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Improving plant photosynthesis and growth by overexpression of cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in chloroplasts

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Introduction

Photosynthetic carbon metabolism in higher plants is thought to be one determining factor in plant growth and crop yield. The Calvin cycle is the primary pathway for carbon fixation. Though some of the enzymes involved in the Calvin cycle are present at levels well in excess of that required to sustain a continued rate of CO_2 fixation, levels of fructose-1,6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase) are extremely low compared to those of the other enzymes in the Calvin cycle (Woodrow and Mott, 1993). It has been reported that transgenic plants with a smaller decrease in the activities of FBPase or SBPase exhibited a reduction in photosynthetic capacity, indicating that the reduction in photosynthesis results from a reduction in the regenerative capacity of the Calvin cycle (Koßmann *et al*., 1994, Harrison *et al*., 1998, Ölçer *et al*., 2001). Furthermore, they catalyze irreversible reactions, and their activities are regulated by light-dependent changes in redox-potential through the ferredoxin/thioredoxin system (Buchanan, 1991). From these facts, it seems likely that FBPase and SBPase in the Calvin cycle are important strategic positions to determine the partitioning of carbon to end products. We have previously demonstrated that fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) from cyanobacterial cells, which can hydrolyze both FBP and SBP with almost equal specific activities (Tamoi *et al*., 1996). In this article, we introduced cyanobacterial FBP/SBPase into tobacco chloroplasts and studied effects of elevated FBPase and SBPase in chloroplast on photosynthesis, assimilate partitioning, and growth rate. (Miyagawa *et al*. in press)

Materials and methods

Plant Material and Growth Conditions. Wild-type tobacco plants (*Nicotiana tabacum* cv. Xanthi) and transgenic tobacco plants overexpressing the cyanobacterial FBP/SBPase gene (T_2 ;TpFS) were hydroponically grown in a growth chamber under a 12-h light (25˚C) and 12-h dark (20˚C) cycle with moderate light intensity (400 μmol m^{-2} s^{-1}), 360 ppm CO_2, and 60% relative humidity. Homozygous T_2 generation plants were used for all experiments.

Determination of enzyme activities, protein and chlorophyll content. The activities of FBPase, SBPase, and other enzymes were according to the cited methods (Stark *et al*., 1992,

Koßmann *et al*., 1993, Leegod, 1993, Haake *et al*., 1998, Tamoi *et al*., 1998, Miyagawa *et al*., 2000). Leaf tissues (1.1 cm^2 x 10 discs) were harvested at 6 h in the light regime and ground to a fine powder in liquid N_2 using a pestle and mortar. For all enzyme assays, except for the Rubisco, leaf tissues were homogenized with 1 ml of a 100 mM Tris-HCl buffer (pH 8.0), 16 mM $MgCl_2$, 1 mM EDTA, 20 mM dithiothreitol (DTT), 2% (W/V) insoluble polyvinylpyrrolidon (PVP), and 2 mM phenylmethylsulfonyl fluoride (PMSF). Preparations and assays of the initial and total activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) were carried out according to the method of Sawada *et al*. (1990). Initial activities of FBPase were measured according to Holaday *et al*. (1992).

Gas-exchange and Chl fluorescence measurements. CO_2 fixation was measured with the portable photosynthesis system LI-6400 (Li-Cor, Lincoln, NE) under the indicated conditions. Chlorophyll fluorescence was measured at 25˚C with a Mini PAM Chlorophyll Fluorometer (Waltz, Efeltrich, Germany) according to the method of Miyagawa *et al*.(2000).

Determination of metabolite and carbohydrate levels. The sugars (hexose and sucrose), as well as ribulose-1,5-bisphosphate (RuBP) and 3-phosphoglycerate (PGA) levels, were measured enzymatically as described by Leegood (1993). The levels of fructose-6-phosphate (F6P), dihydroxyacetate phosphate (DHAP), and glucose-6-phosphate (G6P) were measured according to the methods of Stitt *et al*. (1989). Starch was measured according to Lin *et al*. (1988).

Plant harvest and dry-weight determinations. Root tissue was separated from the growth media by washing the roots with a running stream of water and soaking the roots in water several times. Roots and shoots were dried for 3 days at 130˚C in a forced air-drier.

