Changes in leaf photosynthesis of transgenic rice with silenced *OsBP-73* gene

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Abstract

In comparison with its wild type (WT), the transgenic (TG) rice with silenced *OsBP-73* gene had significantly lower plant height, grain number per panicle, and leaf net photosynthetic rate (P_N). Also, the TG rice showed significantly lower chlorophyll (Chl), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), RuBPCO activase, and RuBP contents, photosystem 2 (PS2) photochemical efficiency (F_v/F_m and $\Delta F/F_m'$), apparent quantum yield of carbon assimilation (Φ_c), carboxylation efficiency (CE), photosynthetic electron transport and photophosphorylation rates as well as sucrose phosphate synthase activity, but higher intercellular CO_2 concentration, sucrose, fructose, and glycerate 3-phosphate contents, and non-photochemical quenching of Chl fluorescence (NPQ). Thus the decreased P_N in the TG rice leaves is related to both RuBP carboxylation and RuBP regeneration limitations, and the latter is a predominant limitation to photosynthesis.

Additional key words: carboxylation efficiency; intercellular CO_2 concentration; net photosynthetic rate; photochemical efficiency; *Oryza*; ribulose-1,5-bisphosphate (RuBP); ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO); RNA interference; RuBP regeneration limitation; stomatal conductance.

Introduction

Constructing mutants is an important means in modern plant science study. Mutants are not only basal materials in research of molecular biology but also new idioplasm resource for genetic improvement of crops and plants. Some scientists have successfully adopted mutants as experimental material to explore the molecular mechanisms for the regulation of photon energy harvesting, utilization, and dissipation in plant photosynthesis. For example, Li *et al.* (2000) used an *Arabidopsis* mutant unable to dissipate photon energy as heat to reveal the function of PsbS protein. Kasahara *et al.* (2002) utilized a mutant lacking chloroplast avoiding light movement to demonstrate the photoprotective role of the movement. Undoubtedly, mutants can also help elucidate the reason for change in leaf net photosynthetic rate (P_N).

OsBP-73, a rice gene, encoding a novel DNA-binding protein with a SAP-like domain, was originally isolated as the putative regulator of the rice *Wx* gene (Chen *et al.* 1999). For investigating the function of *OsBP-73* gene in transcription regulation of *Wx* gene, some strains of TG rice were obtained by using the double-stranded RNA interference (RNAi) technique (Chen *et al.* 2003), an effective way to discover gene function in many organisms (Chuang and Meyerowitz 2000). The TG rice with silenced *OsBP-73* gene had lower plant height, reduced tiller number and panicle size compared with the wild type, WT (Chen *et al.* 2003). In this study leaf photosynthesis and some related parameters were compared in TG rice (two independent lines, 73ds-7 and 73ds-8) and its WT (Zhonghua 11) in order to seek the relationship between the changes mentioned above and photosynthesis in the TG rice, and further understand the regulatory mechanisms of photosynthesis.

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Abbreviations: C_i – intercellular CO_2 concentration; CE – carboxylation efficiency; Chl – chlorophyll; g_s – stomatal conductance; NPQ – non-photochemical quenching; PGA – glycerate 3-phosphate; P_N – net photosynthetic rate; RNAi – RNA interference; RuBP – ribulose-1,5-bisphosphate; RuBPC – ribulose-1,5-bisphosphate carboxylase; RuBPCO – ribulose-1,5-bisphosphate caboxylase/oxygenase; RuBPCO-A – ribulose-1,5-bisphosphate caboxylase/oxygenase activase; SPS – sucrose-phosphate synthase; TG – transgenic; WT – wild type; Φ_c – apparent quantum yield of carbon assimilation.
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Materials and methods

Plants: The seeds of TG rice (73ds-7 and 73ds-8) and its WT (*Oryza sativa* ssp. *japonica* cv. Zhonghua 11) were offered by Prof. Z.-Y. Wang. Plants from these seeds were grown at a photosynthetic photon flux density (PPFD) of about 400 μmol m^{-2} s^{-1} with a 12/12 h light/dark cycle at 28/25 °C in a phytotron. Fully expanded flag leaves at the filling stage and mature seeds were used.

