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ORIGINAL RESEARCH

Reduction in SBPase Activity by Antisense RNA in Transgenic Rice Plants: Effect on Photosynthesis, Growth, and Biomass Allocation at Different Nitrogen Levels

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Abstract Rice cultivar zhonghua11 (*Oryza sativa* L. ssp. *japonica*) plants with decreased sedoheptulose-1, 7-bisphosphatase (SBPase) were obtained by transformation with the rice SBPase antisense gene under the control of the maize ubiquitin promoter. The transgenic and wild-type plants were grown at different nitrogen levels (0.1, 1, or 10 mM NH_4NO_3). Growth rates of the seedlings were measured by the changes in dry weight, and the photosynthetic carbon reduction activities and the potential efficiency of photosystem II were measured by CO_2 assimilation and F_v/F_m, respectively. At low N, there are strong effects on growth and photosynthesis when SBPase was reduced by genetic manipulation. Decreased SBPase activity led to a decrease in the amount of starch accumulated in the leaves at all N levels and the decrease was much more prominent in low N than that in high N, but the starch allocation between shoot and root was unaltered. The analysis of chlorophyll fluorescence and SBPase activity indicated that the decrease of growth and photosynthesis at different N levels were not related to the function of PSII but to the activity of SBPase. Western blot analysis showed the content of SBPase in thylakoid membranes was much more than in the stroma fractions in transgenic plants at low N. Results suggested that low N in addition to a 34% decrease in SBPase activity is sufficient to diminish photosynthesis and limit biomass production. Decreased SBPase activity may reduce the N use efficiency of photosynthesis and growth and alter biomass allocation.

Keywords Antisense RNA · Biomass allocation · Photosynthesis · Sedoheptulose-1, 7-bisphosphatase · Transgenic rice · Nitrogen levels

Abbreviations

FBPase	Fructose-1, 6-bisphosphatase
PRK	Phosphoribulokinase
PSII	Photosystem II
Rubisco	Ribulose-1, 5-bisphosphate carboxylase-oxygenase
RuBP	Ribulose-1, 5-bisphosphate
Ru5P	Ribulose-5-bisphosphate
SBP	Sedoheptulose-1, 7-bisphosphate
SBPase	Sedoheptulose-1, 7-bisphosphatase

Introduction

Many studies reported that the relationship between photosynthesis, growth, and biomass allocation is complex, and growth rate is not well correlated with the rate of photosynthesis on a leaf-area basis in different kinds of plants (Nelson 1988; Poorter et al. 1990). This is partly because some photosynthesis may not contribute to growth due to a nonproductive manner in storage compounds and partly because the rate of growth depends on biomass allocation within the plant and the construction of the leaf (Chapin et al. 1990; Poorter and Remkes 1990). These investigations of the relationship between photosynthesis, growth, biomass allocation, and environment have not provided definitive information on the relationship between

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photosynthesis and growth because of the complex interaction between factors, so it has not been easy to decide whether changes in growth result from changes in photosynthesis or vice versa (Rufty et al. 1989a, b; Poorter et al. 1990; Fichtner and Schulze 1992). The elucidation of the relationship between photosynthesis, growth, and biomass allocation requires specific changes in photosynthesis or growth independent of other changes.

Genetic manipulation of enzymes provides a means to achieve this. Transgenic plants with altered amounts of ribulose-1, 5-bisphosphate carboxylase-oxygenase (Rubisco, EC 4.1.1.39) revolutionized the analysis of photosynthesis and its interaction with the whole plant (Stitt and Schulze 1994; Quick et al. 1991b). However, the content of Rubisco accounts for 15% to 35% of total leaf N in C_3 species, and a decrease in the amount of Rubisco substantially disrupts the N balance of the plant, making it difficult to establish direct links between photosynthesis and growth and allocation (Woodrow and Berry 1988; Paul et al. 1996). The study of phosphoribulokinase (PRK, EC3.1.3.11) has found that only 94% decrease in PRK activity by antisense RNA in transgenic tobacco diminishes photosynthesis at low N level (Banks et al. 1999; Habash et al. 1996). In other series of analogous experiments with the Calvin cycle enzymes fructose-1, 6-bisphosphatase (FBPase, EC 3.1.3.11) (Kossmann et al. 1994) and glyceraldehyde-3-phosphate dehydrogenase (Price et al. 1995), the activities of these enzymes were reduced by 65–90% before an effect on photosynthetic carbon assimilation was observed. These experiments indicated that Rubisco, FBPase, PRK, and glyceraldehydes-3-phosphate dehydrogenase are not close to limiting carbon fixation through this cycle and would not be suitable for use as targets for genetic manipulation to analyze the relationship between photosynthesis, growth, and biomass allocation.

The enzyme sedoheptulose-1, 7-bisphosphate (SBPase, EC 3.1.3.37) functions in the regenerative phase of the primary pathway of the Calvin cycle and is extremely low compared with those of other enzymes in the Calvin cycle. This enzyme is important in the Calvin cycle because it is the branch-point where carbon gets committed to ribulose-1, 5-bisphosphate (RuBP) regeneration and for metabolites leaving the Calvin cycle to move into starch biosynthesis. The investigation of the transgenic plants with reduced SBPase activity has already revealed that SBPase plays an important role in regulating carbon flow in the Calvin cycle, and photosynthesis has been shown to be sensitive to reductions in the levels of the enzymes SBPase (Harrison et al. 1998; Raines et al. 2000). Increased SBPase activity stimulates photosynthesis and growth in transgenic tobacco plants (Miyagawa et al. 2001; Lefebvre et al. 2005) and enhances photosynthesis against stresses in transgenic rice plants (Feng et al. 2007a, b). From these facts, it seems likely that SBPase in the Calvin cycle is an important strategic position to determine photosynthesis and growth and the partitioning of carbon to end products at some environment conditions. However, seldom have these transgenic plants been used to investigate the N economy of photosynthesis. In addition, such antisense transformation has not been undertaken for a major crop. To our knowledge, no report is available on whether SBPase has direct effects on photosynthesis, growth, and allocation of biomass by reducing SBPase activity in transgenic rice plants at different N levels.

We have established the system for antisense-expressing SBPase by genetic engineering of rice plants, and using these transgenic rice plants, we investigated the direct effects of SBPase in vivo on photosynthesis and allocation of biomass and analyzed the relationship between photosynthesis, plant growth, and biomass allocation at different nitrogen levels in this study.

Materials and Methods

Materials and Growth Conditions

A rice cultivar 9311 (*Oryza sativa* L. ssp. *indica*) full-length SBPase cDNA (Chen et al. 2004) was isolated from rice leaves mRNA by RT-PCR. The cDNA was subcloned into the plasmid pMD-T vector confirmed by end-sequencing. A 1.2-kb *Sma*1l–*Xba*l restriction fragment was subcloned into the binary vector pU1301 containing a maize ubiquitin promoter and nopaline synthase terminator sequences, to give the SBPase antisense RNA construct (Fig. 1). The resulting plasmid (pU1301-aS) was introduced into *Agrobacterium tumefaciens* EHA105. The rice cultivar (*Oryza sativa* L. ssp. *japonica*) zhonghua11 was transformed with the resultant plasmid by the standard *Agrobacterium*-mediated method as described previously (Toki 1997). Shoots were regenerated on selective medium containing hygromycin (50 mg l^{-1}), and T_1 plants were obtained by self-pollination of primary transformants (T_0) plants. The resulting T_1 plants were allowed to self-fertilize. The homozygous T_3 plants obtained by self-fertilization of T_2 plants were used in this study. Transgenic plants were selected for hygromycin resistance and verified by PCR or Western blotting.

