*Journal of Experimental Botany*, Vol. 63, No. 8, pp. 3001–3009, 2012
doi:10.1093/jxb/ers004 Advance Access publication 8 February, 2012

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

# Increased fructose 1,6-bisphosphate aldolase in plastids enhances growth and photosynthesis of tobacco plants

**Kimio Uematsu, Nobuaki Suzuki, Tomoko Iwamae, Masayuki Inui and Hideaki Yukawa***

Research Institute of Innovative Technology for the Earth (RITE), 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan

* To whom correspondence should be addressed. E-mail: mmg-lab@rite.or.jp

Received 17 November 2011; Revised 6 January 2012; Accepted 8 January 2012



## Abstract

**The Calvin cycle is the initial pathway of photosynthetic carbon fixation, and several of its reaction steps are suggested to exert rate-limiting influence on the growth of higher plants. Plastid fructose 1,6-bisphosphate aldolase (aldolase, EC 4.1.2.13) is one of the nonregulated enzymes comprising the Calvin cycle and is predicted to have the potential to control photosynthetic carbon flux through the cycle. In order to investigate the effect of overexpression of aldolase, this study generated transgenic tobacco (*Nicotiana tabacum* L. cv Xanthi) expressing *Arabidopsis* plastid aldolase. Resultant transgenic plants with 1.4–1.9-fold higher aldolase activities than those of wild-type plants showed enhanced growth, culminating in increased biomass, particularly under high $CO_2$ concentration (700 ppm) where the increase reached 2.2-fold relative to wild-type plants. This increase was associated with a 1.5-fold elevation of photosynthetic $CO_2$ fixation in the transgenic plants. The increased plastid aldolase resulted in a decrease in 3-phosphoglycerate and an increase in ribulose 1,5-bisphosphate and its immediate precursors in the Calvin cycle, but no significant changes in the activities of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) or other major enzymes of carbon assimilation. Taken together, these results suggest that aldolase overexpression stimulates ribulose 1,5-bisphosphate regeneration and promotes $CO_2$ fixation. It was concluded that increased photosynthetic rate was responsible for enhanced growth and biomass yields of aldolase-overexpressing plants.**

**Key words:** Aldolase, Calvin cycle, *Nicotiana tabacum*, photosynthesis, plastid.

## Introduction

The world population, lately increasing at a remarkably rapid pace, was estimated to exceed 6.9 billion in 2010 (UN Department of Economic and Social Affairs, 2009), bringing total global grain consumption to 2.2 billion tons (Agricultural Outlook: Statistical Indicators, USDA, 2010, http://www.ers.usda.gov/Publications/AgOutlook/AOTables/). The still increasing demand for grain for food, feed, and, more recently, bioethanol, caused grain prices to more than double from 2006 to 2008. To ensure global food security therefore, further improvements in crop production are essential. Breeding crops that grow faster and yield better is one possibly effective solution.

The Calvin cycle is the initial pathway of photosynthetic carbon fixation in $C_3$ plants and comprises reactions catalysed by a total of 11 enzymes localized in chloroplasts (Supplementary Fig. S1, available at *JXB* online). It plays an indispensable role in plant metabolism for the growth and maintenance of plant life, providing intermediates for glycolysis or building blocks for cellular components. In the immediate past three decades, extensive research in higher plants to identify, characterize, and consequently overcome the limitations imposed by the rate-limiting steps of the Calvin cycle has centred on genetic manipulation for the purpose of enhancement of photosynthetic capacity and plant productivity (Furbank and Taylor, 1995; Raines, 2003). These many efforts to improve photosynthesis have been devoted to altering the catalytic properties of the initial carboxylating enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), whose kinetic characteristics were believed to be a major limiting factor of photosynthesis under ambient

conditions (Raines, 2006). Although, as its name suggests, Rubisco is able to catalyse the carboxylation and oxygenation of ribulose 1,5-bisphosphate (RuBP), it has a low affinity for $CO_2$ and a low turnover rate and its carboxylase activity is competitively inhibited by $O_2$ (Hartman and Harpel, 1994). Furthermore, the oxygenation of RuBP results in production of 2-phosphoglycolate, which is then recycled via photorespiratory pathway with concomitant release of $CO_2$ and $NH_3$. To overcome these disadvantages of Rubisco in $CO_2$ fixation, considerable research has been carried out using various approaches such as protein engineering of Rubisco, screening of the natural Rubisco variants which exhibit improved carbon fixation activity, introducing genes encoding $C_4$ pathway enzymes, and engineering of the photorespiratory pathway. However, none of the approaches has been fully successful (Raines, 2006).

