Overexpression of Rubisco Activase Decreases the Photosynthetic CO₂ Assimilation Rate by Reducing Rubisco Content in Rice Leaves

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The effects of overexpression of Rubisco activase on photosynthesis were studied in transgenic rice expressing barley or maize Rubisco activase. Immunoblot and SDS–PAGE analyses showed that transgenic lines from both gene constructs expressed the foreign Rubisco activase at high levels. The activation state of Rubisco in transgenic lines was slightly higher than that in non-transgenic plants (NT). In addition, light activation of Rubisco was significantly more rapid in transgenic lines compared with NT. These findings indicate that the overexpression of Rubisco activase can enhance Rubisco activation. However, despite enhanced activation of Rubisco in these transgenic plants, the CO₂ assimilation rate at ambient CO₂ conditions was decreased. This decrease in CO₂ assimilation rate was observed in both young developing and mature leaves independent of nitrogen nutrition. The contents of nitrogen and Chl did not differ significantly between transformants and NT; however, Rubisco content was substantially decreased in transgenic lines. There was no evidence for reduced transcription of RbcS or RbcL in these transgenic lines; in fact, transcript levels were marginally increased compared with NT. These results indicate that the overexpression of Rubisco activase leads to a decrease in Rubisco content, possibly due to post-transcriptional mechanisms.

Keywords: Overexpression • Photosynthesis • Rice (Oryza sativa L.) • Rubisco, Rubisco activase.

Abbreviations: BRca, barley Rubisco activase; Cab, Chl a/b-binding protein; CABP, carboxy-arabinitol-1,5-bisphosphate; DTT, dithiothreitol; MRca, maize Rubisco activase; NADP-ME, NADP-malic enzyme; NT, non-transgenic rice; PPFD, photosynthetically active photon flux density; RT–PCR, reverse transcription–PCR; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers: BRca, AB564719; and MRca, AB564720.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a stromal protein catalyzing the first step of both the photosynthetic CO₂ assimilation and photorespiratory pathways (Ogren and Bowes 1971). In C₃ plants, photosynthesis is usually limited by Rubisco capacity because of its extremely low catalytic turnover rate and competing oxygenation reaction (Farquhar et al. 1980). Consequently, higher plants accumulate large amounts of Rubisco comprising approximately 15–30% of total nitrogen in leaves (Evans 1989, Makino et al. 1992). These characteristics make Rubisco an attractive target for genetic engineering to improve photosynthesis and nitrogen use efficiency in crop plants.

To become catalytically competent, Rubisco must be activated by the binding of CO₂ to the lysine residue near the active site (carbamylation), followed by the binding of Mg²⁺ (Lorimer 1981, Portis 2003). Rubisco activase is an AAA+ family protein (ATPases associated with a variety of cellular activities) that mediates the activation of Rubisco by facilitating the ATP-dependent removal of various inhibitory sugar phosphates from the Rubisco active site (Portis 2003). This action is necessary for spontaneous carbamylation of Rubisco active sites in vivo and essential for photosynthetic CO₂ assimilation at atmospheric CO₂ concentrations, as evidenced by the observation that plants expressing reduced levels of Rubisco activase show a decrease in photosynthesis and growth rates (Portis 2003). In addition, the rca mutant of Arabidopsis which completely lacks Rubisco activase protein cannot survive in...
Overexpression of activase decreases Rubisco content

Results

Transgenic rice plants overexpressed high levels of Rubisco activase

Three Rubisco activase proteins are encoded by two nuclear genes, \( RcaA \) and \( RcaB \), in barley (Rundle and Zielinski 1991). \( RcaA \) encodes small and large isoforms of Rubisco activase generated by alternative splicing and shares 87% amino acid sequence identity with \( RcaB \). Among those isoforms, the expression level of the small isoform of \( RcaA \) was highest in barley leaves (Rundle and Zielinski 1991). The activity of large isoforms is also believed to be regulated by thioredoxin- \( f \) (Zhang and Portis 1999). For these reasons, we chose to introduce the small isoform of barley \( RcaA \) (designated \( HvRca \) in this study, AB564719) into rice. In contrast, maize contains two \( Rca \) genes both of which encode small isoforms and an identical mature protein (Ayala-Ochoa et al. 2004). One of these coding sequences (designated \( ZmRca \) in this study, AB564720) was introduced into rice. \( HvRca \) and \( ZmRca \) were both expressed in photosynthetic tissues under the control of the rice \( Cab \) (Chl \( a/b \)-binding protein) promoter. The expression of transcripts of \( HvRca \) or \( ZmRca \) was detected in a number of transgenic lines (Fig. 1A; Supplementary Fig. S1A). barley Rubisco activase is highly homologous to rice Rubisco activase and these share 89% identity, whereas the mature protein of barley Rubisco activase after the cleavage of the transit peptide is six amino acids shorter than the rice Rubisco activase. Exploiting this difference in molecular weight, the expression level of barley Rubisco