Amylose content in mature seeds was measured by the colorimetric method with iodine-potassium iodide as described by Juliano (1971). Standard amylose was purchased from *Sigma* (St Louis, MO, USA).

Gas exchange: Leaf P_N was measured *in situ* at 350 μmol(CO_2) mol^{-1} and PPFD of 1 200 μmol m^{-2} s^{-1} using a portable gas analysis system *LI-6400* (*LI-COR*, Lincoln, NE, USA). Air temperature of leaf chamber was maintained at about 30 ºC. Then, five of the measured ten leaves were used to measure apparent quantum yield of carbon assimilation (Φ_c) and carboxylation efficiency (CE) (Farquhar *et al*. 1980, Caemmerer and Farquhar 1981). In Φ_c measurement, CO_2 concentration was kept at 350 μmol(CO_2) mol^{-1}, and PPFD was set at 150, 120, 90, 60, and 30 μmol m^{-2} s^{-1} in turn. For CE measurements, PPFD was kept at 1 200 μmol m^{-2} s^{-1}, and CO_2 concentration was controlled with *LI-COR* CO_2 injection system set at 250, 200, 150, 100, and 50 μmol(CO_2) mol^{-1} in turn.

Chlorophyll (Chl) content and Chl *a* fluorescence: Chl was extracted from fresh leaf segments with 80 % acetone, determined and calculated according to the equations by Arnon (1949). Chl *a* fluorescence was measured with a portable *PAM-2000* fluorometer (*H. Walz*, Effeltrich, Germany) with the standard settings at room temperature (about 28 ºC). The potential photochemical efficiency of photosystem 2 (PS2; F_v/F_m) was measured with fully dark-adapted leaves (through whole night). Then the actual photochemical efficiency of PS2 ($\Delta F/F_m'$) and non-photochemical quenching (NPQ) were measured after the leaves were exposed to PPFD of about 1 200 μmol m^{-2} s^{-1} for 1 h. The calculations were made according to Genty *et al*. (1989) and van Kooten and Snel (1990).

Enzyme contents and activit*ies*: Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) and its activase (RuBPCO-A) contents were measured with a protein detector *ELISA* kit, *ABTS* system (*KPL*, Gaithersburg, MD, USA; Fleming and Pen 1988) as described by Chen *et al*. (2005).

Antibodies to RuBPCO and RuBPCO-A were prepared in our laboratory from the antisera of rabbits immunised with the purified RuBPCO and RuBPCO-A from tobacco leaves, respectively. The standard curve for RuBPCO quantity was made with purified tobacco RuBPCO, and the purification of RuBPCO from tobacco leaf was performed according to Li (1997). The standard curve for RuBPCO-A quantity was made with crude extract of the WT leaves. RuBPCO-A content in the TG rice was expressed as the percentage of its WT. All contents here were based on leaf area.

Sucrose-phosphate synthase (SPS) activity measurement was performed as described by Seneweera *et al*. (1995) with some modifications (Chen *et al.* 2005). Namely, 0.05 cm^3 of the soluble protein extract supernatant mentioned above was added to 0.15 cm^3 of assay buffer including Tris-HCl (50 mM, pH 7.0), $MgCl_2$ (10 mM), Fru-6-phosphate (10 mM), and UDP-Glc (3 mM), and the mixture was incubated at 30 °C for 10 min. Then, 0.05 cm^3 of NaOH (2 M) was added, and incubated at 100 °C for 10 min. After cooling with flowing water, 0.7 cm^3 of 30 % HCl and 0.2 cm^3 of 0.1 % resorcinol (m/v, dissolved in 95 % ethanol) were added, then the mixture was incubated at 80 °C for 10 min. Finally, the optical density of this mixture was measured at 480 nm after cooling. For blanks, the first incubation was performed on ice.

Soluble sugar contents: The leaf segments were baked at 80 ºC for 12 h, ground into fine powder, and preserved in a vacuum at 4 ºC. The soluble sugar was extracted from the fine powder with 95 % ethanol, and the extract was centrifuged. The residue obtained after centrifugation was twice re-extracted with 95 % ethanol. The supernatants were combined. Sucrose, glucose, and fructose contents were measured according to Cardini *et al.* (1955).

