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Biomass Production Is Promoted by Increasing an Aldolase Undergoing Glutathionylation in *Arabidopsis thaliana*

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ABSTRACT

We previously identified a putative fructose-1,6-bisphosphate aldolase (FBA), which was designated as FBA1 (At2g01140.1), as a protein undergoing glutathionylation in *Arabidopsis*. Here we show that increasing FBA1 activity can improve biomass production by enhancing leaf area-based CO_2 assimilation. Transgenic *Arabidopsis* plants with increased accumulation of FBA1 protein (*35S-FBA1* plants) exhibited enhanced photosynthetic CO_2 assimilation rate with increased FBA activity, and yielded more seeds and aerial biomass than wild-type plants under nitrogen nutrient conditions suitable for biomass production. FBA activity and photosynthetic CO_2 assimilation rate decreased in glutathione-deficient mutants, *cad2-1* and *pad2-1*, with decreased glutathione levels, while FBA protein levels were little affected in the mutant plants. An *in vitro* enzyme assay for FBA from mutant and wild-type plants revealed that FBA activity can be increased and recovered by 2.5 mM glutathione, but not by a strong reducing agent, 20 mM dithiothreitol. We found a positive correlation between photosynthetic CO_2 assimilation rate and FBA activity among plants with various levels of FBA protein and of glutathione, and this correlation was strengthened (r^2=0.955) with increasing light intensity from 25 to 1000 μE m^{-2} s^{-1}. On the other hand, there was much less correlation between photosynthetic CO_2 assimilation rate and Rubisco protein (r^2=0.013) or total activity (r^2=0.005), which have been generally considered to be a major limiting factor of photosynthesis. These results indicate that FBA activity is a limiting factor for photosynthetic CO_2 assimilation and biomass production, even at the light intensity at which Rubisco is fully activated, and that it is likely to be regulated by glutathione.

Keywords: Aldolase, Calvin cycle, CO_2 assimilation, glutathione, Rubisco

Abbreviations: **A**, CO_2 assimilation rate; **Chl**, chlorophyll; **DTT**, dithiothreitol; **γ-ECS**, γ-glutamylcysteine synthetase; **FBA**, fructose-1,6-bisphosphate aldolase; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **Rubisco**, ribulose-1,5-bisphosphate carboxylase/oxygenase; **WT**, wild type

INTRODUCTION

Glutathione is a ubiquitous tripeptide that is synthesized from cysteine, glutamate and glycine through two reactions. One reaction is catalyzed by γ-glutamylcysteine synthetase (γ-ECS), the rate-limiting enzyme of glutathione biosynthesis, and the other by glutathione synthetase in plants (Noctor and Foyer 1998; Noctor *et al.* 2002; Ogawa *et al.* 2004). Glutathione has numerous physiological functions including detoxification of xenobiotics (Marrs 1996) and of heavy metals (Cobbett 2000), regulations of growth and development (Vernoux *et al.* 2000; Henmi *et al.* 2001; Ogawa *et al.* 2001, 2004; Stasolla *et al.* 2004; Yanagida *et al.* 2004; Ogawa 2005; Cairns *et al.* 2006), and translational and transcriptional regulation (Kan *et al.* 1988; May *et al.* 1998; Baena-González *et al.* 2001; Yosef *et al.* 2004), and enzymatic regulation (Ito *et al.* 2003; Zaffagnini *et al.* 2007; Palmieri *et al.* 2010) in plants.

Plant growth is greatly restricted by an aberration of the light-harvesting system in the *Arabidopsis* mutant *chlorina1-1* (*ch1-1*), in which growth is partially or completely restorable by supplementation with glutathione (Ogawa *et al.* 2004). This suggests that glutathione might regulate the efficiency of converting captured light energy into carbohydrates. Identification of target proteins of glutathionylation during the photosynthetic processes may be a cue to understand the mechanism underlying the phenomenon that glutathione can compensate the defect of light harvesting.

To date, some proteome analysis to identify glutathionylated proteins have been reported in photosynthetic organisms, *Arabidopsis* (Ito *et al.* 2003; Dixon *et al.* 2005) and *Chlamydomonas* (Michelet *et al.* 2008; Zaffagnini *et al.* 2012). A putative plastidic fructose-1,6-bisphosphate aldolase (FBA), designated as FBA1 (At2g01140.1), has been identified as a protein undergoing glutathionylation in the suspension-cultured cells of *Arabidopsis* (Ito *et al.* 2003). Although functional analysis of glutathionylated proteins is intensively performed in *Chlamydomonas* (Rouhier *et al.* 2008; Zaffagnini *et al.* 2012), but there has been little progress in functional analysis of glutathionylated proteins in *Arabidopsis* or land plant species. Therefore, in order to deepen the understanding of the physiological significance of protein glutathionylation in plants, it is essential to perform functional analysis of glutathionyled proteins in land plant species.

The Calvin cycle consists of 13 reactions catalyzed by 11 enzymes including FBA (Buchanan 1980; Haake *et al.* 1998; Raines 2004). In the cycle, four enzymes, fructose-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SBPase), NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GAPDH), and phosphoribulokinase (PRK), are redox-regulated by the ferredoxin-thioredoxin system (Buchanan 1980) as well as is Rubisco activase, which is required for the light activation of Rubisco (Zhang *et al.* 1999). However, there has so far been no evidence that the Calvin-cycle reactions catalyzed by FBA are redox-regulated by glutathione or thioredoxin system. Conventional knowledge (Haake *et al.* 1998) suggests

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that FBA-catalyzed reaction might limit the rate of the Calvin cycle, but there has been no evidence that overproduction of FBA isozymes can promote growth or photosynthesis in plants. Based on the amino acid sequence conserved among plastid FBAs, other FBA isozymes, which are different from FBA1, have so far been considered to participate in the Calvin cycle.