Fig. 1 Expression of barley Rubisco activase in leaves of transgenic rice. (A) Semi-quantitative RT–PCR. Expression of the actin gene was examined as an internal control. (B) SDS–PAGE and western blotting. Proteins extracted from leaves (3.2 \( \mu g \)) were separated by 10% SDS–PAGE. Protein bands were detected by Coomassie Blue staining (CBB) or western blotting probed with anti-rice Rubisco activase antibody (WB). \( OsRcaI \), rice Rubisco activase large isoform; \( OsRcall \), rice Rubisco activase small isoform; \( HvRca \), barley Rubisco activase; \( Rbcl \), Rubisco large subunit; NT, Non-transgenic rice; \( BRca3 \), \( BRca6 \); transgenic rice that overexpress barley Rubisco activase.
rubisco activase protein in transgenic rice was analyzed by semi-quantitative immunoblotting (Fig. 1B). As expected, the mobility of barley Rubisco activase in SDS–PAGE reflected the predicted molecular weight of the mature protein, larger than that of rice Rubisco activase. In homoyzogous transgenic lines BRca3 and BRca6, the band of barley Rubisco activase was easily detected just below the band of the small isoform of rice Rubisco activase (OsRcaII). In maize, the difference in mobility on SDS–PAGE was very small between ZmRca and OsRcaII, and as a result these two bands could not be separated by SDS–PAGE (Supplementary Fig. S1). However, the intensity of the band corresponding to the total amount of the small isoform of Rubisco activase was significantly increased in transgenic lines MRca5 and MRca6. We also detected unknown immunoreactive bands just below the small isoforms in all plants analyzed. These bands are presumed to be functional Rubisco activase cleaved by limited proteolysis near the N-terminus, a phenomenon previously reported in maize (Vargas-Suárez et al. 2004). In our immunoblot analysis, an antiserum raised against rice Rubisco activase (Fukayama et al. 2010) was used to detect the Rubisco activase protein. As quantitative cross-reactivity of this antiserum with recombinant activase could not easily be determined, underestimation of the expression levels of introduced Rubisco activase is likely. Expression levels of the introduced Rubisco activase were high enough in the transgenic lines for the protein band to be detected by Coomassie Blue staining (Fig. 1B; Supplementary Fig. S1B). Judging from the band intensity at the position of HvRca or ZmRca, the expression levels of Rubisco activase in transgenic rice were roughly estimated to be 2- to several fold higher than that in non-transgenic rice. Comparing these transgenic lines, the expression level of Rubisco activase in BRca6 was higher than that of BRca3, and the expression of MRca6 was the highest among transgenic lines. These results clearly indicate that Rubisco activase was overexpressed at physiologically significant levels in the transgenic rice described here.

**Activation of Rubisco in transgenic rice**

In order to ascertain whether the light-dependent activation of Rubisco was affected in our transgenic rice overexpressing Rubisco activase, the activation state of Rubisco was studied in BRca lines under three different light intensities (Fig. 2A). The activation levels of Rubisco in BRca lines were high under high light intensity (1,000 μmol m⁻² s⁻¹) and decreased under low light intensity (250 μmol m⁻² s⁻¹) and dark conditions similar to what was observed in non-transgenic rice. These findings indicate that expressed barley Rubisco activase did not have a major effect on light activation of Rubisco in transgenic rice. Although the differences were not statistically significant, the activation levels of Rubisco in BRca lines were slightly higher than that in non-transgenic rice, suggesting that overexpression of Rubisco activase may stimulate the activation level of Rubisco in vivo, although to a limited extent. The potential effects of overexpression of Rubisco activase in rice were examined by measuring the rate of photosynthetic activation after a sudden increase in light intensity. This activation is substantially limited by the activation rate of Rubisco mediated by Rubisco activase (Fukayama et al. 1998, Hammond et al. 1998). The relaxation time of this photosynthetic activation was studied to ascertain whether the overexpression of barley Rubisco activase can enhance the activation rate of photosynthesis (Fig. 2B). The relaxation times were significantly lower in BRca lines compared with non-transgenic rice, indicating that overexpression of Rubisco activase significantly enhances the activation rate of photosynthesis after a step change in irradiance. Similar results for Rubisco activation and relaxation time were also observed in MRca lines.
(Supplementary Fig. S2). Thus, these results are considered to be general and reproducible.