Four independent lines of transgenic rice plants (L1, L2, L3, L4) were selected for this study. Line1 (L1) had activities of SBPase that was on average 15% of the wild type; Line2 (L2), 37%; Line3 (L3), 53%; Line4 (L4), 74%. Control line (C) was transformed in the same way as the others, but with the SBPase sequence omitted from the construct, which gave wild-type activities of SBPase. The seeds of these transgenic plants were allowed to germinate on agar in the presence of

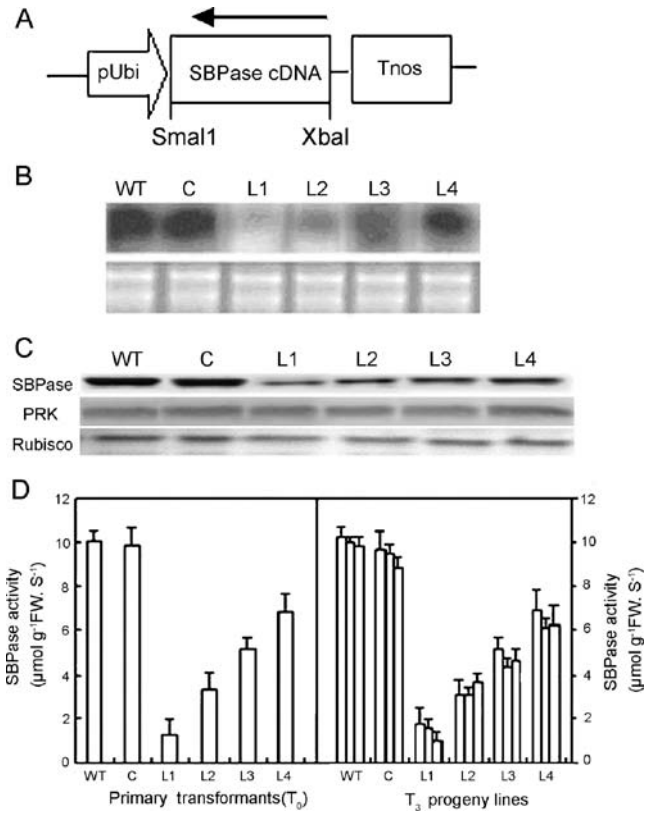

Fig. 1 Production and selection of antisense SBPase transgenic rice transformants. **a** SBPase antisense gene construct: *pUbi*, maize ubiquitin promoter; *Tnos*, nopaline synthase terminator; the rice SBPase cDNA fragment (1,179 bp) was subcloned in the reverse orientation between the maize Ubi promoter and the Nos terminator in the binary vector pU1301. **b** RNA blot gel analysis of SBPase mRNA from wild type, control plant, and four independent transformants expressing the SBPase antisense construct. The *bottom panel* showed ethidium bromide (EtBr) staining of total RNA to confirm equal loading in each line. **c** Protein blot analysis of wild-type and individual transformants (T_3). The same samples were separated by SDS-PAGE and polyclonal antibodies used to detect PRK and monoclonal antibodies used to detect Rubisco proteins. Each lane represents a sample taken from one individual line. **d** Total SBPase activity in individual transformants (T_0) and the corresponding T_3 progeny lines. *WT* wild type (untransformed line), *C* control plants (transformed with the SBP sequence omitted from the construct), *L1–L4* independent transformant lines. The values are the mean ± SE of five independent experiments

50 μg l^{-1} hygromycin. The seeds of wild-type plants were allowed to germinate on agar in the absence of hygromycin. After growth for 2 weeks, plants were transferred to the pots full with the mixture of sand and soil. The plants were grown in a greenhouse at 25±2°C with photosynthetic photon flux density of 300 μmol $m^{-2}s^{-1}$, a relative humidity of 70% to 80%, and a photoperiod of 14:10-h light/dark. Then, they were irrigated to field capacity with nutrient solution (Yoshida et al. 1976) with the exception to contain different N concentrations (0.1, 1, or 10 mM NH_4NO_3). Plants were harvested after 60, 45, and 30 days of growth at 0.1, 1, or 10 mM NH_4NO_3, respectively, which was a compromise between suitable size for measurements of photosynthesis for the smallest plants and the onset of flag-leaf of plant lines. Gas exchange and biochemical assays were carried out on the youngest fully expanded leaves.

RNA Blot Analysis

Total RNA was extracted using TRIzol (Gibco BRL) from the fully expanded leaves and separated on 1.4% denaturing agarose gel. Then, 10 μg of total RNA was transferred onto a nylon membrane and hybridized with ^{32}P-labeled rice SBPase cDNA probe in 5× SSC, 5× Denhardt's mixture, 1% SDS at 65°C, and washed three times with 0.1× SSC, 0.1× SDS at 65°C.

Protein Gel Blot Analysis

About 0.1 g leaf tissue was harvested from the youngest fully expanded leaves and quickly ground to a fine power in liquid nitrogen, and 0.5 ml extraction buffer (50 mM Tris–HCl, pH7.5, 2% β-mercaptoethanol, 10% glycerol) was added. After vigorous vortex, the crude extract was clarified by centrifugation (10 min at 14,000×*g*, 4°C), and the supernatant was removed for protein estimation and protein gel blot analysis. Protein estimation was determined according to the Bradford method with BSA as a standard. Equal amounts of proteins were separated on 12% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed using antibodies against SBPase, PRK, Rubisco, and Rubisco activase. Proteins were detected using alkaline phosphatase conjugated to the secondary antibody. Polyclonal antibody raised against SBPase was a gift from Christine A. Raines (Department of Biological Sciences, University of Essex, UK); polyclonal antibodies against PRK, a gift from Henry Miziorko (Department of Biochemistry, Medical College of Wisconsin, USA); and monoclonal antibody against Rubisco was a gift from Martin Parry (Biochemistry and Physiology Department, IACR, Rothamsted, Harpenden, UK).

Analysis of Photosynthetic Gas Exchange

Measurements of net photosynthetic gas exchange were made on a fully expanded leaf of rice seedlings using a portable open gas exchange system (CI-340, a hand-held portable photosynthesis system, 4845NW Camas Meadows Drive, Camas, WA 98607, USA). The light-saturating photosynthetic rate was made at a 360-μl l^{-1} CO_2 concentration and at temperature of 25°C with relative humidity 80% and saturating light (800–1,000 μmol $m^{-2}s^{-1}$). The

apparent quantum yield and carboxylation efficiency of photosynthesis were determined as the slope of photosynthesis light and CO_2 response curves, respectively. The measurements on these photosynthetic parameters lasted approximately 10 min, during which no significant recovery was observed on these parameters.