Results

Generation of transformants. In order to target the FBP/SBPase (fbp-I product) onto chloroplasts of the tobacco plant (*Nicotiana tabacum* cv. Xanthi), we constructed a plasmid containing the FBP/SBPase gene from *Synechococcus* PCC 7942 with a tomato *rbcS* promoter and transit peptides (Fig. 1A). Fifteen antibiotic-resistant tobacco transformants carrying the *fbp-I* transgene were generated using an *Agrobacterium tumefaciens*-mediated gene transfer system. We confirmed the localization of FBP/SBPase in transgenic plants by Western blot analysis of protein extracts from isolated chloroplasts using an antibody raised against S. 7942 FBP/SBPase (Fig. 1B).

Plant growth. At 12 weeks after planting, transgenic plants grew significantly larger than wild-type plants (Fig. 2B). After 18 weeks, TpFS-6 and TpFS-3 increased approximately 1.4- and 1.5-fold in height compared to the wild-type plants, respectively (Figs. 2A and 2B). The total dry weight of TpFS-6 and TpFS-3 increased approximately 1.4- and 1.5-fold compared to that of the wild-type plants, respectively (Fig. 2C).

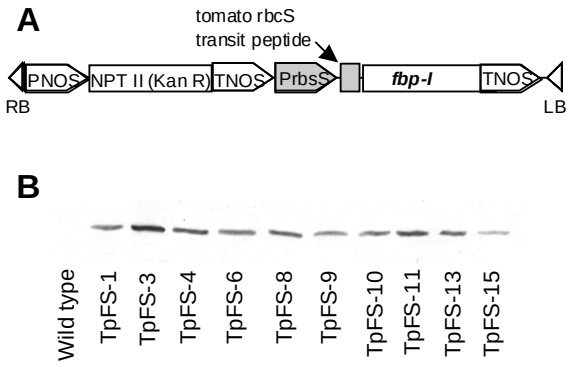

Figure 1. Creation of the transgenic plants. (A) Construct of the plasmid DNA. (B) Protein levels of FBP/SBPase.