The rate of photosynthesis is limited by not only the carboxylation reaction of Rubisco but also RuBP regeneration capacity, which is determined by the capacity of electron transport chain to supply ATP and NADPH to support the regenerative reactions of the Calvin cycle (Caemmerer and Farquhar, 1981). For example, under high $CO_2$ concentration or low irradiance, RuBP regeneration capacity determines the photosynthetic rate. In fact, analysis of antisense plants with reduced activity of Rubisco revealed that the control coefficient on photosynthesis varied from 0.1 to 0.9 depending on the experimental and growth conditions (e.g. light intensity and $CO_2$ concentration) (Stitt and Schulze, 1994), suggesting that Rubisco activity does not always dominate the rate of photosynthesis and that other rate-limiting steps exist. To evaluate the contribution of other enzymes comprising the Calvin cycle to photosynthesis, transgenic plants with decreased activity of individual enzymes were generated and used to investigate the effects of each enzyme on photosynthesis as well as that of Rubisco (Koßmann *et al.*, 1994; Paul *et al.*, 1995; Price *et al.*, 1995; Harrison *et al.*, 1997; Haake *et al.*, 1998; Henkes *et al.*, 2001). As a result of these analyses, sedoheptulose 1,7-bisphosphatase (SBPase), transketolase (TK), and fructose 1,6-bisphosphate aldolase (aldolase) were found to have significantly higher control coefficient on photosynthesis than other Calvin cycle enzymes and were therefore candidate targets of engineering to improve photosynthetic carbon fixation (Raines, 2003). This idea was supported by the fact that transgenic plants overexpressing plant SBPase or cyanobacterial fructose 1,6-/sedoheptulose 1,7-bisphosphatase (FBP/SBPase) have shown demonstrably enhanced photosynthesis and growth (Miyagawa *et al.*, 2001; Lefebvre *et al.*, 2005; Tamoi *et al.*, 2006; Feng *et al.*, 2007).

Aldolase (EC 4.1.2.13) is one of the six nonregulated enzymes (its activity is not regulated by effectors or post-translational modification, but by expressional regulation or protein degradation) in the Calvin cycle (Graciet *et al.*, 2004) and catalyses the reversible conversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate to fructose 1,6-bisphosphate on the one hand and erythrose 4-phosphate and dihydroxyacetone phosphate to sedoheptulose 1,7-bisphosphate on the other (Supplementary Fig. S1). Analyses of antisense plants (Haake *et al.*, 1998, 1999; Henkes *et al.*, 2001) and mathematical modelling of the metabolic pathway (Zhu *et al.*, 2007) suggest that non-regulated enzymes in the Calvin cycle (e.g. TK, aldolase) have the potential to control photosynthetic carbon flux through the Calvin cycle. However, the enzymes have not received their due attention, with not even a single report in the literature on the analysis of plants overexpressing genes encoding these enzymes. In order to investigate the effect of overexpression of aldolase, this study generated transgenic tobacco (*Nicotiana tabacum* L. cv Xanthi) expressing *Arabidopsis* plastid aldolase. Elevated plastid aldolase activity accelerated RuBP regeneration and resulted in increased photosynthetic capacity, growth rate, and biomass yield.