Measurements of Chlorophyll Fluorescence

Chlorophyll fluorescence was measured with a CI-510CF chlorophyll fluorescence module (CID Inc. USA) under atmospheric conditions. After a dark adaptation period of 30 min, minimum fluorescence (F_0) was determined by a weak red light. Maximum fluorescence of dark-adapted state (F_m) was measured during a subsequent saturating light pulse (8,000 μmol $m^{-2}s^{-1}$ for 0.8 s). The measurements were performed on the leaves of rice seedlings. The maximal efficiency of PSII photochemistry was determined as the ratio of variable to maximal chlorophyll fluorescence (F_v/F_m; Krause and Weis 1991).

Measurements of SBPase, PRK, and Rubisco Activities

To determine light-dependent activation of SBPase, PRK, and Rubisco, the whole plants were irradiated with saturating light 800–1,000 μmol $m^{-2}s^{-1}$ for 10 min at 25°C to promote full activation of SBPase, PRK, and Rubisco at different N concentrations. After illumination, leaf tissues were harvested immediately for the determination of the initial and total SBPase, PRK, and Rubisco activities.

SBPase activity measurements were determined by phosphate release (Harrison et al. 1998). After photosynthesis measurement, 0.1-g leaves were ground to a fine powder in liquid nitrogen in 1 ml extraction buffer (50 mM HEPES, pH8, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 2 mM benzamidine, 0.5 mM phenylmethylsulfonylfluoride, 10 mM DTT), and the resultant was centrifuged for 5 min at 14,000×*g*, 4°C. The resulting supernatant was stored in −80°C refrigerator. For the assay, the reaction was started by adding 20 μl of extract to 80 μl of assay buffer (50 mM Tris–HCl pH8.3, 15 mM $MgCl_2$, 1.5 mM EDTA, 10 mM DTT, 2.5 mM Ca $(NO_3)_2$, 2 mM SBP) and incubated at 25°C for 5 min. SBP was purchased from the University of Essex (UK). The reaction was stopped by adding 50 μl of 1 M perchloric acid and centrifuged for 10 min at 14,000×*g*, 4°C. Sample (10 μl) and standards (10 μl, PO_3^- 0.125–4 nmol) were incubated for 30 min at 25°C with 100 μl of malachite green solution (0.035% malachite green, 0.35% polyvinyl alcohol), and the A620 was measured using a microplate reader.

Activity of PRK was assayed at 25°C by coupling the formation of ADP to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase in the presence of 20 mM DTT to ensure full activation of PRK (Kagawa 1982). Initial activities of PRK were measured as described by Leegood (1990). Rubisco was assayed according to the method of Banks et al. (1999). Initial activities were determined from 20 μl of undiluted extract and assayed for 1 min before quenching with 100 μl of 10 mol/l formic acid. Total Rubisco activity was determined after preincubation of extract in assay buffer minus RuBP for 3 min. The assay was then started with RuBP and quenched with 10 mol/l formic acid after 1 min. The incorporation of ^{14}C label was determined by scintillation counting. The activation state of Rubisco and PRK was calculated as the relative ratio of initial to total activities.

Isolation of Thylakoids and Chloroplast

Thylakoids were isolated from the leaves of wild-type and transgenic rice plants. Fresh leaves were homogenized in a medium containing 0.4 M Suc and 50 mM Tricine, pH7.6, and the homogenate was filtrated through four and then eight layers of gauze. The filtrate was centrifuged at 800×*g* for 2 min to remove large debris. The supernatant was centrifuged at 3,000×*g* for 10 min. The pellet was washed twice by the buffer (50 mM Tricine, 10 mM NaCl, 5 mM $MgCl_2$, pH7.6) at 10,000×*g* for 10 min. The resulting washed pellet was thylakoid membranes. All procedures were carried out at 0°C to 4°C.

Intact chloroplasts were isolated by centrifugation on a Percoll density gradient according to the protocol of Mullet and Chua (1983). The percentage of intact chloroplasts was determined by measuring ferricyanide photoreduction before and after osmotic shock. Chlorophyll content was determined in 80% (*v*/*v*) acetone according to Porra et al. (1989).

Determination of Growth Parameters

After harvested, fresh weights (FW) of whole plants were determined. The dry weights (DW) were determined after oven drying at 80°C for 24 h. Leaf relative water content was calculated as: (FW−DW)/(TW−DW)×100, where FW is the fresh weight, TW is the turgid weight after rehydrating samples for 24 h, and DW is the dry weight after oven drying samples at 80°C for 24 h. Relative growth rate (RGR) for the single plants was calculated from the mean of the seedling DW, final plant DW, and days of growth.

Determination of Metabolite and Carbohydrate Levels

Plant tissues (0.1 g FW) were placed into the liquid nitrogen immediately and then ground in liquid nitrogen using a pestle and mortar with 1 ml of 5% $HClO_4$. The

levels of sucrose and phosphorylated metabolites (RuBP) were measured enzymatically as described by Leegood (1993). The level of starch was measured as described by Lin et al. (1988).

Results

Production and Selection of Rice Transformants

A SBPase antisense gene construct was prepared by inserting rice SBPase cDNA fragment in the binary vector pU1301 between a maize ubiquitin promotor and nopaline synthase terminator (Fig. 1a). A total of 119 primary transformants (T_0) were rooted on hygromycin-containing medium subsequently transferred to soil and grown until maturity. Expression of the transgenic plants in the T_0 plants was confirmed by reverse transcription-PCR (data not shown). Following this screening step, in order to identify the transgenic lines expressing reduced levels of the SBPase gene, RNA blot gel analysis was utilized to determine the transcript levels of the transgenic plants, and the results revealed that different transcript levels varied in different lines, and the control line (transformed with the SBP sequence omitted from the construct) has the same transcript level of wild type (untransformed line; Fig. 1b). Western blot analysis of T_3 plants from four selected SBPase antisense-expressing lines (L1, L2, L3, L4) and control line (C) confirmed that no significant changes in the levels of the Calvin cycle enzymes Rubisco and PRK had occurred (Fig. 1c). Analysis of total SBPase activities in newly fully expanded leaves of T_0 and T_3 progeny of transgenic plants revealed that plants with a range of SBPase activities had been produced and that these were maintained in subsequent generations (Fig. 1d). SBPase expression segregated in all the lines of both the T_0 and T_3 progenies with some plants having SBPase activities close to wild type, although in others a decrease of as much as 15% was evident.