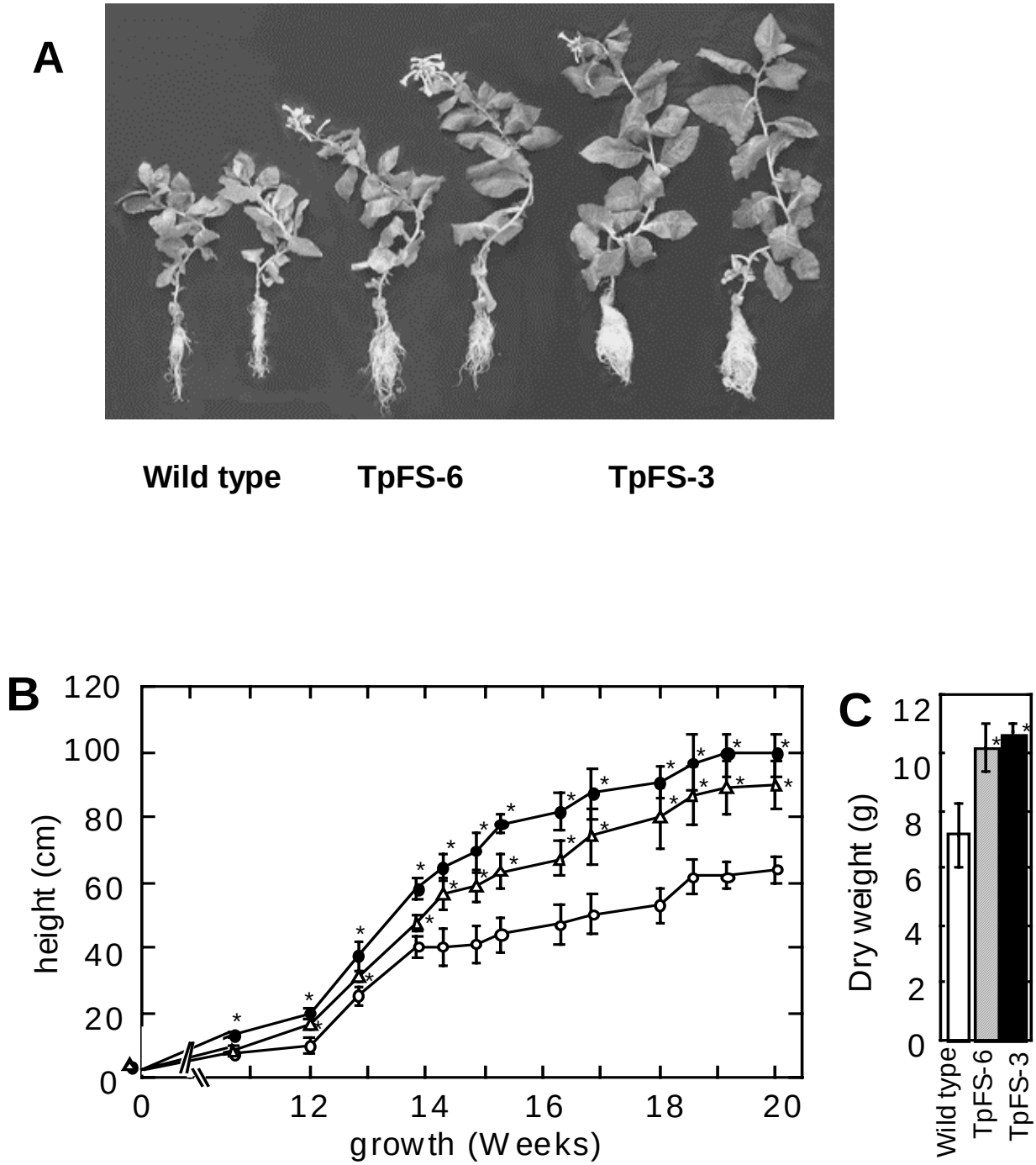

Figure 2. (A) Wild-type and transformed tobacco plants after 18 weeks of culture. (B) Growth rate of wild-type (○), TpFS-6 (□) and TpFS-3 (●) plants. (C) The total dry weight of wild-type and transformed tobacco plants after 18 weeks of culture.

The Calvin cycle enzymes and chlorophyll. The total SBPase activity derived from plastidic SBPase and cyanobacterial FBP/SBPase in TpFS-3 and TpFS-6 was 2.3 ± 0.4 and 1.7 ± 0.1-fold higher than that in the wild-type plants, respectively. The FBPase activity derived from plastidic and cytosolic FBPase and cyanobacterial FBP/SBPase in TpFS-3 and TpFS-6 was 1.6 ± 0.2 and 1.2 ± 0.1 -fold higher than that in the wild-type plants, respectively.

The ratio of the initial activity, reflecting the degree of activation *in vivo*, to the total activity of plastidic FBPase was approximately 65% in wild-type plants. The substantial FBPase activity in the chloroplast of TpFS-3 and TpFS-6 was approximately 2.7 and 1.6-fold higher than that in wild-type plants. Total extractable activities of phosphoribulokinase (PRK), NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GAPDH), aldolase, total Rubisco remained unaltered in the transformants (Fig. 3). Interestingly, the initial activity of Rubisco in TpFS-3 and TpFS-6 was approximately 1.2- and 1.1-fold higher than that in wild-type plants, respectively, indicating that the *in vivo* activation state of Rubisco in transgenic plants was increased. There were no differences in total extractable activities of transketolase (TK), ADP-glucose pyrophosphorylase (AGPase), and sucrosephosphate synthase (SPS) between wild-type and TpFS plants (data not shown). There was no difference between the TpFS-3 plant leaves and the wild-type plant leaves with regard to chlorophyll contents per square meter.

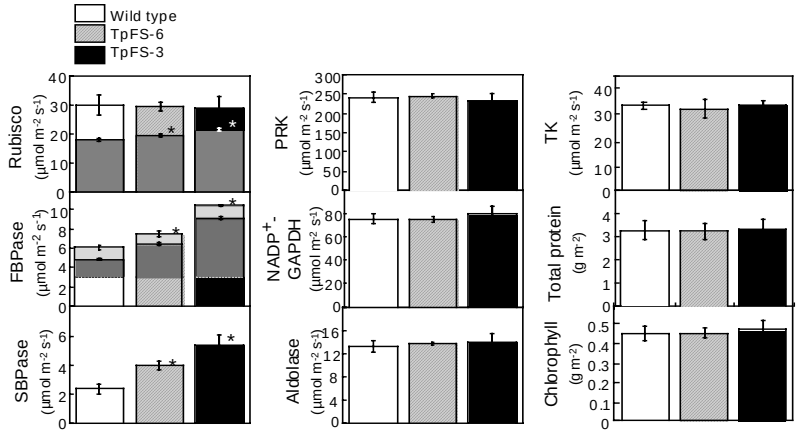

Figure 3. Activities of the Calvin cycle enzymes and other enzymes involved in carbon metabolism. The results are given as the mean ± SD of twelve extracts, each from a separate plant. In Rubisco, each shaded bar represents the initial activity. In FBPase, the dark grey bar represents the initial activity of the plastidic FBPase and/or the activity of FBP/SBPase, while the grey bar shows the in vivo inactivation state of the plastidic FBPase.