In order to deepen the physiological importance of protein glutathionylation in land plant species, the aim of the present study was to investigate the relationship between FBA1 and photosynthesis in the model plant *Arabidopsis thaliana*. We constructed and analyzed transgenic *Arabidopsis* plants with several levels of FBA1 protein to investigate the photosynthetic characteristics and consequent biomass production. We also used glutathione-deficient mutant plants to know the relationship between FBA activity and glutathione in view of photosynthesis. Based on the results, we here show the photosynthetic importance of FBA1 in land plant species and the possibility to develop a novel biotechnology increasing crop and biomass yields by genetic modification of FBA1 levels.

MATERIALS AND METHODS

Plant materials and growth conditions

We used an ecotype of *Arabidopsis thaliana*, Columbia-0 (Col), as the wild-type (WT) plant. *Arabidopsis* plants were grown 1-3 plants in square plastic pots (6.5 × 6.5 × 5 cm) filled with two volumes of vermiculite (Asahi-Kogyo, Okayama, Japan) at the bottom, one volume of Kureha soil (Kureha-Engei-Baido, KUREHA Co., Tokyo, Japan) in the middle layer, and one volume of vermiculite on the top, under a long-day (16-h photoperiod) condition at a light intensity of 100 μE m^{-2} s^{-1} with white fluorescent lamps.

Construction of transgenic plants

Total RNA was isolated from the WT plants by using RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). Then, RT-PCR was carried out by using ProSTAR first strand RT-PCR kit (Stratagene, La Jolla, CA, USA), so that a cDNA was produced. The following sets of specific primers (1F-1: 5′-GGATCCTATGGCGTC TGCTAG-3′ and 1R-1: 5′-ATCTGCAACGGTCTCGGGAGA-3′; 1F-2: 5′-GTGTGGTCCGAGGTGTTCTTCT-3′ and 1R-2: 5′-GAG CTCGAGTAGGTGTAACCCTTG-3′) which were designed based on a cDNA sequence (GenBank accession number: NM_126176) of *FBA1* (*At2g01140*) were used so that two fragments of full-length cDNA were amplified by PCR. Then, each of the fragments was TA-cloned to a pGEM-T Easy vector (Promega, Madison, WI, USA). The two fragments were fused at a *Bst*PI site, so that a vector (pGEM-FBA1) including full-length cDNA was constructed. For the purpose of producing a transformed plant, the pGEM-FBA1 was processed by restriction enzymes *Bam*HI and *Sac*I, and then the β-glucuronidase gene of the binary vector pBI121 was replaced by the fragment. The pBI121 expression vector thus prepared in accordance with the foregoing procedure was introduced to *Arabidopsis* WT plants by using the *Agrobacterium* method (Clough and Bent 1998), so that a transformed plant was produced.

Evaluation of *Arabidopsis* biomass and seed yield

Three *Arabidopsis* plants were grown in square plastic pots as described in plant materials. Four pots were put on a square plastic dish and 100 ml of NH_4NO_3 at a concentration of 3, 9, 18, 45, or 90 mM was supplied to the dish at 7, 14, 21, 28 and 35 days after seed imbibition. As a control, water only was supplied to the dish at the same time as was a solution of NH_4NO_3. Water supply continued until the silique color of plants began to change to yellow. The total aerial part was carefully harvested and dried up in a drying room (temperature, 22°C; relative humidity, 10%) for more than one week to measure the total aerial biomass. After the measurement of the total aerial biomass, seeds were collected to measure the weight per pot using a Sartorius ME235S analytical balance (Sartorius AG, Göttingen, Germany).

Western blot analysis

Leaf blades detached from each plant were frozen and ground to a powder in liquid nitrogen with tungsten beads. One hundred mg of the leaf powder was homogenized with 100 μL of protein extraction buffer containing 50 mM Tris (pH 8.0), 80 mM $MgCl_2$, 0.2 mM EDTA, 10% (w/v) glycerol. An aliquot of the homogenate was used for quantification of chlorophyll (Chl). The remaining homogenates were centrifuged at 10,000 × *g* for 10 min. The supernatants were collected and adjusted to the same Chl equivalent with the extraction buffer. Dilution was adequately done with the same buffer. An aliquot of the sample solution (adequately diluted supernatant) was mixed with the same volume of a buffered solution containing 100 mM Tris (pH 6.8), 4% (w/v), SDS, 20% (w/v) glycerol, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% [w/v]). Subsequently, the resolved proteins were blotted onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK) followed by immuno-detection using anti-FBA1, FBA2 and FBA3 rabbit antisera. Anti-rabbit IgG linked to horseradish peroxidase (GE Healthcare, Buckinghamshire, UK) was used as a secondary antibody specific for the primary antibodies. Cross-reactive protein bands were developed using an ECL plus protein gel blot analysis kit (GE Healthcare, Buckinghamshire, UK). Rabbit antisera were raised against a synthetic polypeptide corresponding to the Ser337 to Asp353 sequence of FBA1 protein, the Thr349 to Leu365 sequence of FBA2 protein and the Thr348 to Leu364 sequence of FBA3 protein.

Photosynthetic CO_2 assimilation

Plants were grown under a long-day condition (16-h photoperiod; 100 μE m^{-2} s^{-1}) for photosynthetic CO_2 assimilation analysis. Photosynthetic CO_2 fixation was measured with a Li-Cor LI-6400 (Lincoln, NE, USA) apparatus with the following measurement cell (LI-6400-40 Leaf Chamber Fluorometer) parameters: 22°C, 370 ppm CO_2, 10% blue component in the photosynthetically active radiation, and an air flow rate of 500 μmol s^{-1}.