Photosynthetic rate in transgenic rice

The photosynthetic rate in rice leaves varies with leaf age and nitrogen status. Thus, the effect of overexpression of Rubisco activase on the CO2 assimilation rate was investigated in leaves of different age and nitrogen supply using BRca lines (Fig. 3). In the nitrogen treatment experiment, slow release fertilizer (coated urea) was used as a nitrogen source, instead of chemical fertilizer, to avoid physiological damage to roots under high nitrogen conditions. In general, the CO2 assimilation rates tended to be decreased in leaves of BRca lines. The decreases in CO2 assimilation rate in BRca lines were marked in young developing or mature leaves, whereas the decreases were barely detectable in senescent older leaves. The decrease in CO2 assimilation rate was also observed in the uppermost fully expanded leaves grown under varying levels of nitrogen nutrition (Fig. 3B). The reduction in photosynthesis seen in BRca lines was maximal under low nitrogen conditions. The CO2 assimilation rate of MRca lines was only measured in plants grown with normal nutrition using chemical fertilizer. Consistent with the BRca line, the CO2 assimilation rate tended to decrease in MRca lines (Supplementary Fig. S2).

The overexpression of barley Rubisco activase affected the content of other protein components in rice leaves (Fig. 4). Remarkably, Rubisco contents were significantly decreased in the mature ninth leaves of BRca lines. Significant decreases in Rubisco content in BRca lines were also found in the uppermost fully expanded leaves regardless of nitrogen supply. Although soluble proteins were decreased in mature ninth leaves and Chl was increased in the seventh leaves, overall changes in nitrogen, soluble protein and Chl were not so marked during leaf aging and with different nitrogen supplies compared with changes in Rubisco content. This significant decrease in Rubisco content was also observed in MRca lines (Supplementary Fig. S3). These results indicate that the down-regulation of Rubisco by overexpression of Rubisco activase is consistent, and suggest that it has a greater impact on Rubisco content compared with the other leaf constituents.

The CO2 assimilation rate was positively correlated with nitrogen content in non-transgenic rice and in BRca lines; however, this relationship was shifted in BRca lines compared with non-transgenic rice (Fig. 5). In contrast, the CO2 assimilation rate was highly correlated with Rubisco content and the regression line was quite similar between non-transgenic rice and BRca lines. These findings suggest that the CO2 assimilation rate was largely determined by Rubisco content in tested plants with different Rubisco activase levels and the decreases in the CO2 assimilation rate observed in the transgenic rice analyzed here were mainly due to the reduction in Rubisco content.

Expression of the RbcS gene family and Rbcl

As described above, the overexpression of Rubisco activase decreased Rubisco protein levels in leaves. Quantitative reverse transcription–PCR (RT–PCR) was carried out to clarify whether this decrease was due to down-regulation of the expression of RbcS gene family and Rbcl or due to post-transcriptional processes. Rice contains five RbcS genes (OsRbcS1–OsRbc5) in the nuclear genome and one Rbcl gene in the chloroplast genome (Suzuki et al. 2007). Although the differences were not statistically significant, the expression levels of most of these genes were either unaffected or in fact marginally increased in BRca lines compared with non-transformed material (Fig. 6). This unexpected trend was also observed in MRca lines (Supplementary Fig. S4). These results suggest that decreases in Rubisco content by overexpression of Rubisco activase did not result from down-regulation of expression of the RbcS gene family or Rbcl at the transcriptional level.

Effects of overexpression of Rubisco activase on growth

To examine the effects of Rubisco activase overexpression on growth, young seedlings (4.5 leaf stage) were transplanted, and
shoot length, tiller number and leaf number of the main stem were measured until heading (84 d after transplanting; Fig. 7).