Photosynthetic electron transport and photophosphorylation: Chloroplasts were isolated from fresh and pre-chilled rice leaves with STN solution (0.4 M sucrose, 10 mM NaCl, and 50 mM Tris-HCl, pH 7.6) at 4 ºC as described by Wei *et al*. (1988). Photophosphorylation activity was measured based on Wei *et al.* (1998) with some modifications. Chloroplast photophosphorylation reactions were carried out in 1 cm^3 reaction mixtures containing 50 mM Tricine-NaOH (pH 8.0), 5 mM NaCl, 5 mM $MgCl_2$, 10 mM Na_2HPO_4, 1 mM ADP, 1 mM FeCy for non-cyclic photophosphorylation, and chloroplasts containing 20 μg of Chl. ATP content was measured by the luciferin/luciferase luminescence assay (Allnutt *et al*. 1991).

The electron transport rates of the whole chain were assayed with H_2O as an electron donor and ferricyanide (FeCy) as an electron acceptor according to Shen and Shen (1962). The remainder of the reaction mixture was identical to the above photophosphorylation reaction.

PS2 electron transport rate of thylakoids was measured as described in Baena-González *et al.* (2003) with some modification. Thylakoids were isolated from

leaves (Hong and Xu 1999), the rate was measured at saturating irradiance (1 200 μmol m^{-2} s^{-1}) by a Clark-type oxygen electrode, using 1,4-benzoquinone (0.5 mM) as an electron acceptor and NH_4Cl (20 mM) as an uncoupler.

RuBP and glycerate 3-phosphate (PGA) contents: RuBP was extracted according to Vu *et al.* (1997) with some modifications. RuBP was extracted from the liquid N_2-frozen leaf powder with 0.5 M HCl. After the extract was centrifuged at 16 000×*g* and 2 ºC for 5 min, the supernatant was adjusted to pH 8.2 with 4 M KOH and then 2 M Tris-Base as micro-adjustment. Then, RuBP was transformed into PGA by adding the pH-adjusted supernatant to an assay buffer containing 0.1 M Tris-HCl (pH 8.2), 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, and 150 μg RuBPCO. PGA content was determined according to Seemann and Sharkey (1986).

Statistical analysis of all data including means, standard errors, and *t*-tests was made with *Sigma Plot 8.0* (*SPSS*, USA).

Results

Phenotype of TG rice: In comparison with WT plants, the growth of the TG rice was strikingly inhibited, resulting in dwarf plants with reduced values of actual grain number per panicle, filled spikelet percentage, and seed amylose content (Table 1).

Table 1. The phenotype and seed amylose content of transgenic (TG) rice and its wild type (WT). Each value in this table is the mean of 8 plants with SE. The significant levels of difference between transgenic rice and its WT are shown by asterisks $^{**}p<0.01$.

	WT	73ds-7	73ds-8
Plant height [cm]	83.6±1.1	62.8±1.6**	66.6±1.0**
Actual grain number per panicle	87.0±2.7	59.2±3.9**	72.0±4.1**
Filled spikelets [%]	76.0±1.3	60.8±3.3**	72.6±3.1
Seed amylose content [%]	100	88.3	93.2

Table 2. Photophosphorylation [μmol(ATP) m^{-2} s^{-1}] and electron transport [μmol(FeCy) m^{-2} s^{-1} for the whole chain and relative amount for photosystem 2 (PS2)] rates as well as P/O ratio (formed ATP/evolved O_2, an index describing the coupling efficiency of photophosphorylation and non-cyclic electron transport) in the chloroplasts isolated from flag leaves of transgenic (TG) rice and its wild type (WT). Each value except for P/O ratio in this table is the mean and standard error of three replicates. The significant levels of differences between TG rice and its WT are shown by asterisks $^{*}p<0.05$ or $^{**}p<0.01$.