Photosynthesis. The photosynthetic activity of the transgenic plants increased significantly compared with that of the wild-type plants at irradiances above 200 µmol m^{-2} s^{-1} under atmospheric conditions (360 ppm CO_2). Under 1,500 µmol m^{-2} s^{-1}, the photosynthetic activities of TpFS-3 and TpFS-6 were 1.24- and 1.20-fold higher than those of wild-type plants, respectively (Fig. 4A). The response of the CO_2 assimilation to a range of intercellular CO_2 concentrations (Ci) was assessed by taking *in vivo* measurements of CO_2 uptake at irradiance (400 µmol m^{-2} s^{-1}) (Fig. 4B), indicating that the photosynthetic activity of TpFS-3 increased approximately 1.50-fold more than that of wild-type plants under saturated CO_2 conditions. The PS II activities (F_v/F_m) of TpFS-3 and TpFS-6 were almost equal to those of wild-type plants (data not shown).

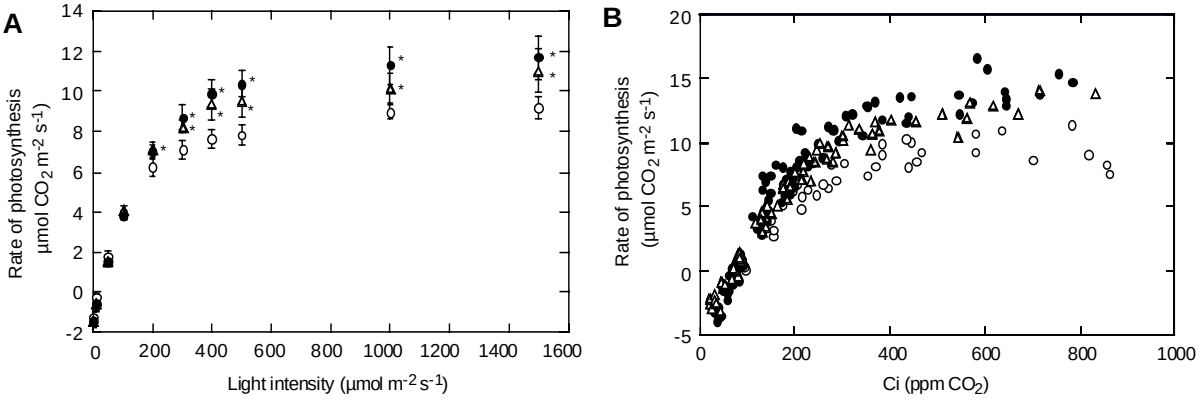

Figure 4. (A) Effect of increasing irradiance on CO_2 assimilation at 360 ppm of CO_2. (B) Response of CO_2 assimilation rates to increasing intercellular CO_2 concentrations (Ci) at irradiance (400 µmol m^{-2} s^{-1}). Wild-type (○), TpFS-6 (□), and TpFS-3 (●) plants.

Phosphorylated metabolites and carbohydrates. The content of RuBP in TpFS-6 and TpFS-3 plants was 1.5- and 1.8-fold larger than that in wild-type plants, respectively. In TpFS-6 and TpFS-3 plants, the content of PGA was 1.3- and 1.4-fold higher than that in the wild-type plants, respectively. In TpFS-3 plants, the contents of DHAP, F6P, and G6P were 1.2- to 1.5-fold higher than those in wild-type plants. In TpFS-6 plants, the contents of PGA, DHAP, F6P, and G6P were 1.0- to 1.4-fold higher than those in wild-type plants. In the upper leaves of TpFS-3 plants, hexose, sucrose, and starch contents were approximately 2.5-, 2.0-, and 1.6-fold higher than those in wild-type plant leaves, respectively. In TpFS-6 plants, these levels were approximately 1.7-, 1.5-, and 1.5-fold higher than those in wild-type plant leaves, respectively. Total starch amount in the roots of TpFS-3 was 3-fold larger than that in the root of wild-type plants because the fresh weight of roots in TpFS-3 was 3-fold larger that that in wild-type plants.