Assay of FBA activity

The activity of cleavage reaction of fructose-1,6-bisphosphate (FBP) into dihydroxyacetone-phosphate (DHAP) and glyceroldehyde-3-phosphate (GAP) was assayed according to Haake *et al.* (1998). The rate of DHAP production was spectrophotometrically monitored at 340 nm, which is an oxidation rate of NADH following DHAP consumption by glycerol-3-phosphate dehydrogenase (GPDH). Fully expanded mature leaf blades detached from plants grown for four weeks were frozen and then ground into a powder in liquid nitrogen with tungsten beads. The frozen leaf powder was extracted with a buffer of 50 mM Tris (pH 8.0), 80 mM $MgCl_2$, 0.2 mM EDTA, 10% (w/v) glycerol. After centrifuging the extract for 10 min, FBA activity was assayed at 25°C in a buffer of 25 mM Tris-HCl buffer (pH 8.5), 0.35 mM NADH, 3.45 units per ml of GPDH, 0.5 mM FBP and the enzyme solution, i.e., the crude extract from the leaf. The activity was calculated from the initial linear rate. To investigate effects of GSH or DTT on FBA activity, FBA activity was assayed following incubation of the extract in the presence of 2.5 mM GSH or 20 mM DTT at 25°C for 10 min.

Assay of Rubisco activity

The activity of carboxylation of ribulose 1,5-bisphosphate to 3-phosphoglycerate of Rubisco was assayed according to the method of Sage *et al.* (1993). The activity was determined by spectrophotometrically monitoring the decrease in absorbance at 340 nm. The activation state of Rubisco was determined by comparing the initial with the total activity of Rubisco according to the method of Sage *et al.* (1993). Fully expanded mature leaf blades detached from plants grown for four weeks were frozen and then ground to a powder in liquid nitrogen with tungsten beads. Frozen powdered leaves were extracted with a buffer of 50 mM HEPES-NaOH (pH 8.0), 20 mM $MgCl_2$, 10 mM DTT. After centrifuging the extract for 15 sec, the initial activity of Rubisco was assayed at 25°C in a

buffer of 100 mM Bicine-NaOH (pH 8.0), 20 mM $MgCl_2$, 5 mM DTT, 10 mM $NaHCO_3$, 5 mM phosphocreatine, 5 mM ATP, 0.2 mM NADH, 0.5 mM ribulose 1,5-bisphosphate, 5 U/ml of creatine phosphokinase, 10 U/ml of 3-phosphoglyceric phosphokinase, 20 U/ml of GAPDH and the enzyme solution, i.e., the crude extract from the leaf. Total activity was assayed following incubation of the extract in the presence of 20 mM $NaHCO_3$ at 25°C for 5 min.

Rubisco and chlorophyll contents

The same leaf extract that Rubisco activity was assayed was used for determination of Chl and Rubisco contents. An aliquot of the homogenate was used for quantification of Chl. The quantification of Chl was performed according to the method of Arnon (1949). The amount of Rubisco protein was measured by using Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Hachioji, Japan).

Nitrogen content

Following measurement of leaf CO_2 assimilation rate, the leaf blade was detached from the plant and dried up with a lyophilizer. The dried leaf blade was subjected to analysis of nitrogen content with a Vario Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

HPLC analysis for GSH and GSSG

Leaf blades from three plants were frozen and then ground to a powder in liquid nitrogen. The leaf powder (per mg) was homogenized with 10 µl extraction buffer consisting of 0.0425% (v/v) phosphoric acid, 0.05 M $NaClO_4$, 0.05 M HCl. The homogenate was centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was centrifuged in a microconcentrator Microcon YM-3 (Amicon, Inc., Beverly, MA, USA) at 14,000 × *g* at 4°C. The filtrates were used in the assays for reduced (GSH) and oxidized glutathione (GSSG). GSH and GSSG were detected by monitoring ultraviolet absorbance at 195 nm with an LaChrom UV-VIS detector L-7420 (Hitachi, Tokyo, Japan) following a Hitachi LaChrom L-7000 HPLC system (L-7100 pump, 0.05 ml/min [0-2'] and 0.2 ml/min [2'-18']; L-7300 column oven, 40°C) equipped with a Shiseido Capcel Pac18 reverse phase C18 column (AQ 5 mm, φ 2 mm × 250 mm; Tokyo, Japan). GSH and GSSG were determined using authentic GSH and GSSG. The mobile phase consisted of 0.1 M $NaClO_4$, 0.085% (v/v) phosphoric acid, 1% (v/v) acetonitrile.

Statistical analyses

Statistical analysis was performed by t-tests for comparison of two means, one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test or multivariate analysis of variance (MANOVA) for multiple comparisons using the SYSTAT 11 statistical software (SYSTAT Inc., Evanston, Illinois).

RESULTS

Construction of transgenic *Arabidopsis* plants overexpressing *FBA1* gene

We designated an FBA undergoing glutathionylation as FBA1 (At2g01140.1). In order to investigate the function of FBA1 in plants, we generated transgenic *Arabidopsis* plants (*35S-FBA1*) overexpressing the *FBA1* gene with an additional artificial sequence under the control of the cauliflower mosaic virus *35S* promoter. The transgene-derived FBA1 protein was distinguished from the genome-derived one (**Fig. 1A**). Based on accumulation levels of FBA1, we selected three lines, 9-2, 11-5, and 15-1, for further analysis.