Effects of Nitrogen Levels on Growth of Seedlings and Water Content of Leaves

Growth rates of seedlings were measured by the changes of relative dry weight accumulation. Figure 2 shows that the effects of nitrogen levels on growth of seedlings of wild-type and four transgenic lines. Results showed that low N compared with high N produced smaller plants. At low N, plants with the lowest SBPase activities were 61% (1 mM N) or 93% (0.1 mM N) smaller by the end of the experiment than the control line. The growth rate was significantly decreased in control plants and four transgenic lines at low N; however, the decrease in the growth rate was much greater once SBPase was decreased below 70% of the wild-type activity. Stronger effects on growth of L1 compared with the control plant were observed. At high N, the decrease in the growth rate was slight in different lines. To explain these differences in dry weight, we measured the shoot-to-root ratio and the water of the leaves. Figure 2 and Table 1 showed that there was no consistent effect on the shoot-to-root ratio and leaf relative water content of wild-type, control line, and transgenic lines at different N levels.

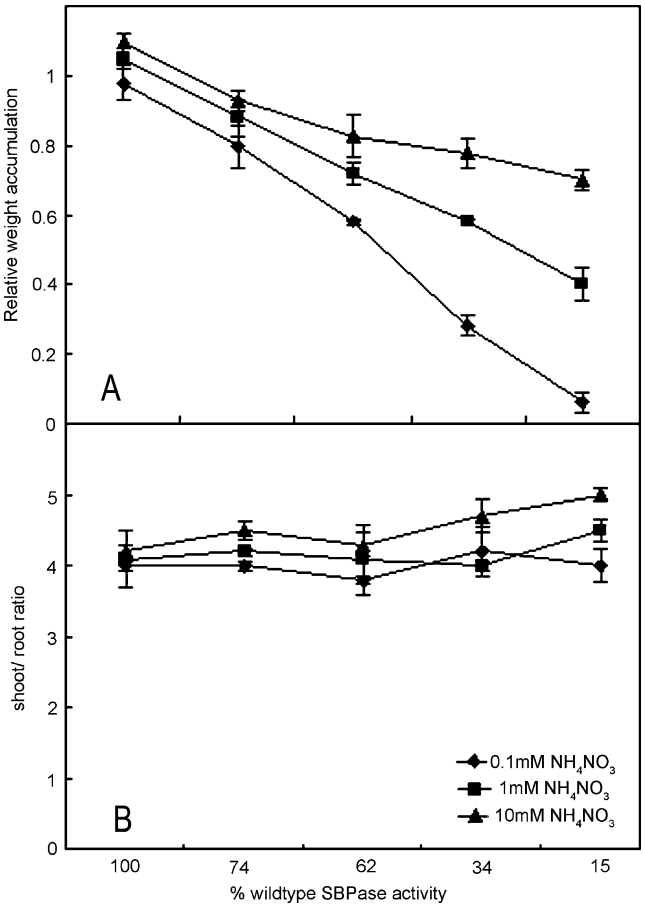

Fig. 2 Effects of nitrogen levels on the growth of seedlings (**a**) and shoot-to-root ratio (**b**) of control plants and transgenic plants. The growth rate of seedlings was calculated as the relative increase in dry weight assimilation per seedling after the plants were harvested. For measurements of dry weight, the seedlings were dried at 80°C for 2 days. Shoot-to-root ratio was calculated in dry weight (shoot or root) after the plants were harvested. The values are the mean ± SE of five independent experiments

Effects of Nitrogen Levels on Co_2 Assimilation

We investigated the effects of nitrogen levels on CO_2 assimilation in the seedlings of wild-type and transgenic lines. Figure 3 shows that a decrease in SBPase activity had an effect on CO_2 assimilation in four lines, especially in L1

Table 1 Effects of nitrogen levels on leaf relative water content (RWC) in wild-type plants and transgenic plants

RWC (%)	NH_4NO_3 concentration (mM)		
	0.1	1	10
L1	97.5±0.6	92.8±0.8	90.3±0.2
L2	97.1±0.3	93.8±0.2	90.8±0.2
L3	97.4±0.9	98.5±0.1	91.1±0.1
L4	98.2±0.2	96.8±0.1	93.6±0.7
C	98.1±0.4	97.8±0.9	95.2±0.1
WT	97.5±0.4	90.8±0.9	89.8±0.6

The values are the mean ± SE of five to seven independent experiments

WT wild type (untransformed line), *C* control plants (transformed with the SBP sequence omitted from the construct), *L1–L4* independent transformant lines

and L2 at different N levels. At high N, the rates of CO_2 assimilation of leaves decreased significantly when SBPase activities were below 60% of wild-type SBPase activity. However, at low N, the decrease of CO_2 assimilation in the youngest expanded leaves of transgenic lines was much greater than that at high N, especially in which rates of photosynthesis in L1 were less than 10% of the rates in the control plant. Similar results were also observed in the carboxylation efficiency of photosynthesis and the apparent quantum yield. These results showed that low N in addition to low SBPase activity significantly limited the rate of CO_2 assimilation in the youngest expanded leaves of transgenic lines.

Effects of Nitrogen Levels on Metabolite (RuBP) and Carbohydrate Levels

The effects on metabolite (RuBP) and carbohydrate (sucrose and starch) levels in seedlings of wild-type and transgenic rice plants at different nitrogen levels were further investigated. Figure 4 showed that RuBP started to decrease significantly when SBPase activities were decreased below 60% of wild-type plants at different nitrogen levels. At low N, RuBP in both wild-type and transgenic rice plants decreased significantly than at high N. However, the decrease was much greater in L1 and L2, which have less SBPase activity than in other lines. Similar results were also observed in the starch and sucrose contents. L1 with the lowest SBPase activity showed the lowest accumulation of metabolite (RuBP) and carbohydrate at low N. These results indicated that the metabolite (RuBP) and carbohydrate (starch and sucrose) content at low N were significantly lower in transgenic plants than that in wild-type plants.

Effects of Nitrogen Levels on SBPase, PRK, and Rubisco

To explain the physiological basis for decreased growth and CO_2 assimilation at low N induced by antisense-expressing SBPase, we firstly investigated the effects of nitrogen levels on PSII photochemistry (F_v/F_m) in seedlings of wild-type and transgenic rice plants. Our results showed that there were no significant changes in the maximal efficiency of PSII photochemistry (F_v/F_m) in wild-type and transgenic plants at different nitrogen levels, suggesting that the decreased CO_2 assimilation at low N had nothing to do with the changes in the function of PSII (Fig. 5a). Thus, we further investigated whether the decrease of CO_2 assimilation rate at low N was