	Photophosphorylation	Electron transport		P/O ratio
		whole chain	PS2 [%]	
WT	26.72±2.42	40.92±0.77	100.00	1.3
73ds-7	10.07±0.94**	17.86±0.11**	61.19±4.45*	1.1
73ds-8	20.63±0.34*	33.20±0.63**	55.94±1.28**	1.2

Leaf P_N of TG rice was significantly lower than that of its WT (Fig. 1*A*). In comparison with the WT, the TG rice had a similar stomatal conductance (g_s) (Fig. 1*B*) and a remarkably higher intercellular CO_2 concentration (C_i) (Fig. 1*C*), suggesting that the decrease in P_N of the TG rice leaves was not due to a decrease in g_s. In consonance with decreased P_N, the carboxylation efficiency (CE) of TG rice leaves significantly declined (Fig. 1*D*), implying that the capacity of RuBP carboxylation decreased in TG rice leaves.

The apparent quantum yield of carbon assimilation (Φ_c) was significantly lower in the TG rice than in its WT (Fig. 1*E*).

Chl content and Chl *a* fluorescence: Compared with the WT, the Chl content in TG rice leaves was significantly decreased (Fig. 2*A*). Also, the TG rice showed a significantly lower PS2 photochemical efficiency (F_v/F_m and $\Delta F/F_m'$) and a higher NPQ (Fig. 2*B*).

Sugar contents: Compared with the WT, the contents of soluble sugars such as sucrose and fructose were significantly increased but glucose content was decreased in the TG rice leaves (Fig. 3*A–C*).

Enzyme contents and activities: The soluble protein, RuBPCO, RuBPCO activase contents, and sucrose-phosphate synthase (SPS) activity in TG rice leaves were also remarkably decreased compared with the WT (Fig. 3*D–G*). This is consistent with the results of some

previous studies showing that the declined P_N was always accompanied by a down-regulated SPS activity (*e.g.* Chen *et al*. 2005).

Photosynthetic electron transport and photophosphorylation: The whole chain and PS2 electron transport and photophosphorylation rates were significantly reduced in chloroplasts isolated from TG rice leaves (Table 2), implying that the capacity of the assimilatory power (ATP and NADPH) formation is declined in the TG rice leaves.

RuBP and PGA contents: In comparison with WT, RuBP pool size was significantly reduced while PGA content was increased in TG leaves (Fig. 4). Hence the capacity of RuBP regeneration was down-regulated in TG rice leaves.

Discussion

A positive correlation between leaf P_N and crop yield is the reflection of the essential relationship between photosynthesis and crop yield (Xu and Shen 2001). The lowered values of plant height, actual grain number per panicle, and filled spikelet percentage in TG rice must be linked to its deduced P_N (Table 1).

The significantly lower P_N in TG rice leaves may be mainly attributed to a lower photosynthetic capacity of mesophyll cells rather than g_s because their significantly lower P_N was accompanied by significantly higher C_i compared with WT leaves (Fig. 1). The lower photosynthetic capacity of mesophyll cells in TG rice may be explained by a lower RuBP carboxylation capacity. The RuBP carboxylation reaction catalysed by RuBPCO is widely accepted as the ultimate rate-limiting step in photosynthetic carbon assimilation at saturating irradiance and atmospheric CO_2 concentration (Jensen 2000). Some studies indicate that P_N is positively correlated with the activity and/or activated amount of RuBPC (Hesketh *et al*. 1981, Evans 1986). Furthermore, RuBPCO loss or RuBP carboxylation limitation has been considered as the main cause of photosynthetic down-regulation at elevated CO_2 concentration (Bowes 1991, Stitt 1991, Rogers *et al*. 1996, Moore *et al*. 1999). In TG rice leaves, the significantly decreased CE (Fig. 1) and RuBPCO and RuBPCO-A contents (Fig. 3) indicate a declined RuBP carboxylation capacity, supporting the above explanation.

The decrease in RuBPCO and its activase contents may be due to higher accumulation of soluble sugars such as sucrose and fructose in TG rice (Fig. 2). Increased hexose contents lead, *via* hexokinase-related signalling, to repression of expression of *RbcS* and other genes, resulting in the decrease of amounts of RuBPCO and other proteins (Jang and Sheen 1994, van Oosten *et al*. 1994).