In general, the transgenic rice plants overexpressing Rubisco activase exhibited a normal phenotype, with shoot length, tiller number and leaf expansion rate broadly similar to those of non-transgenic plants regardless of nitrogen treatments. Only shoot length up to 42 d and leaf age up to 70 d after transplanting were marginally decreased in transgenic rice. At 84 d after transplanting, all of these growth parameters of non-transgenic rice and BRca lines finally attained the same levels. Panicle and straw dry weight were also studied using the same set of plants at harvest time (Fig. 8). Although the differences were not always significant, BRca lines showed slightly lower panicle and straw dry weight. These results indicate that the overexpression of Rubisco activase does not...
greatly perturb growth rate and yield in rice, apart from a small negative effect.

Discussion

In this study, we successfully overexpressed foreign Rubisco activase from closely related species at high levels in rice (Fig. 1; Supplementary Fig. S1). The expressed foreign Rubisco activase was readily detected by SDS–PAGE with Coomassie Brilliant Blue staining. Both barley and maize constructs were effective in attaining high level expression. To date, we have overexpressed various photosynthetic genes in rice (Matsuoka et al. 2001, Taniguchi et al. 2008, Ishikawa et al. 2011). In the case of NADP-malic enzyme (NADP-ME), rice NADP-ME and maize C4-specific NADP-ME were overexpressed using the same expression cassette and expression levels were compared in transgenic rice (Tsuchida et al. 2001). Transgenic rice carrying the maize gene accumulated a several fold higher level of NADP-ME than those carrying the rice gene. In addition to this, the photosynthetic enzymes from C4 plants were always expressed at significantly high levels in transgenic rice (Matsuoka et al. 2001, Taniguchi et al. 2008, Ishikawa et al. 2011). These observations suggested that the overexpression
of the native rice gene was partially suppressed, potentially by some endogenous regulation mechanism, while that of the foreign gene escaped such suppression. This could be a reason for attaining effective high level expression of Rubisco activase in this study.

Interaction of Rubisco activase with Rubisco has been suggested to be species specific (Wang et al. 1992). It is well known from in vitro experiments that Rubisco activase from the family Solanaceae cannot activate Rubisco from other families and Rubisco activase from non-Solanaceae plants fails to activate Solanaceae Rubisco (Wang et al. 1992). Thus, it was a matter for concern whether the introduced foreign Rubisco activase could functionally interact with rice Rubisco in vivo. According to Li et al. (2005), amino acid residues K311 and V314 (numbering based on spinach Rubisco activase) are considered to be important substrate recognition sites in spinach Rubisco activase (non-Solanaceae) while in tobacco Rubisco activase (Solanaceae) the corresponding residues are D311 and L314. In rice, barley and maize, V314 is conserved, but K311 is replaced by R311 in maize (Supplementary Fig. S5). However, this may not be significant as both lysine and arginine are basic amino acids and show similar biochemical characteristics. In this study, it was shown that barley and maize Rubisco activase can mediate and enhance the activation of rice Rubisco in vivo (Fig. 2; Supplementary Fig. S2). These findings imply that Rubisco activase can interact with and effectively promote activation of Rubisco from closely related species in vivo. In a previous study using transgenic Arabidopsis expressing only the small isoforms of Rubisco activase, Rubisco activation did not respond to light intensity and remained at a high level (>80%) under low light conditions (Zhang et al. 2002). Although the small isoforms from different species were significantly over-expressed in this study, Rubisco activation was decreased under low light intensity to a similar extent with non-transgenic rice. These results suggest that the activity of small isoforms over-expressed in rice is coordinately regulated by the native large isoform in response to light intensity.