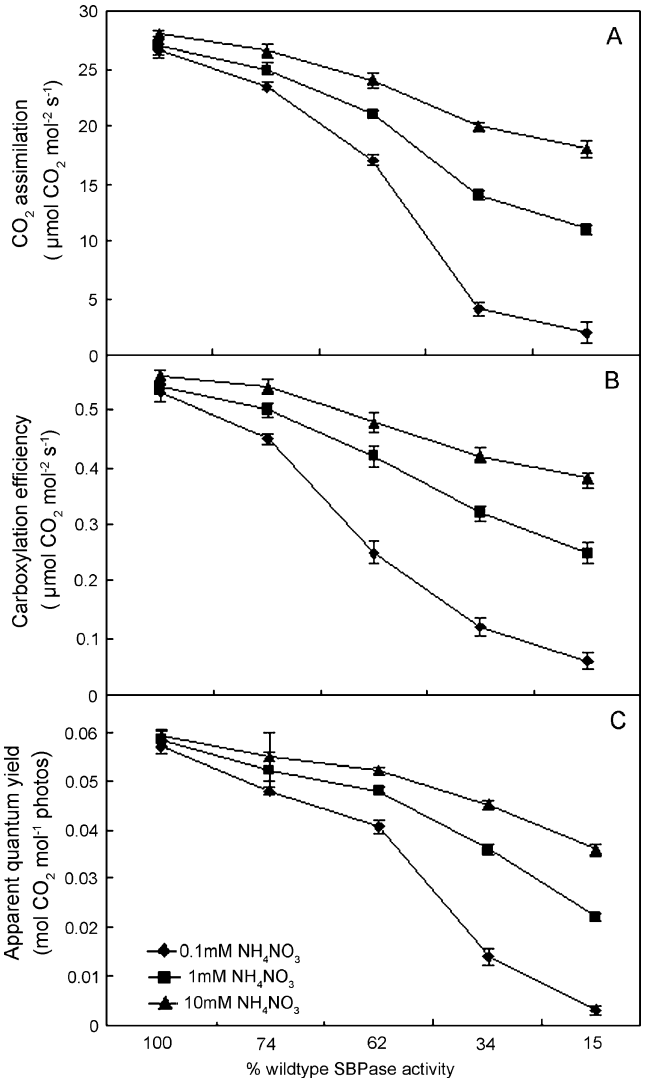

Fig. 3 Effects of nitrogen levels on CO_2 assimilation rate (**a**), the carboxylation efficiency (**b**), and the apparent quantum yield (**c**) of photosynthesis in wild-type plants and transgenic plants. The photosynthetic gas exchange parameters were determined on the expanded leaves in control plants and transgenic plants. Values are the mean ± SE of five to seven independent experiments

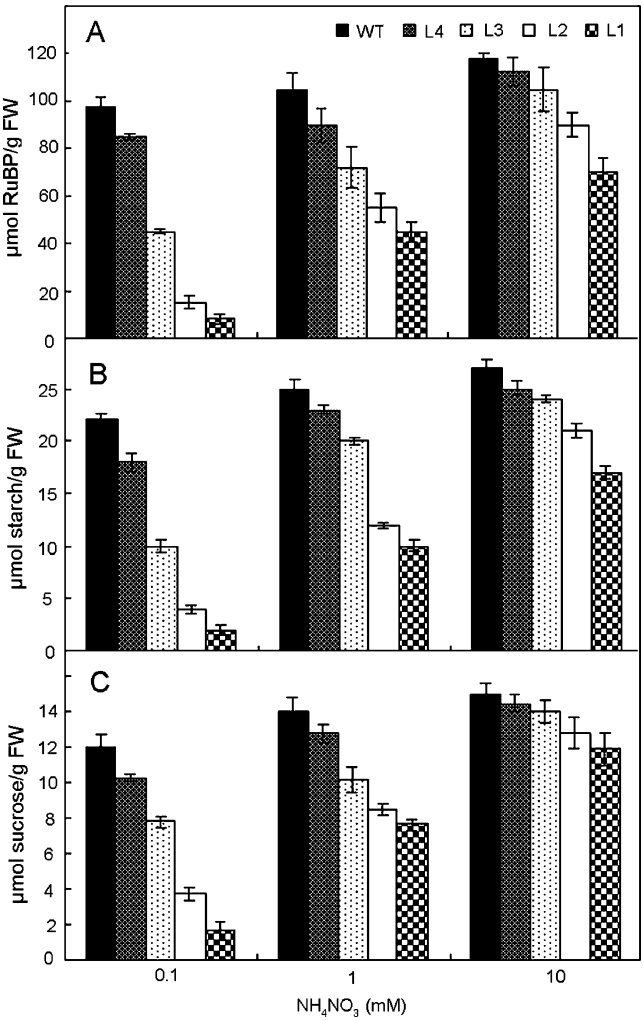

Fig. 4 Effects of nitrogen levels on RuBP (**a**), starch (**b**), and sucrose (**c**) in wild-type plants and transgenic plants. The contents of RuBP and starch and sucrose were determined on the expanded leaves in control plants and transgenic plants. *WT* wild type (untransformed line), *L1–L4* independent transformant lines. Values are the mean ± SE of five to seven replications

due to the inhibition of the Calvin cycle reaction. Figure 5b showed that SBPase activity started to decrease at low N (1 mM) in wild-type plants and transgenic plants. When nitrogen levels were lower than 1 mM, SBPase activity in both types of plants decreased significantly. However, these decreases in SBPase activity were much greater in transgenic plants than those in wild-type plants. These results suggested that the decrease of CO_2 assimilation is due to a lower level of SBPase activity in transgenic plants than in wild-type plants at the low N level.

Then, we investigated the effects of nitrogen level on SBPase association in the soluble fractions and the thylakoid fractions in wild-type and transgenic plants. Figure 5c showed that the content of SBPase in soluble fractions decreased at low N, and this decrease was more notable in L1 plants than in wild-type plants. On the other hand, the content of SBPase in thylakoid fractions increased at low N, and this increase was more pronounced in L1 plants than in wild-type plants (Fig. 5d). These results indicated that low N caused association of SBPase with thylakoids from the stroma fractions and such association was much more pronounced in transgenic plants than in wild-type plants.

We also compared the effects of nitrogen on the activity of Rubisco between wild-type plants and transgenic plants. Because PRK synthesizes RuBP from ribulose-5-phosphate (Ru5P) and is a potential site of metabolic regulation (Laing et al. 1981; Gardemann et al. 1983), we also compared the effects of nitrogen on the activity of PRK between wild-type plants and transgenic plants.

In this study, we observed that the total Rubisco activity and the total PRK activity were not affected in different