To precisely evaluate how much increase in leaf FBA activity was brought by increasing leaf FBA1, we immunologically quantified not only FBA1 but also other plastidic FBA isozymes, FBA2 (At2g21330.1) and FBA3 (At4g38970.1), using appropriate specific rabbit antisera and recombinant proteins expressed in *E. coli*. FBA3 accounted for 70% of the total plastid FBA protein level. By

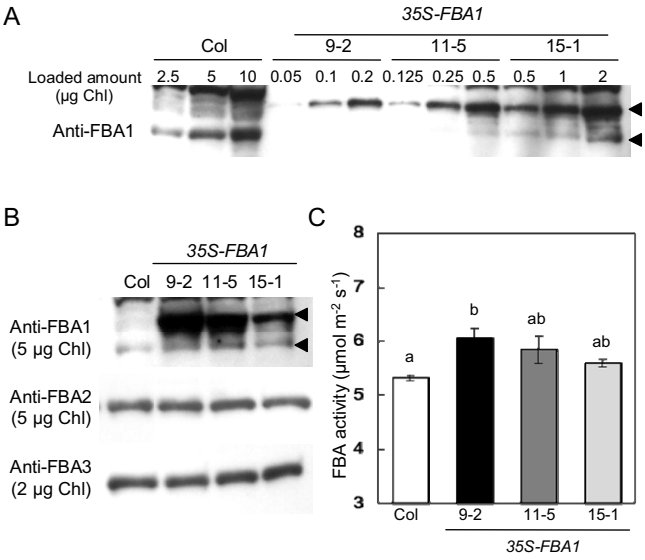

Fig. 1 FBA protein accumulation and activity in *35S-FBA1* overexpressing transgenic plants. (**A**) Western-blot analysis on FBA1 in *35S-FBA1* and WT plants. Samples were normalized by the Chl content in each plant. The amount loaded into each lane was indicated by the Chl equivalent shown above the lane. The upper and lower arrowheads indicate the bands corresponding to the transgene- and genome-derived FBA1 protein, respectively. (**B**) Western-blot analysis on FBA1, FBA2 and FBA3 in *35S-FBA1* and WT plants. Used anti-serum is indicated on the left-hand side of the Western-blot image. The Chl equivalent amount loaded into each lane is indicated below the used anti-serum name. (**C**) Leaf FBA activity of *35S-FBA1* and WT plants. Plants were grown for four weeks. Each value represents the mean ± SE of 4 measurements. Comparisons of FBA activity among transgenic and WT plants were performed by means of analysis of Tukey's *post-hoc* test. Different letters indicate significant differences at $P < 0.05$.

comparison, FBA2 and FBA1 accounted for 28 and 2%, respectively, of the total plastid FBA protein level. The protein accumulation level of transgene-derived FBA1 in the lines 9-2, 11-5 and 15-1 of *35S-FBA1* plants were estimated to be approximately 22-, 17-, and 10-fold of the genome-derived level, respectively. The levels of the genome-derived FBA1, FBA2 and FBA3 were little affected among *35S-FBA1* and WT plants (**Fig. 1B**). FBA activity in 9-2, 11-5 and 15-1 was increased by 14, 10, and 5%, respectively, compared to the WT plants (**Fig. 1C**).

Improvement of biomass production by increasing FBA1

To investigate effects of FBA1 overproduction on biomass production, we chose a transgenic line, 11-5. Plants were grown under a long-day condition at a light intensity of 100 µE m^{-2} s^{-1} and various nitrogen nutrient conditions controlled by subirrigation with ammonium nitrate solutions. The *35S-FBA1* line 11-5 appeared to grown better than the WT plants 6 weeks after sowing (**Fig. 2A**), and yielded more seeds and aerial biomass than the WT plants (**Fig. 2B, 2C**).

Improved photosynthesis in *35S-FBA1* plants

To determine whether improvement of growth and seed yield by overproduction of FBA1 is attributed to enhancement of photosynthesis, we measured leaf CO_2 assimilation rate. Compared to WT plants, *35S-FBA1* plants exhibited enhanced leaf area-based CO_2 assimilation rate (**Fig. 3A**) and leaf N-based CO_2 assimilation rate (**Fig. 3B**).

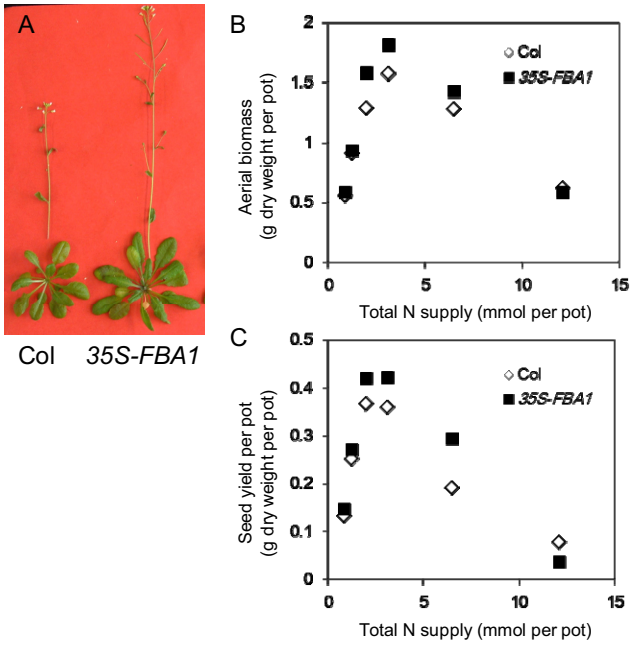

Fig. 2 Growth, aerial biomass and seed yields of *35S-FBA1* and WT plants. (**A**) A comparison of growth between *35S-FBA1* and WT plants. Plants were grown for six weeks. (**B**) Aerial biomass yield at the seed harvest under various nitrogen nutrient conditions. Each value indicates the mean of the results from 4 pots (12 individual plants). There was a significant difference ($P < 0.05$, MANOVA) in aerial biomass yield between *35S-FBA1* and WT plants. (**C**) Seed yield under various nitrogen nutrient conditions. Each value indicates the means of the results from 4 pots (12 individual plants). There was significant difference ($P < 0.05$, MANOVA) in seed yield between *35S-FBA1* and WT plants.