Non-steady state photosynthesis in antisense Rubisco activase transgenic plants after sudden increases in light intensity is well correlated with Rubisco activase content above the wild-type level, and the photosynthetic rate is considered to be largely limited by Rubisco activase under these conditions (Hammond et al. 1998). In addition to this report, overexpression of Rubisco activase reduced the relaxation time for photosynthetic activation (Fig. 2), an indication that Rubisco activase does not accumulate in excess even in wild-type plants and evidence that the overexpression of Rubisco activase can enhance the non-steady state photosynthesis after step increases in light intensity. In contrast, the reduction of Rubisco activase contents up to 70–90% were needed to affect the steady-state photosynthetic rate in antisense transgenic plants (Jiang et al. 1994, Mate et al. 1996, Eckardt et al. 1997), suggesting that wild-type plants contain an excess of Rubisco activase above that required for steady-state photosynthesis. Since photosynthesis is considered to be limited by Rubisco capacity under low CO₂ conditions, the measurement of the initial slope of the A/Ci response of photosynthesis can be a useful means to screen for Rubisco activase limitation (Sage et al. 2008). Our previous study indicated that the content of Rubisco activase was more closely correlated with the initial slope of the A/Ci curve than that of Rubisco in rice (Uchida et al. 1995). These findings imply that Rubisco activase makes a more important contribution to the potential for photosynthesis in rice than we previously thought. In this study, the increase in Rubisco activase contents apparently did not enhance the steady-state photosynthesis; rather it reduced the photosynthetic rate (Fig. 3). These unexpected results could be important for understanding the mechanism of optimization of photosynthesis and in considering a strategy for the improvement of photosynthesis.

Rubisco activations in transgenic rice were slightly higher than that in non-transgenic rice (Fig. 2; Supplementary Fig. S2). However, the photosynthetic rate was highly correlated with Rubisco content (Fig. 5), and both the photosynthetic rate and Rubisco content were decreased in transgenic lines (Figs. 3, 4; Supplementary Fig. S2). Thus, the decreases in the photosynthetic rate could be largely explained by the reduction of Rubisco. It has been reported that the reductions of Rubisco activase by antisense RNA increased the Rubisco content in three different plant species: tobacco, Arabidopsis and rice (Mate et al. 1993, Eckardt et al. 1997, Jin et al. 2006). It is possible that reductions of other Calvin cycle enzymes have similar effects on Rubisco content; however, increases in Rubisco were not significant in transgenic plants containing
reduced amounts of chloroplastic glyceraldehyde-3-phosphate dehydrogenase (He et al. 1997). Similarly, inhibition of the expression of other Calvin cycle enzymes such as transketolase (Henkes et al. 1998) and aldolase (Haake et al. 1999) showed similar or lower total Rubisco activity. Thus, it is likely that the effect of Rubisco activase on Rubisco content is specific to activase rather than general to other components of photosynthesis. In contrast, the reduction of Rubisco content by antisense RNA did not influence the Rubisco activase content (Jiang and Rodermel 1995). These observations led us to conclude that Rubisco activase levels may be an important factor regulating Rubisco content, and not vice versa.

Reduction of Rubisco content under long-term exposure to elevated CO2 is a well known phenomenon reported in many plant species (Long et al. 2004). In rice, expression of the RbcS gene family was down-regulated under elevated CO2, whereas that of Rubisco activase was up-regulated under elevated CO2 (Fukayama et al. 2009). It is commonly accepted that the down-regulation of Rubisco is triggered by carbohydrate accumulation in leaves. However, considering the results obtained in this study, it is also likely that the reduction of Rubisco observed under elevated CO2 could be partly due to up-regulation of Rubisco activase if it can negatively regulate Rubisco content.

Suppression of Rubisco activase content to levels <5–30% of controls was required before reductions in photosynthetic rate and growth were observed in antisense Rubisco activase plants (Jiang et al. 1994, Mate et al. 1996, Eckardt et al. 1997), suggesting that Rubisco activase is basically present in excess in photosynthetic tissues. In contrast, the flux control coefficient for Rubisco is estimated to be about 0.7, the highest among Calvin cycle enzymes (Raines 2003), an indication that the photosynthetic rate can be predominately limited by Rubisco content though the extent of this limitation will depend on environmental conditions. In this study, it is proposed that the expression level of Rubisco activase affects Rubisco contents. This effect of Rubisco activase on Rubisco potentially affects limitation of photosynthetic flux by Rubisco. Thus, Rubisco activase might play a more important indirect role in determining potential photosynthetic capacity than was previously thought and should be re-evaluated as a potential key regulatory point in determining photosynthetic flux in C3 plants.