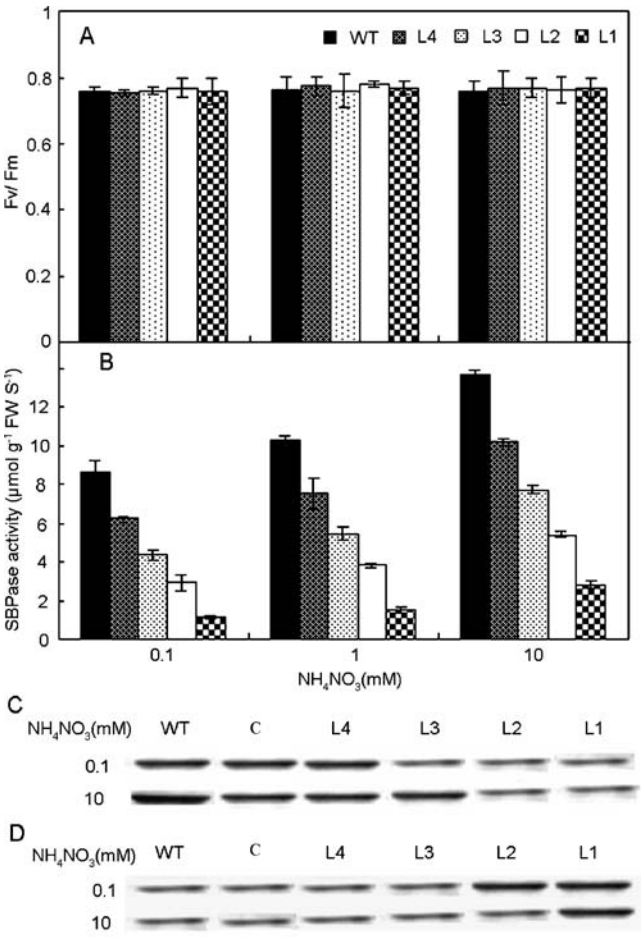

Fig. 5 Effects of nitrogen levels on F_v/F_m, SBPase activity, and association in wild-type plants and transgenic plants. **a** F_v/F_m. **b** SBPase activity. **c** Content of SBPase in the soluble fractions of extracts of leaves. **d** Content of SBPase in the thylakoid fractions of extracts of leaves. *WT* wild type (untransformed line), *C* control plants, *L1–L4* independent transformant lines. The values are the mean ± SE of five independent experiments

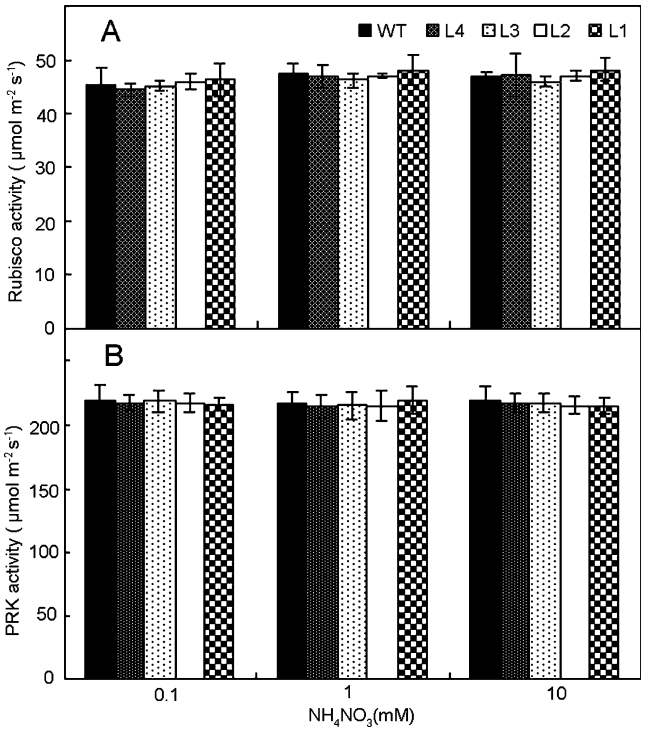

Fig. 6 Effects of nitrogen levels on Rubisco (**a**) and PRK (**b**) activities in wild-type plants and transgenic plants. *WT* wild type (untransformed line), *L1–L4* independent transformant lines. The values are the mean ± SE of five to seven independent experiments

nitrogen levels in wild-type and transgenic plants (Fig. 6). Analysis of Western blotting also showed that there was no change in the content of Rubisco and PRK in wild-type and transgenic plants at different nitrogen levels (data not shown). On the contrary, the initial Rubisco activity and the initial PRK activity clearly decreased with decreasing nitrogen. Thus, decreased nitrogen level resulted in a significant decrease of the Rubisco and PRK activation state in wild-type and transgenic plants. Figure 7 also showed the effects of nitrogen on the Rubisco and PRK activation state in both wild-type and transgenic plants. Low N caused a progressive inhibition of Rubisco and PRK activation state in both wild-type and transgenic rice plants. However, the decrease of the Rubisco and PRK activation state occurred significantly below 1 mM in transgenic plants. Moreover, at low N (0.1 mM), the inhibition of the Rubisco and PRK activation state was more prominent in transgenic plants than in wild-type plants. These results suggested that the decreased SBPase activation resulted in the decrease of Rubisco and PRK activation state at low N, thus decreasing the CO_2 assimilation rate in transgenic rice plants.

We further investigated the cause of the decrease of the Rubisco activation state and PRK activation state to low N in transgenic plants. The activation states of Rubisco and PRK were calculated as the relative ratio of initial to total activities. We examined whether the inhibitions of Rubisco and PRK activation state were associated with the decrease in the content of RuBP and Rubisco activase. We also analyzed the content of RuBP in the soluble fractions and the thylakoid fractions in wild-type and transgenic plants. Figures 8a and 9a, c showed that the contents of RuBP and Rubisco activase in soluble fractions decreased at low N, and these decreases were more pronounced in transgenic L1 plants than in wild-type plants.

On the other hand, the contents of RuBP and Rubisco activase in thylakoid fractions increased at low N, and these increases were more pronounced in transgenic L1 plants than in wild-type plants (Figs. 8b and 9b, d). Thus, the results suggested that the decrease of the Rubisco activation state at low N in transgenic plants was due to lower content of RuBP and Rubisco activase in the soluble stroma fractions, which was linked to increased association of RuBP and Rubisco activase to thylakoid membranes from the soluble stroma fractions at low N.

Discussion

In this study, our data showed that antisense-inhibiting SBPase gene resulted in the decreased growth and

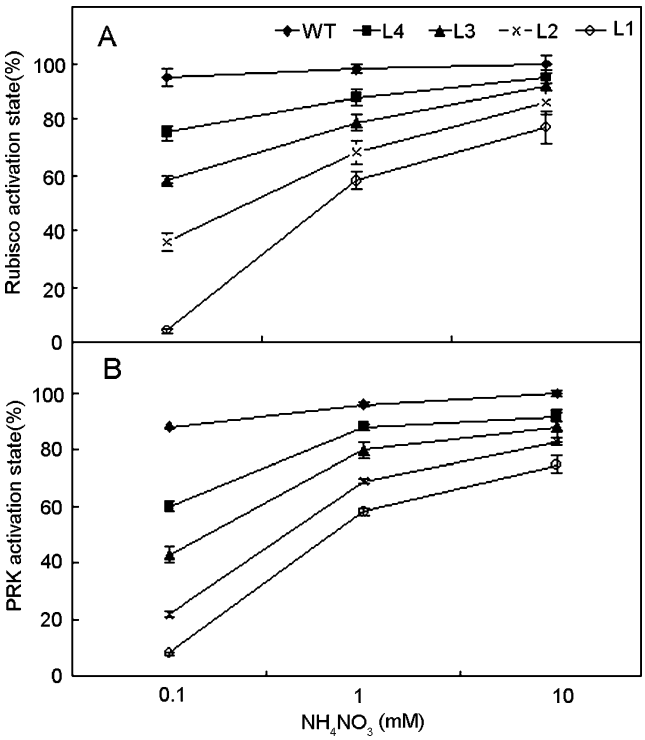

Fig. 7 Effects of nitrogen levels on Rubisco (**a**) and PRK (**b**) activation states calculated as the relative ratio of initial to total Rubisco and PRK activities in wild-type plants and transgenic plants. *WT* wild type (untransformed line), *L1–L4* independent transformant lines. The values are the mean ± SE of five to seven independent experiments