Correlation between FBA1 and photosynthesis

Leaf area-based CO_2 assimilation rate correlated to FBA activity among *35S-FBA1* and WT plants, and the correlation was strengthened with increasing the light intensity from 25 to 1000 μE m^{-2} s^{-1} (**Fig. 4A**). FBA activity correlated strongly to FBA1 accumulation level among *35S-FBA1* and WT plants (**Fig. 4B**).

Relationship among FBA activity, glutathione level and photosynthesis

Glutathione is synthesized in a manner dependent on the light intensity (Ogawa *et al.* 2004). To investigate the relationship between FBA activity and glutathione level in light of photosynthetic CO_2 assimilation rate, we used glutathione-deficient mutants, *cad2-1* and *pad2-1*. A mutant, *cad2-1*, has a six nucleotide deletion on *GSH1* gene encoding γ-ECS (Cobbett *et al*. 1998) and another mutant, *pad2-1*, has the substitution of Ser to Gln at amino acid position of 298 on γ-ECS (Parisy *et al.* 2007). Compared to WT plants, the glutathione-deficient mutants *cad2-1* and *pad2-1* exhibited 60% and 50% glutathione levels, respectively, with little difference in glutathione redox state (ratio of reduced glutathione to total glutathione). The mutants showed lower CO_2 assimilation rates than did the WT plants (**Fig. 5A**). FBA activity was decreased in the glutathione-deficient mutant (**Fig. 5B**), whereas the protein levels of FBA1, FBA2, and FBA3 were little affected among mutant and WT plants (**Fig. 5C**). The decreased FBA activity in the mutants was recoverable up to that of WT plants by reduced glutathione (GSH) but not by a strong reducing agent, DTT (**Fig. 5B**).

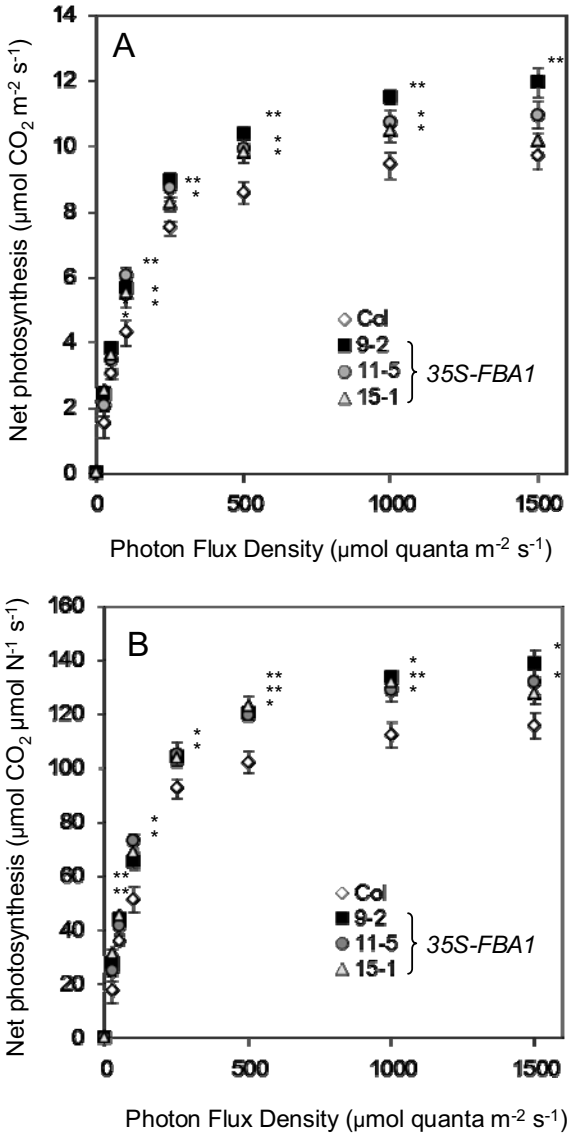

Fig. 3 CO_2 assimilation rates of *35S-FBA1* plants. Net CO_2 assimilation rates per leaf area (**A**) and per leaf nitrogen (**B**) in *35S-FBA1* and WT plants grown for four weeks. Each value represents the mean ± SE of 6 measurements. Comparisons of CO_2 assimilation rates between transgenic and WT plants were performed by means of analysis of *t*-test. Asterisks indicate significant differences (**, $P < 0.01$; *, $P < 0.05$) between transgenic and WT plants at each light intensity.

Photosynthetic characteristics on *35S-FBA1* and glutathione-deficient mutants

To investigate affects of other photosynthetic factors on photosynthesis in *35S-FBA1* plants and glutathione-deficient mutants, we compared photosynthetic characteristics among transgenic, mutant and WT plants (**Tables 1, 2**). There was no significant difference between *35S-FBA1* and WT plants in total leaf nitrogen, Chl, and Rubisco protein (**Table 1**) or between glutathione-deficient mutant and WT plants (**Table 2**). When the leaf was exposed to a light intensity of 250 μE m^{-2} $s^{-1}$for assay of Rubisco, there was no difference between *35S-FBA1* and WT plants in the content, initial and total activity, and activation state (ratio of the initial activity to the total activity) of Rubisco (**Table 3**). There were no differences between mutant and WT plants in the initial, total activities, or activation state of Rubisco (**Table 4**). The activation state showed nearly 100% in all plants tested, a light intensity of 250 μE m^{-2} s^{-1} was enough for light-activation of Rubisco activity.