Alternatively, the lower photosynthetic capacity in TG leaves may be also explained by a lower RuBP regeneration capacity. Leaf P_N at saturating irradiance is potentially limited either by carboxylation or by regeneration of RuBP (Farquhar *et al.* 1980). Also, the decrease of the RuBP regeneration capacity is associated with reduced electron transport capacity (Farquhar *et al.* 1980, Farquhar and Caemmerer 1982, Ruuska *et al*. 2000) and we obtained similar results. In the TG rice leaves, the significantly declined apparent Φ_c (Fig. 1*E*), PS2 photochemical efficiency (Fig. 2*B*), photosynthetic electron transport, and photophosphorylation rates (Table 2) as well as RuBP content (Fig. 4) imply that the

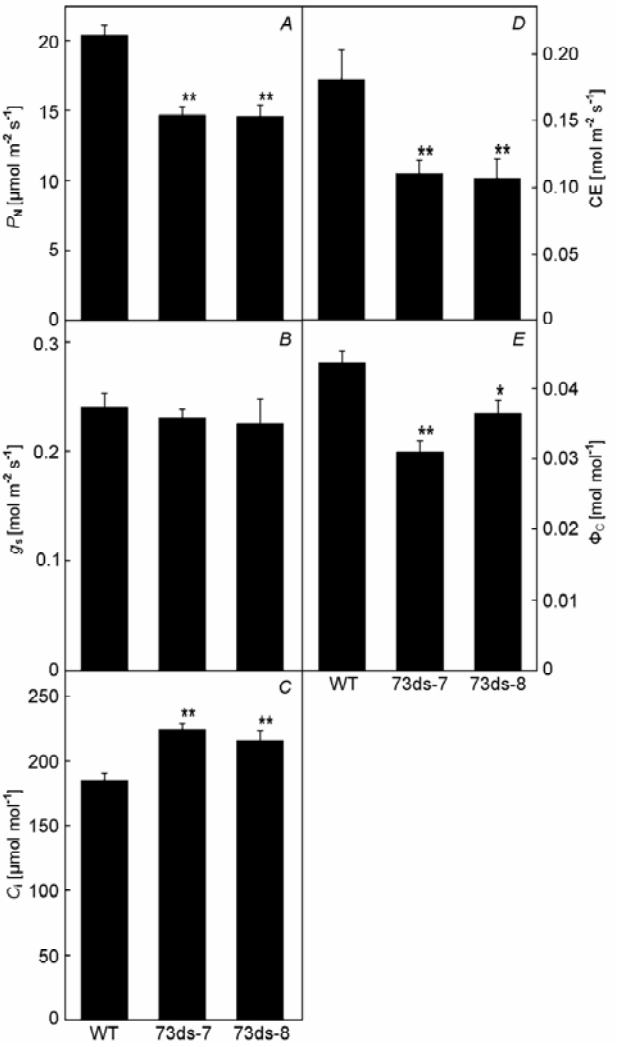

Fig. 1. Net photosynthetic rate, P_N (*A*), stomatal conductance, g_s (*B*), intercellular CO_2 concentration, C_i (*C*), carboxylation efficiency, CE (*D*), and apparent quantum yield of carbon assimilation, Φ_c (*E*) in flag leaves of transgenic (TG) rice and its wild type (WT). Each mean value was calculated using ten (*A*–*C*) or five (*D*, *E*) leaves; SE is expressed as a vertical bar. The significant differences between TG rice and WT are shown by asterisks: $^{*}p<0.05$ or $^{**}p<0.01$.

TG rice leaves must have a lower RuBP regeneration capacity compared with the WT leaves.

Both low RuBP carboxylation capacity and low RuBP regeneration capacity may be responsible for the declined P_N in the TG rice leaves. Which of them is then the predominant factor leading to the lowered P_N in the TG rice leaf? Change in leaf RuBP content may answer this question.