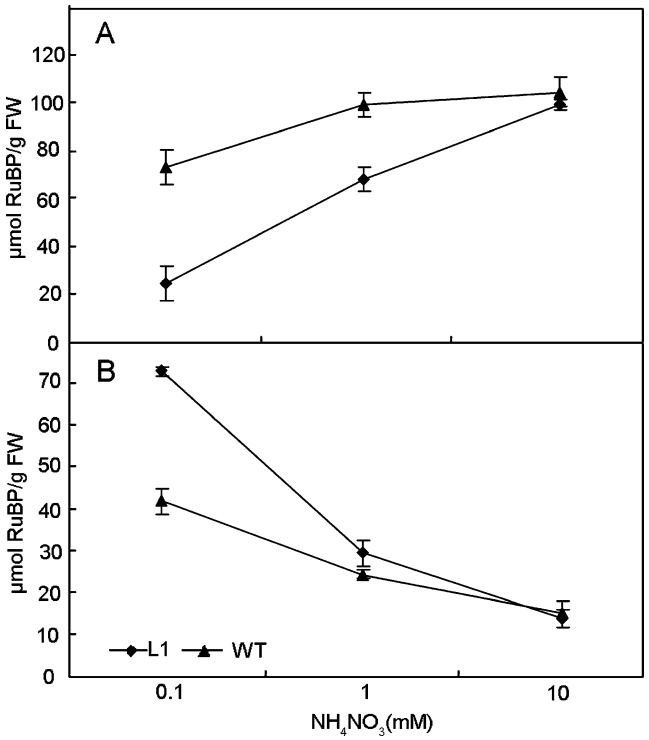

Fig. 8 Content of RuBP in the soluble fractions (**a**) and the thylakoid fractions (**b**) of extracts from the leaves of wild-type plants and transgenic L1 plants after being harvested. The values are the mean ± SE of five to seven independent experiments

photosynthesis in transgenic rice plants and showed the physiological basis of SBPase in vivo for such decrease at low N. The results suggested an important physiological role of SBPase in vivo, i.e., affecting the N use efficiency photosynthesis and growth.

The effect on photosynthesis, growth, and biomass allocation by decreasing SBPase activity at different nitrogen levels has been proposed significantly. Although decreased Rubisco activity in transgenic tobacco reduced photosynthesis, however, the content of Rubisco accounts for 15% to 35% of total leaf N in C_3 species, and a decrease in the amount of Rubisco substantially disrupts the N balance of the plant, making it difficult to establish direct links between photosynthesis and growth and allocation (Woodrow and Berry 1988). Sufficient to diminish photosynthesis and limit biomass production need a 94% decrease in PRK at low N (Banks et al. 1999). Some studies have shown that reduced SBPase levels in transgenic tobacco lead to decreased photosynthetic capacity and alter carbohydrate accumulation (Harrison et al. 1998). However, our results further showed photosynthetic capacity, and carbohydrate accumulation is not only related with SBPase activity, but also with nitrogen levels. Our data suggested that low N in addition to a 34% decrease in SBPase activity is sufficient to diminish photosynthesis and limit biomass production. SBPase therefore represents a more ideal target for studies of this nature than other enzymes in Calvin cycle such as Rubisco and PRK. SBPase activity was affected predictably by antisense in different transgenic lines at all N levels further validating the use of the plant material to examine direct effects between photosynthesis, growth, and allocation.

To further understand why decreasing SBPase activity in vivo can inhibit growth and photosynthesis of transgenic plants at low N, we investigated whether such an inhibition of CO_2 assimilation was associated with RuBP and Rubisco activase-mediated activation of Rubisco. Rubisco is active when a specific lysine residue within its catalytic site is

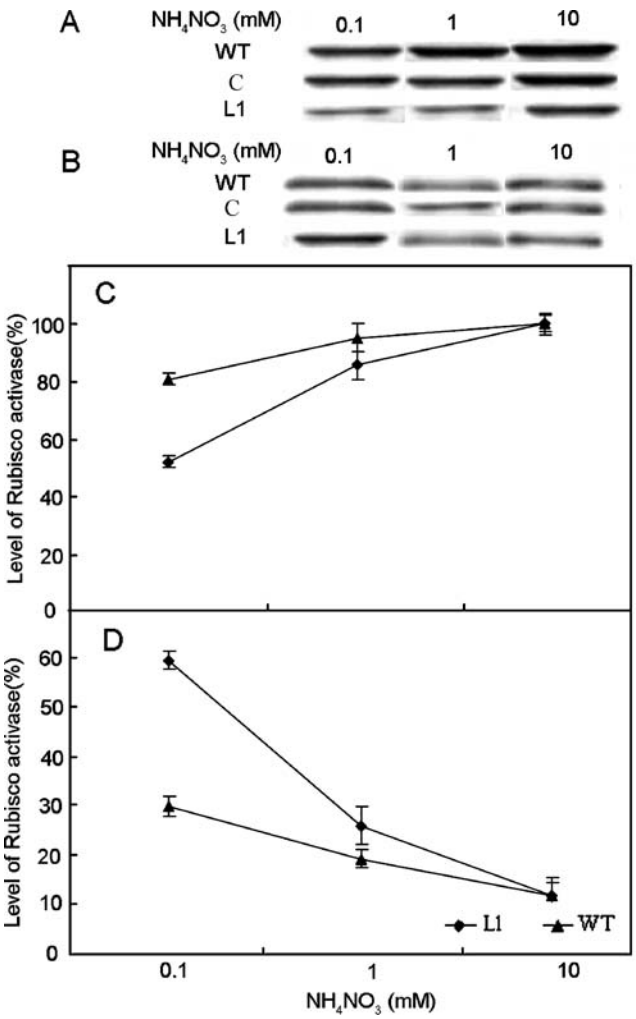

Fig. 9 **a** Content of Rubisco activase in the soluble fractions of extracts from the leaves of wild-type plants and control plants and transgenic L1 plants. **b** Content of Rubisco activase in the thylakoid fractions of extracts from the leaves of wild-type plants and control plants and transgenic L1 plants after harvested. **c** Quantification of results in **a**. **d** Quantification of results in **b**. The values are the mean ± SE of five independent experiments