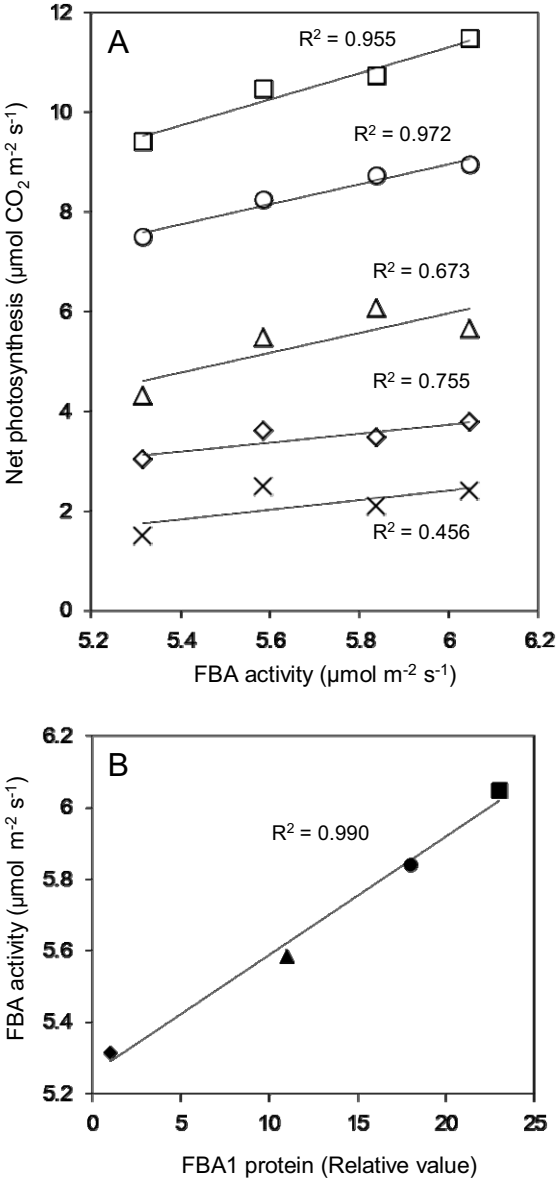

Fig. 4 Relationships among FBA1, FBA activity and photosynthesis. (**A**) Values of FBA activity taken from **Fig. 1C** and of CO_2 assimilation rate at each light intensity taken from **Fig. 3A** were replotted: Light intensity of 25 (cross), 50 (open diamond), 100 (open triangle), 250 (open circle), 1000 (open square) μE m^{-2} s^{-1}. (**B**) FBA1 protein levels taken from **Fig. 1A** and FBA activities taken from **Fig. 1C** were replotted: WT plants (filled diamond); *35S-FBA1* lines, 9-2 (filled square), 11-5 (filled circle), 15-1 (filled triangle) plants.