What is the underlying mechanism of the decrease in Rubisco content in our transgenic rice? It is possible that the overexpression of Rubisco activase temporarily enhances the photosynthetic rate by the stimulation of Rubisco activation, leading to a compensatory down-regulation of Rubisco as is often observed under long-term elevated CO2 treatment. If the overexpression of Rubisco activase enhances photosynthesis, it raises an alternative possibility that the senescence of individual leaves was also accelerated in transgenic lines. However, if this were the case, the expression of RbcS and RbcL would be down-regulated in Rubisco activase-overexpressing lines. In this study, RbcS and RbcL were slightly up-regulated by the overexpression of Rubisco activase (Fig. 6), suggesting that down-regulation by sugar accumulation or accelerated senescence could not be the main cause of the decrease in Rubisco. Rubisco content was significantly increased in mature leaves of anti-activase tobacco plants, and this increase in Rubisco was marked in older leaves (He et al. 1997). These observations suggested that Rubisco activase levels have a much greater impact on Rubisco content in older leaves. However, in this study, the changes in Rubisco activase levels did not influence the Rubisco content in older leaves and they tended to be more significant in young leaves in Rubisco activase-overexpressing lines (Fig. 4), also suggesting that differences in progression of senescence between transgenic and non-transgenic plants were not responsible for this phenomenon. It has been reported that the activation state of Rubisco affects the susceptibility of Rubisco to proteolysis (Khan et al. 1999). It is possible that the levels of Rubisco activase influence the stability of Rubisco, which may affect the content of Rubisco in rice leaves.

In this study, it is proposed that Rubisco content can be negatively regulated by Rubisco activase. This decrease in Rubisco content is probably not a result of the down-regulation of RbcS and RbcL transcription, and the detailed mechanism remains to be determined. Understanding this regulatory mechanism could be crucial to the success of strategies to enhance photosynthetic capacity and provide a better understanding of the limiting factors for photosynthetic flux and biomass production.

Materials and Methods

cDNA cloning, constructions and transformation of rice

Total RNA was isolated from leaves of barley (Hordeum vulgare L. cv. Kashima) and maize (Zea mays L. cv. Golden Cross Bantam) using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instruction. The first-strand cDNA was synthesized from the total RNA with an oligo(dT)18 primer using a PrimeScript II first-strand cDNA synthesis kit (TAKARA). The full-length cDNAs of the Rubisco activase small isoform of barley (HvRca, AB564719) and maize (ZmRca, AB564720) were amplified by RT–PCR using gene-specific primers (listed in Supplementary Table S1) as described previously (Fukayama et al. 2001). The amplified cDNAs were fused to the rice Cab promoter and cloned into a binary vector pIG121Hm containing a hygromycin resistance gene. The constructs were introduced into calli derived from rice (Oryza sativa L. cv. Nipponbare) via Agrobacterium-mediated gene transfer. Transgenic plants were regenerated from hygromycin-resistant calli and planted in soil. The levels of introduced protein in the leaves were screened by immuno-blotting or SDS–PAGE with Coomassie Blue staining as described below. Primary transgenic plants (T1) exhibiting a segregation ratio of around 1:3 were selected. Plants with the highest protein level in the progeny were taken as homozygous and used for further analysis.
Plant growth conditions

Rice plants were grown in soil under natural light conditions in a temperature-controlled greenhouse (28°C day and 23°C night). For analysis of leaf development and aging, rice seedlings at the 4.5-leaf age were transplanted into 1 liter pots supplemented with a chemical fertilizer (N:P:K = 8:8:8) at 0.3 g N per pot. At panicle initiation stage, the same chemical fertilizer was applied at a rate of 0.1 g N as top dressing. For analysis of the effects of nitrogen, the seedlings were transplanted at the same leaf stage in 1/10,000-are pots with three different nitrogen levels (0.1, 0.3 and 0.6 g per pot as low, medium and high N, respectively) applied as coated urea (LP-70, Chisso Asahi Fertilizer Co. Ltd.). P2O5 and K2O were applied at 0.3 g per pot as a basal dressing. At the panicle initiation stage, chemical fertilizer was applied as top dressing (0.05 and 0.2 g per pot for low, medium and high N, respectively).

Barley and maize plants were planted into 4 liter pots filled with soil containing the same chemical fertilizer at 1.0 g N per pot and grown under the same condition as for rice.

