-specific, light-regulated expression in a C₃ plant (rice). Plant J. 6 (3), 311–319. https://doi.org/10.1046/j.1365-313X.1994.0600311.x.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

We wish to thank Efren Bagunu, Florencia Montecillo, Juyi 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.

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

Chirag Maheshwari: Resources, Investigation, Writing - original draft, Writing - review & editing. Robert A. Coe: Methodology, Writing - review & editing. Shanta Karki: Resources. Sarah Covshoff: Resources, Writing - review & editing. Ronald Tapia: Resources. Aruna Tyagi: Conceptualization. Julian M. Hibberd: Conceptualization & Resources. Robert T. Furbank: Conceptualization. W Paul Quick: Conceptualization, Supervision, Writing - review & editing. Hsiang-Chun Lin: Conceptualization, Resources, Investigation, Supervision, Writing - review & editing.
Morita, K., Hatanaka, T., Misoo, S., Fukayama, H., 2014. Unusual small subunit that is not expressed in photosynthetic cells alters the catalytic properties of Rubisco in rice. Plant Physiol. 164 (1), 69–79. https://doi.org/10.1104/pp.113.228015.

Ogawa, S., Suzuki, Y., Yoshizawa, R., Kanno, K., Makino, A., 2012. Effects of reduced carbonic anhydrase activity on CO2 assimilation rates in Setaria viridis: a transgenic analysis. J. Exp. Bot. 63 (2), 299–310. https://doi.org/10.1093/jxb/erq257.

Quick, W.P., Schurr, U., Fichtner, K., Schulze, E.D., Rodermel, S.R., Bogorad, L., Stitt, M., 1991. The impact of decreased Rubisco on photosynthesis, growth, allocation and storage in tobacco plants which have been transformed with antisense rbcS. Plant J. 1 (1), 51–58. https://doi.org/10.1046/j.1365-313X.1991.00051.x.

Rodermel, S., 1999. Subunit control of Rubisco biosynthesis—a relic of an endosymbiotic past? Photosyn. Res. 59 (2–3), 105–123. https://doi.org/10.1023/A:1006122619851.

Rodermel, S.R., Abbott, M.S., Bogorad, L., 1988. Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell 55 (4), 673–681. https://doi.org/10.1016/0092-8674(88)90226-7.

Sasanuma, T., 2001. Characterization of the rbcS multigene family in wheat: subfamily classification, determination of chromosomal location and evolutionary analysis. Mol. Genet. Genom. 265 (1), 161–171. https://doi.org/10.1007/s004380000404.

Sheehy, J.E., Ferrer, A.B., Mitchell, P.L., Elmido-Mabilangen, A., Publico, P., Dionora, M. J.A., 2007. How the rice crop works and why it needs a new engine. In: Sheehy, J.E., Mitchell, P.L., Hardy, B. (Eds.), Charting New Pathways to C4 Rice. International Rice Research Institute, Los Banos, Philippines, pp. 3–26. https://doi.org/10.1142/9789812709523_0001.

Sudo, E., Suzuki, Y., Makino, A., 2014. Whole-plant growth and N utilization in transgenic rice plants with increased or decreased Rubisco content under different CO2 partial pressures. Plant Cell Physiol. 55 (11), 1905-1911. https://doi.org/10.1093/pcc/pcu119.