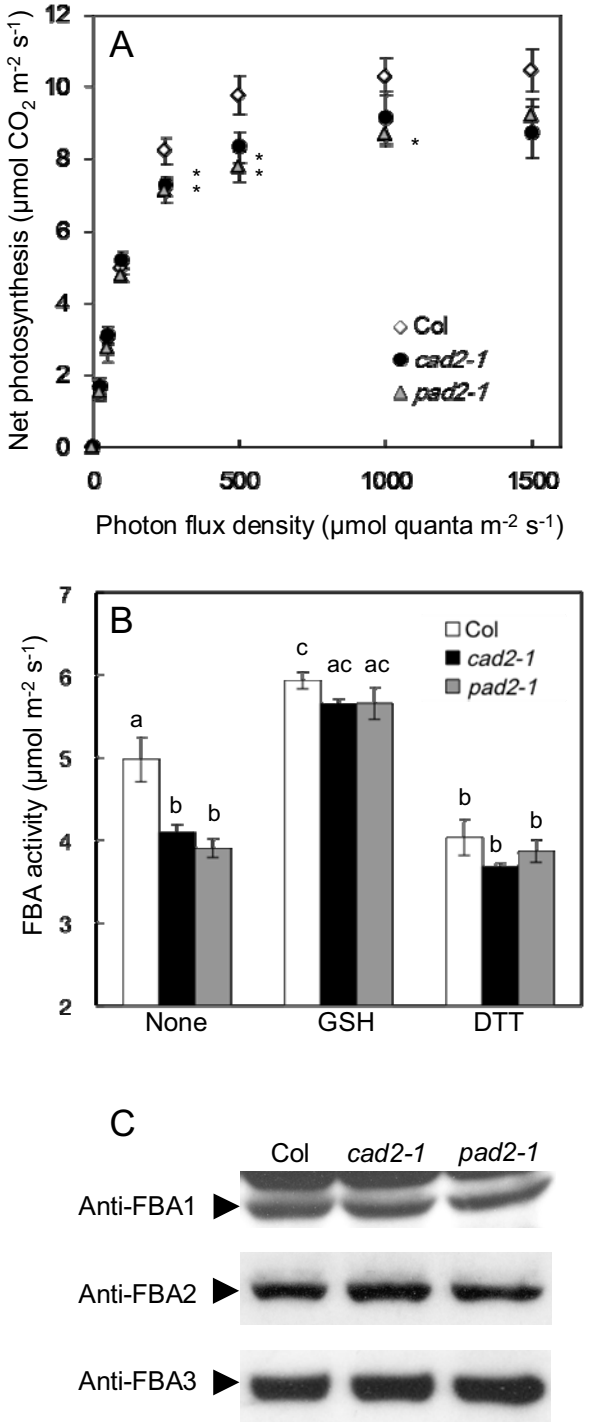

Fig. 5 CO_2 assimilation rates, activities and protein levels of FBA in glutathione-deficient mutants, *cad2-1* and *pad2-1*. (**A**) Light-photosynthesis curve for mutant and WT *Arabidopsis*. Net CO_2 assimilation rates in mutant and WT plants grown for four weeks were plotted. Each value represents the mean ± SE of 5 measurements. Comparisons of CO_2 assimilation rates at the same light intensity among mutant and WT plants were performed by means of analysis of t-test. Asterisks indicate the mean values that are significantly different (**, $P < 0.01$; *, $P < 0.05$) between mutant and WT plants. (**B**) The FBA activities without (described as "None" in panel) and with supplementation of 2.5 mM GSH or 20 mM DTT in mutant and WT plants grown for four weeks. Each value represents the mean ± SE of 4 measurements. Comparisons among values were performed by means of analysis of Tukey's *post-hoc* test. Different letters indicate significant differences at $P < 0.05$. (**C**) Protein levels of FBA1, FBA2, and FBA3 analyzed by Western blot analysis. The amount of the Chl-based sample that was loaded for FBA1, FBA2 and FBA3 are 20, 2, and 2 μg Chl, respectively.

DISCUSSION

FBA1 is one of the rate-limiting enzymes in the Calvin cycle

In the present study, we demonstrated that increasing accumulation of FBA1 can improve photosynthetic CO_2 assimilation (**Fig. 3**) and consequent biomass production (**Fig. 2**) in *A. thaliana*, although FBA1 was a quantitatively minor FBA isozyme in WT chloroplasts. Photosynthetic CO_2 assimilation rate correlated strongly to FBA activity (**Fig. 4A**), which was mainly attributed to the accumulation level of FBA1, not of FBA2 or FBA3 (**Fig. 4B**). The correlation between photosynthetic CO_2 assimilation and FBA activity was strengthened with increasing light intensity (**Fig. 4A**). Considering that Rubisco was fully activated at a light intensity of 250 μE m^{-2} s^{-1} (**Tables 3, 4**), this strongly suggests that the maximal photosynthetic CO_2 assimilation relies on FBA1 activity.

The Calvin cycle involves 11 enzymes. Intensive efforts have been made to be clear the potential importance of each enzyme in determining the rate of the Calvin cycle. According to Stitt's group studies on antisense plants, FBA

Table 1 The photosynthetic characteristics of *35S-FBA1* and wild-type plants. Each value represents the mean ± SE of 8 measurements. There are no significant differences ($P > 0.05$) in photosynthetic characteristics among transgenic and wild-type plants. Comparisons among values in each photosynthetic characteristic performed by means of analysis of Tukey post hoc test with $P = 0.05$.

Genotype	Leaf N	Rubisco protein	Rubisco-N/leaf N	Chl	Chl *a/b* ratio
	mmol m^{-2}	g m^{-2}	%	mmol m^{-2}	
Col	83.9 ± 1.8	1.61 ± 0.11	21.8 ± 1.5	0.25 ± 0.00	2.99 ± 0.05
35S-*FBA1* 9-2	86.1 ± 3.1	1.74 ± 0.05	21.2 ± 0.6	0.24 ± 0.01	3.11 ± 0.09
35S-*FBA1* 11-5	83.2 ± 3.2	1.68 ± 0.09	22.7 ± 1.2	0.26 ± 0.01	2.99 ± 0.09
35S-*FBA1* 15-1	79.5 ± 2.3	1.74 ± 0.13	24.3 ± 1.8	0.25 ± 0.00	3.16 ± 0.07

Table 2 The photosynthetic characteristics of glutathione-deficient mutant and wild-type plants. Each value represents the mean ± SE of 8 measurements. Comparisons in photosynthetic characteristics between mutant and wild-type plants were performed by means of analysis of Tukey's post hoc test. Different letters indicate significant differences at $P < 0.05$.

Genotype	Leaf N	Rubisco protein	Rubisco-N/leaf N	Chl	Chl *a/b* ratio
	mmol m^{-2}	g m^{-2}	%	mmol m^{-2}	
Col	84.7 ± 1.2 a	1.44 ± 0.09 b	19.1 ± 1.2 c	0.26 ± 0.01 e	3.07 ± 0.15 f
cad2-1	84.3 ± 1.7 a	1.59 ± 0.03 b	21.1 ± 0.4 c	0.27 ± 0.01 e	3.01 ± 0.07 f
pad2-1	77.6 ± 3.5 a	1.67 ± 0.12 b	24.1 ± 1.8 d	0.27 ± 0.01 e	2.94 ± 0.13 f

Table 3 Rubisco activity and activation state in *35S-FBA1* and wild-type plants. Each value represents the mean ± SE of 4 measurements. There are no significant differences ($P > 0.05$) among transgenic and wild-type plants. Comparisons among values were performed by means of analysis of Tukey's post hoc test.