carbamylated and bound with Mg^{2+}. The change in the carbamylation state in vivo is mediated by another stroma protein, Rubisco activase (Salvucci et al. 1985, 1996). Under physiological conditions, Rubisco activase functions in the chloroplast stroma by removing inhibitory sugar phosphates from the active site of Rubisco in an ATP-dependent manner, and the decrease in the content of Rubisco activase in the chloroplast stroma would result in a decrease in the activation of Rubisco and thus a decrease in the CO_2 assimilation rate (Portis 1992). In the absence of RuBP, Rubisco activase has no effect on the activation state of Rubisco, indicating that Rubisco activase may bind Rubisco only when the active site is occupied with RuBP (Portis 1995; Salvucci and Ogren 1996). Our study found that the content of RuBP and Rubisco activase in the soluble stroma fractions decreased significantly at low N, and such a decrease was more pronounced in transgenic plants than in wild-type plants. We further observed that the association of RuBP and Rubisco activase with the thylakoid membranes increased with increasing N levels in wild-type and transgenic plants. However, the increase in RuBP and Rubisco activase content in the thylakoid membrane fractions in wild-type plants was much less than in transgenic plants (Figs. 8 and 9). Thus, our results showed that RuBP and Rubisco activase were mostly sequestered to the thylakoid membrane from the soluble stroma fraction at low N. It is suggested that compared to wild-type plants, the reduction of CO_2 assimilation in transgenic plants at low N was associated with decreased RuBP and Rubisco activase content, which resulted in more association of RuBP and Rubisco activase with the thylakoid membranes from the soluble stroma fraction. SBPase is thought to be responsible for the regeneration of the CO_2 acceptor, RuBP, and the regenerative capacity of RuBP responds linearly to reductions in SBPase activity (Harrison et al. 2001). Our results showed that SBPase retained lower activity in transgenic plants than in wild-type plants at low N because of an association of SBPase with thylakoids from the soluble stroma, and such an association was much more pronounced in transgenic plants than in wild-type plants. Since SBPase functions in the chloroplast stroma under physiological conditions (Raines et al. 1999), the lower accumulation of the metabolite (RuBP) in transgenic plants was mainly due to the lower activity and content of SBPase in the soluble stroma.

The function of PSII measured by F_v/F_m describes its potential activities, i.e., healthiness and resistance to stress factors, which is different from the productivity measured for a certain period of time. Our results also showed that PSII was not affected at different N levels, suggesting that decreased photosynthesis in transgenic plants at low N was not related to the function of PSII (Fig. 5). However, it has been reported that photosynthetic electron transport is the functional limitation of photosynthesis in field-grown Pima cotton plants (*Gossypium barbadense*) under stress conditions (Wise et al. 2004). Some stress induces a reversible thylakoid membrane leakiness (Mitsuya et al. 2003; Yamane et al. 2003; Pastenes and Horton 1996; Schrader et al. 2004) and induces a cyclic electron transport around PSI that can balance the loss of protons due to membrane leakiness so that a sizeable pH can be maintained despite membrane leakiness (Schrader et al. 2004). It has been proposed that the deactivation of Rubisco can be an adaptive mechanism in response to the decreased capacity for electron transport under stress conditions so that the thylakoid energy is maintained and the accumulation of high levels of photorespiratory intermediates is reduced (Schrader et al. 2004). Thus, the binding of RuBP and Rubisco activase to thylakoid membranes observed in our study can be explained by an adaptive mechanism in response to the decreased photosynthetic electron transport, which may result from membrane leakiness and increased cyclic electron transport at low N. It should be pointed out that the reduced Rubisco activation state should have no effect on the apparent quantum yield of photosynthesis. However, the results in this study showed that low N reduced the apparent quantum yield of photosynthesis (Fig. 3). As discussed above, nitrogen stress (low N) may induce membrane leakiness and increase cyclic electron transport, which leads to a decrease in photosynthetic electron transport although low N induced no damage to PSII. Thus, the reduced apparent quantum yield may be explained by the decreased photosynthetic electron transport due to membrane leakiness and increased cyclic electron transport at low N. The decrease in the quantum yield of PSII electron transport would result in the decrease in the apparent quantum yield of photosynthesis. Therefore, the decrease in the apparent quantum yield observed in this study could also be explained by the down-regulation of PSII activity in response to an inhibition of photosynthetic carbon metabolism.

Therefore, the results presented here strongly suggested a role for SBPase at low N which reduced Rubisco activation state by providing less RuBP and decreased the association of RuBP and Rubisco activase with the thylakoids from the chloroplast stroma, then reduced the N use efficiency of photosynthesis and growth rate. Many investigations reported that N deficit many affect plants via inhibition of meristem growth (Tolley Hendry and Radin 1986; Bastow-Wilson 1988; Rufty et al. 1989a, b) and inhibition of photosynthesis in mature leaves. Our results suggested that it is a possible important role for SBPase elucidating the N availability and the availability of photosynthesis to both regulate storage and allocation of biomass to optimize resource utilization.

The changes observed by Fichtner et al. (1993) in the shoot-to-root ratio in plants with altered Rubisco content were probably caused by the general disruption of plant N balance (Scheible et al. 1997). Accumulation of starch at low N is a well-established phenomenon (Rufty et al. 1988; Paul and Driscoll 1997) and is interpreted as being a symptom of sink-limited growth, i.e., the capacity to assimilate and produce outweighs the capacity to use it, with surplus assimilate accumulating as starch. Therefore, following this logic, a decrease in the rate of photosynthesis should be less important to overall biomass production under sink-limited conditions at low N, at which there is much C. However, the opposite was true with SBPase-limited photosynthesis at low N, which had a larger effect on growth than at high N but has no direct effects on the shoot-to-root ratio. This was partly because the interaction of low N plus antisense pushed SBPase activity lower than at high N, which more markedly affected photosynthesis, and it also indicates that source and sink are less out of balance at low N than was assumed in hypothesis.

It is worth comparing C allocation in our results with those of similar experiments carried out on Rubisco transgenic plants (Quick et al. 1991a; Fichtner et al. 1993). There was almost complete allocation of C away from starch in transgenic plants with decreased Rubisco activity at low N. Starch content responded more strongly to the changed source/sink balance because of Rubisco antisense. Allocation of C away from starch probably accounted for the observation that structural dry weight did not differ between the wild-type and the Rubisco transgenic plants. A possible explanation for the difference in C allocation may be that a decrease in Rubisco content, at least in the early phase of N deficiency, releases a large amount of N for use elsewhere in the plants (Paul and Stitt 1993). A decrease in the amount of SBPase, however, will not result in a large redistribution of N because SBPase accounts for only a fraction of the N within a leaf (Evans 1989). Thus, decreased photosynthesis in SBPase antisense plants occurs without any N redistribution. Strong interactions between starch metabolism and N metabolism have been demonstrated (Scheible et al. 1997). N balance was clearly perturbed in Rubisco transgenic plants, which in itself may have attached starch metabolism. It is also possible that internal N signals generated through mobilization of Rubisco influence the way long-term stores are managed, and the extension of this in Rubisco transgenic plants may have ensured the more effective partitioning of C away from starch in these plants. In conclusion, genetic decrease of SBPase goes beyond the normal acclimation in low N, and the resulting decrease in photosynthesis limits biomass production under conditions normally regarded as sink-limited.

The results suggested a new physiological function of antisense SBPase involved in photosynthesis and plant growth at nitrogen levels. These results would be valuable to understand the relationship between photosynthesis and growth and allocation in rice plants at different N levels and optimize the C/N metabolism in future studies.

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