RT–PCR analyses

Total RNA was isolated from the uppermost fully expanded leaves using the RNase Plant Mini Kit (Qiagen). The first-strand cDNA was synthesized from 1.2 μg of total RNA with oligo(dT)18 and random hexamer as primers using a PrimeScript II 1st Strand cDNA Synthesis Kit (TAKARA). Semi-quantitative RT–PCR was performed using a Quick Taq HS DyeMix (Toyobo) with 5% dimethylsulfoxide (DMSO) and the primers listed in Supplementary Table S1. The DNA polymerase was first activated at 94°C for 2 min, and PCR was carried out for 25 cycles of 30 s at 94°C, 30 s at 60°C and 2 min at 68°C, followed by a final extension step for 4 min at 68°C. Quantitative RT–PCR was performed essentially as described by Suzuki et al. (2009) using the gene-specific primers listed in Supplementary Table S1. PCR was carried out using SYBR Premix Ex Taq GC (TAKARA) and Thermal Cycler Dice TP800 (TAKARA) according to the manufacturer’s instruction. Expression of the actin gene (Rac1, AB047313) was examined as an internal control.

SDS–PAGE and immunoblotting

Segments of about 3 cm were harvested from the mid-section of the uppermost fully expanded leaves at 11:00–12:00 h and immediately frozen in liquid nitrogen until use. Total leaf soluble protein extraction, protein determination and SDS–PAGE were carried out as described previously (Ishikawa et al. 2011). After SDS–PAGE, the gel was stained with Coomassie Brilliant Blue R-250 or subjected to immunoblotting using antisera raised against the rice Rubisco activase (Fukayama et al. 2010). Immunoreactive bands were visualized using alkaline phosphatase as described previously (Fukayama et al. 2006).

Gas exchange measurements

Gas exchange of the uppermost fully expanded leaf was measured with an open gas exchange system LI-6400 (Li-Cor) as described previously (Tsuchida et al. 2001). Unless stated otherwise, the measurements were performed at a leaf temperature of 28°C, CO2 partial pressure of 35 Pa, 21% O2, a photosynthetically active photon flux density (PPFD) of 1,500 μmol m–2 s–1 and a vapor pressure deficit at the leaf surface of 1.0–1.2 kPa. To examine the activation of CO2 assimilation after an increase in light intensity, the measurements were performed at a CO2 partial pressure entering the leaf chamber of 25 Pa. The relaxation time for the activation of CO2 assimilation was calculated as described previously (Ishikawa et al. 2011). Gas exchange parameters were calculated according to the equations of von Caemmerer and Farquhar (1981).

Determination of Rubisco activity and catalytic site

Leaf tissues were harvested after 30 min of illumination (PPFD of 0, 250 or 1,000 μmol m–2 s–1) and immediately frozen in liquid nitrogen. The leaf tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle. The leaf powder was suspended in extraction buffer [50 mM Bicine-NaOH, 0.1 mM EDTA, 5 mM diethiothreitol (DTT), pH 7.8] which had been prepared CO2 free and briefly centrifuged for 2–3 s. The supernatant was used for measurement of Rubisco initial activity and to quantify catalytic sites. Rubisco activity was determined at 28°C using [14C]NaHCO3 by assaying the incorporation of 14C into acid-stable products, as described previously (Ishikawa et al. 2009). Rubisco catalytic site concentrations were determined by measuring the stoichiometric binding of [14C]carboxy-arabinitol-1,5-bisphosphate (CABP) as described previously (Ishikawa et al. 2009). Rubisco total activity was estimated using the catalytic site concentrations of each sample and the catalytic turnover rate of Rubisco (1.39 mol mol–1 s–1). The activation state of Rubisco was calculated as a percentage of the initial activity divided by total activity.

Determination of Rubisco, Chl, total soluble protein and nitrogen

Leaf tissues were homogenized in extraction buffer [50 mM HEPES-KOH, 5 mM MgCl2, 1 mM EDTA, 5 mM DTT, 4 mM amino-n-caproic acid, 0.8 mM benzamidine, 0.05% (v/v) Triton X-100, 5% (w/v) glycerol, 0.1% (w/v) polyvinylpyrrolidone, pH 7.4] using a chilled mortar and pestle with a small amount of quartz sand. The homogenate was then centrifuged at 15,000 × g for 5 min at 4°C. The supernatant was used for the determination of Rubisco and total soluble proteins.

Rubisco content was determined using [14C]CABP as described above and calculated by assuming a stoichiometry of 6.5 molecules of CABP bound per molecule of Rubisco. For determination of Chl, a portion of homogenate was taken from the mortar and extracted with 80% (v/v) acetone. Chl was
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Supplementary data are available at PCP online.

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