Genotype	Rubisco activity		Rubisco activation state
	Initial	Total	
	μmol m^{-2} s^{-1}	μmol m^{-2} s^{-1}	%
Col	36.9 ± 3.2	36.8 ± 1.2	100 ± 7
35S-*FBA1* 9-2	34.9 ± 0.5	34.6 ± 1.0	101 ± 3
35S-*FBA1* 11-5	38.9 ± 1.8	37.6 ± 0.2	104 ± 5
35S-*FBA1* 15-1	37.3 ± 1.1	36.4 ± 1.1	103 ± 7

Table 4 Rubisco activity and activation state in glutathione-deficient mutant and wild-type plants. Each value represents the mean ± SE of 4 measurements. There are no significant differences ($P > 0.05$) among mutant and wild-type plants. Comparisons among values were performed by means of analysis of Tukey's post hoc test.

Genotype	Rubisco activity		Rubisco activation state
	Initial	Total	
	μmol m^{-2} s^{-1}	μmol m^{-2} s^{-1}	%
Col	42.6 ± 1.4	42.1 ± 1.0	101 ± 2
cad2-1	42.3 ± 1.6	41.6 ± 1.8	102 ± 2
pad2-1	42.7 ± 1.1	42.7 ± 1.7	100 ± 3

has been recognized as a potential limiting factor of the Calvin cycle (Haake *et al.* 1998). In addition to FBA, several enzymes have been suggested to be potential limiting factors in the Calvin cycle: Rubisco (Rodermal *et al.* 1988; Hudson *et al.* 1992), transketolase (Henkes *et al.* 2001), and sedoheptulose-1,7-bisphosphatase (SBPase) (Harrison *et al.* 1998), fructose-1,6-bisphosphatase (FBPase) (Raines 2003; Tamoi *et al.* 2005). Overexpression of a bifunctional cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) in tobacco plants promoted CO_2 assimilation, growth, and final biomass production (Miyagawa *et al.* 2001). Overexpression of *Arabidopsis* SBPase also promoted CO_2 assimilation and growth but consequent biomass production was not increased (Lefebvre *et al.* 2005). No report has so far been published to show that overexpression of transketolase or FBA promotes CO_2 assimilation and growth. This study, however, shows that overexpression of one plastidic FBA isozyme, FBA1, can enhance CO_2 assimilation, growth, and consequent biomass and seed yields. FBA1 is a quantitatively minor isozyme and the amino acid sequence of FBA1 resembles those of conventional FBA isozymes, FBA2 and FBA3. These situations have probably hidden the importance of FBA1 in CO_2 assimilation, in spite of the phylogenic difference in the amino acid sequence between FBA1 and the conventional Calvin cycle FBA isozymes.

Glutathione-dependent regulation of CO_2 assimilation

FBA activity was decreased by GSH deficiency and this was recovered by supplementation of GSH (**Fig. 5**). This effect was quantitatively independent of FBA1, FBA2 or FBA3 (**Fig. 5**). Taking into consideration that the light-saturated photosynthetic CO_2 assimilation rate is quantitatively correlated with FBA1, not FBA2 or FBA3, these imply that photosynthetic CO_2 assimilation is regulated by glutathione-dependent regulation of FBA1. It should be noted that FBA activity is decreased by DTT, likely to suggest that FBA activity might be regulated via glutathionylation of FBA1, the covalently binding of glutathione to FBA1. Upon illumination, glutathione biosynthesis is promoted by ATP production depending on pH gradient formation across the thylakoid membrane during photosynthesis. Since glutathione is labile to oxidation at high pH, the formation of the disulfide bridge between FBA1 and glutathione is assumed to be promoted by pH changes following photosynthesis. Indeed, the glutathione-deficient mutants, *cad2-1* and *pad2-1*, showed decreased FBA activities and its activities were restored by GSH, not by DTT.

Glutathionylation has recently been considered to be important in the regulation of photosynthesis (Ogawa 2005; Rouhier *et al.* 2008). Intensive survey on glutathionylated proteins in the model photosynthesis organism *Chlamydomonas reinhardtii* has been performed (Zaffagnini *et al.* 2012). Based on their obtained results, it has been postulated that glutathionylation of proteins including FBA functions in preventing irreversible inactivation of the proteins from photooxidative stress and is involved in oxidative stress signaling. Thioredoxin *f*, which activates several enzymes involved in the Calvin cycle, has a redox sensitive cysteine residue undergoing glutathionylation at sites different from the catalytic cysteine residues (Michelet *et al.* 2005), supporting the above idea regarding the role of protein glutathionylation. However, phylogenically unique cysteine residues in *Arabidopsis* FBA1 and probable orthologues of other plant species are not conserved in *Chlamydomonas* FBAs. This may suggest that the mechanism underlying FBA1-dependent CO_2 assimilation is an evolutional advantage for land plants. In addition, this study may also suggest that protein glutathionylation function in facilitating or activating metabolic activities. We have failed, but continue, to determine conditions for analyzing recombinant FBA1 because it is very unstable and easy to lose its activity. We cannot further understand the metabolic importance of this isozyme until we succeed in finding appropriate conditions for recombinant FBA1.

New potential booster of the Green Revolution

Nitrogen nutrient conditions strongly influence photosynthesis, growth and seed yield. As far as we know, there has been no report showing that any biotechnology increases nitrogen-based photosynthetic CO_2 assimilation. In this respect, genetic increasing of FBA1 in plants is a potentially novel technology for increasing biomass and crop yields. Appropriate nitrogen fertilizing increases biomass and seed yields, which is one of constitutive factors of the "Green Revolution". This study further evidences overexpression of *FBA1* improves biomass and seed yields even at the optimal nitrogen nutrient conditions (**Fig. 2B, 2C**). Planting density we used had been optimized based on biomass production before investigating differences in responses to nitrogen nutrient conditions. Taken together, this implies that overexpression of FBA1 in plants is able to enhance the Green Revolution to ameliorate agricultural production.

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