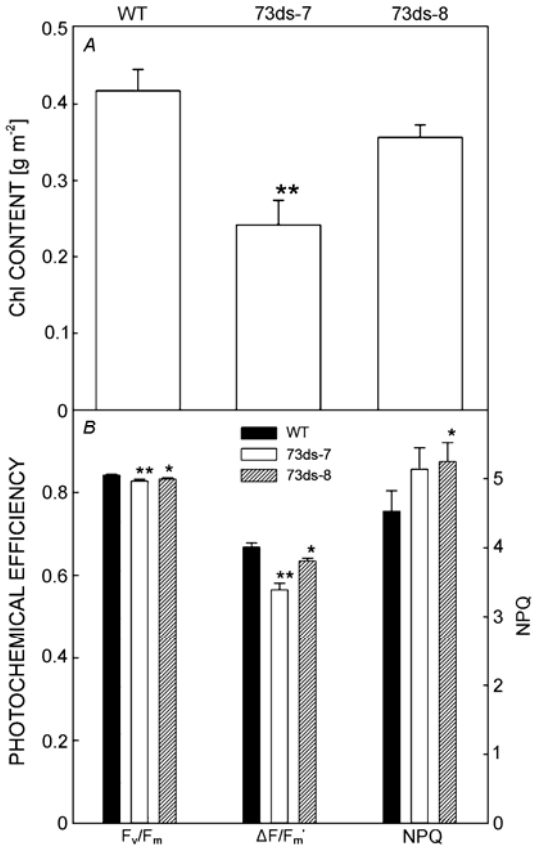

Fig. 2. Chlorophyll content (*A*), PS2 photochemical efficiency (F_v/F_m and $\Delta F/F_m'$) and non-photochemical quenching (NPQ) (*B*) in flag leaves of transgenic (TG) rice and its wild type (WT). Each mean value was calculated using six leaves; SE is expressed as a vertical bar. The significant differences between TG rice and WT are shown by asterisks $^{*}p<0.05$ or $^{**}p<0.01$.

RuBP content in a photosynthesizing leaf is a net result of RuBP production through regeneration from triose phosphates and RuBP consumption through carboxylation and/or oxygenation catalysed by RuBPCO. Thus, when the consumption and the regeneration dynamically balance each other, an unchanged RuBP content should be observed. If the RuBP regeneration does not match its consumption, the balance between them will be broken. RuBP content will increase when photosynthesis is limited by RuBP carboxylation (Ziska and Teramura 1992, Hussain *et al*. 1999), while RuBP content will decrease when photosynthesis is limited by RuBP regeneration. When the two limitations exist simultaneously, the changed direction of RuBP content will be dependent on the predominant one. Therefore, the significantly declined RuBP content in TG rice leaves (Fig. 4) reported here indicates that the declined RuBP regeneration capacity is the predominant one leading to decrease of photosynthesis in the TG rice leaves.

Additionally, a lowered RuBP content may cause RuBPCO decarbamylation so that the RuBP