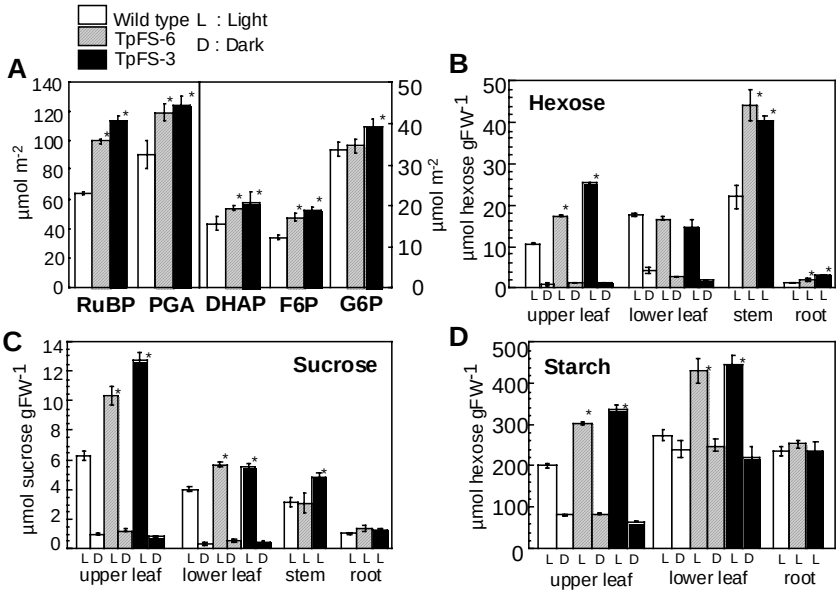

Figure 5. Levels of photosynthesis intermediates of the Calvin cycle (A) and contents of hexose (B), sucrose (C), and starch (D) in wild-type, TpFS-6, and TpFS-3 grown for 12 weeks in hydroponic culture. The levels of phosphorylated metabolites were measured in the fourth leaf from the top at 6 h after illumination, while the levels of carbohydrates were measured in the forth leaf from the top (upper), third leaf form the bottom (lower), stem, and root at 12 h in the light regime (L) and 12 h in the dark regime (D). Mean ± SD values for twelve plants are presented in each case.

Discussion

Transgenic plants expressing FBP/SBPase grew significantly faster and larger than wild-type plants under the atmospheric condition (Fig. 2). In both transgenic plants (TpFS-3 and TpFS-6), their photosynthetic activities and metabolite contents depended on the respective increased levels of both enzymes (Figs. 4 and 5A). These results suggest that the regenerative stage of the Calvin cycle is one of the limiting steps of photosynthetic carbon fixation, leading to its influence on carbon allocation. Furquhar *et al.* (1980) have reported that the photosynthetic rate is limited by the Rubisco capacity at relatively low CO_2 concentration. Interestingly, the initial activity of Rubisco in TpFS-3 plants, reflecting the degree of activation *in vivo*, was 1.2-fold higher than that in wild-type plants; however, there were no differences in the total activity of Rubisco between wild-type and TpFS plants (Fig. 3). The content of RuBP in TpFS-3 plants was 1.8-fold larger than that in wild-type plants. Rubisco

activase has no effect on the activation state of Rubisco in the absence of RuBP, suggesting that Rubisco activase may bind Rubisco only when the active site is occupied with RuBP (Salvucci *et al*., 1996, Portis, 1995). Accordingly, the increase in RuBP might result in the high activity of Rubisco activase, leading to the increase in the initial activity of Rubisco and the increase in photosynthesis in the transgenic plants.
In the present study, overexpression of cyanobacterial FBP/SBPase in the chloroplasts of tobacco plants leads to increased sugar and starch contents under normal conditions (Fig. 5B). It has been proposed that the starch accumulation directly inhibits photosynthesis in source leaves, that the rate of sucrose and starch synthesis limits photosynthesis, and that sugar accumulation in the source leaves triggers changes in gene expression, resulting in a decrease in Rubisco and photosynthetic activity under high CO_2 condition (Ludewig *et al*., 1998). The inhibition of the carbon metabolism in sink organs is often accompanied by an accumulation of carbohydrates in the respective source leaves (von Schaewen *et al*., 1990). However, TpFS plants with increased levels of starch and sucrose did not show any significant downregulation of photosynthesis and the enzyme activities involved in the Calvin cycle in source leaves (Figs. 3 and 4). This was because the photosynthetic rate might not have exceeded the capacity of phloem loading and sink to utilize the photosynthesis for growth in transgenic plants.

The data reported here lead us to the conclusion that FBPase and/or SBPase involved in the regeneration of RuBP are one of the limiting factors that participate in the regulation of the carbon flow through the Calvin cycle and the determination of the partitioning of carbon to end products.

Acknowledgments

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