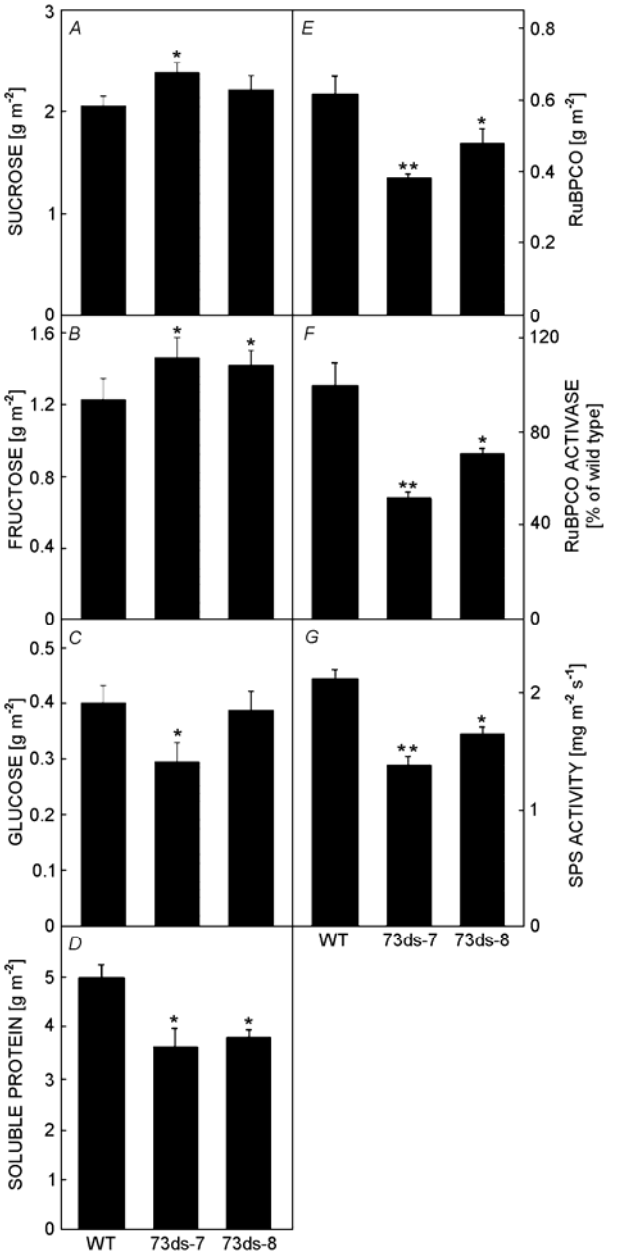

Fig. 3. Contents of sucrose (*A*), fructose (*B*), glucose (*C*), soluble protein (*D*), RuBPCO (*E*), and RuBPCO activase (*F*) and SPS (*G*) activities in flag leaves of transgenic (TG) rice and its wild type (WT). Each mean value was calculated using six (*A–C*) or eight (*E–G*) leaves; SE is expressed as a vertical bar. The significant differences between TG rice and its WT are shown by asterisks $^{*}p<0.05$ or $^{**}p<0.01$.

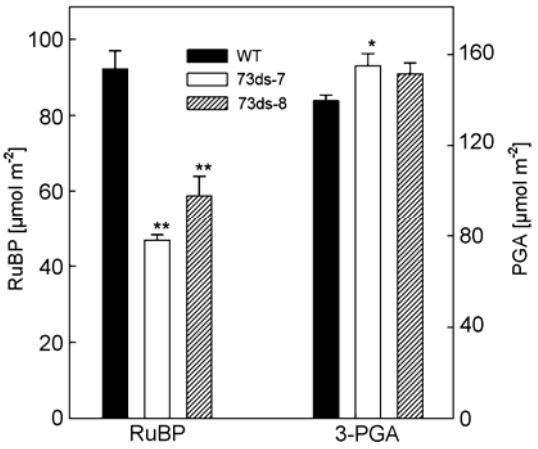

Fig. 4. RuBP and PGA contents in flag leaves of transgenic (TG) rice and its wild type (WT). Each mean value was calculated using five measurements; SE is expressed as a vertical bar. Three leaves were used in each measurement. The significant differences between TG rice and its WT are shown by asterisks $^{*}p<0.05$ or $^{**}p<0.01$.

consumption rate is matched with its regeneration rate (Mott *et al*. 1984, Sage 1990). Besides the decreased RuBPCO and RuBPCO-A contents (Fig. 3) the declined RuBP content (Fig. 4) or RuBP regeneration capacity may be partially responsible for occurrence of RuBP carboxylation limitation of photosynthesis in the TG rice leaves.

Rice *Wx* gene is specifically expressed only in pollen and endosperm but not in other organs (Hirano and Sano 2000), while *OsBP-73* gene was originally isolated as the putative regulator of the rice *Wx* gene. It is weakly expressed in root, leaf, and immature seed (Chen *et al.* 2003). Therefore, the decrease in P_N of the TG rice leaves is unlikely to be related to the expression level of the *Wx* gene. Perhaps the *OsBP-73* gene, as a transcription factor, also regulates the expression of some genes encoding some components of the photosynthetic apparatus. The pathway or mechanism that by silencing *OsBP-73* gene through RNAi technique leads to the decreased P_N in TG rice leaves is worth further studying.

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