## Materials and methods

### *Construction of vector*

Total RNA was isolated with RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). cDNA was synthesized by reverse transcription of the total RNA with ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA) and random hexamer. *Arabidopsis thaliana* plastid aldolase (AtptAL) gene (accession no. AF428455) omitting the region encoding plastid targeting signal was amplified from *A. thaliana* seedling cDNA using two primers (AtptAL-Fw, 5′-CAACGGCGGAAGAGTGGCTTCCTCCTACGCCGATGA-3′, and AtptAL-Rv, 5′-GAGCTCTCAATAGGTGTACCCTTTGAC-3′). The sequence encoding the plastid targeting signal of *N. tabacum* Rubisco small subunit (accession no. M32419) was amplified from *N. tabacum* seedling cDNA using two primers (rbcS-Fw, 5′-TCTAGAATGGCTTCCTCAGTTCTTTC-3′, and rbcS-Rv, 5′-TCATCGGCGTAGGAGGAAGCCACTCTTCCGCCGTTG-3′). *N. tabacum* Rubisco small subunit fragment was fused to the *A. thaliana* plastid aldolase gene by PCR using rbcS-Fw and AtptAL-Rv. This chimeric gene was inserted into pIG121-Hm (Ohta *et al.*, 1990) by replacing the plasmid's GUS coding region and the constructed expression vector was designated pIG121-AtptAL.

### *Generation and growth of transgenic plants*

pIG121-AtptAL or an empty vector plasmid derived from pIG121-Hm was introduced into *Agrobacterium tumefaciens* (LBA4404) by electroporation. *Agrobacterium*-mediated transformation (Horsch *et al.*, 1985) was done using wild-type tobacco leaf discs taken from a stable population. Transformants were regenerated on selection medium (MS medium (Murashige and Skoog, 1962) containing ($l^{-1}$) 30 g sucrose, 2 g gellan gum, 1 mg 6-benzylaminopurine, 100 μg 1-naphthaleneacetic acid, 500 mg carbenicillin and 250 mg kanamycin and then were rooted on hormone-free selection medium. All plants were grown in soil and cultured in controlled environmental chambers at 25 °C under a 16/8 light/dark regime with photon flux density of 400 μmol $m^{-2}$ $s^{-1}$, 370 (ambient) or 700 ppm $CO_2$, and 60% relative humidity. Plants were fertilized with diluted (1:500, v/v) HYPONeX (Hyponex Japan, Osaka). In order to avoid positional biases, plants were placed randomly in environmental chambers and their positions were changed every day. $T_0$ plants were selfed to obtain $T_1$ seeds. Single-copy transformants were selected by Southern blot analysis and segregation analysis of kanamycin resistance of the $T_1$ and $T_2$ generations. Six homozygous $T_2$ transgenic lines were obtained and used for experiments. Untransformed wild-type and empty vector-transformed plants did not show any differences in all experiments. Therefore, only data from wild-type plants are presented in figures.

### Northern blot analysis

Total RNA (10 μg) was run in a 1% (w/v) agarose/formaldehyde gel and transferred onto a nylon membrane. rRNA bands visualized by ethidium bromide staining were used as a loading control. The preparation of specific probes, hybridizations, and signal detection were carried out with AlkPhos Direct Labeling and Detection System and CDP-Star (GE Healthcare, Buckinghamshire, UK), following the manufacturer's instructions.

### Protein analysis

Intact chloroplasts were isolated from leaves of 10-week-old plants by Percoll density gradient centrifugation as reported previously (Hasunuma *et al.*, 2008). Isolated intact chloroplasts were suspended in chloroplast extraction buffer (20 mM TRIS-HCl pH 7.5, 10 mM $MgCl_2$) and sonicated. The extracts were centrifuged at 12,000 *g* for 5 min at 4 °C. The supernatant was electrophoresed on a 12.5% SDS-polyacrylamide gel. The protein bands were visualized with SYPRO Ruby gel stain (Bio-Rad, CA, USA). For the Western blot analysis, proteins were separated on a SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. An affinity-purified anti-AtptAL antibody, which was raised in rabbits against synthetic peptides (ESEEAKEGMFVKGYTY and GAESKDRATPEQV) corresponding to part of the C-terminus of AtptAL, was prepared and then conjugated with horseradish peroxidase. The blots were probed with the horseradish peroxidase-conjugated rabbit anti-AtptAL antibody and then detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare). Protein concentration was determined using Bradford's method (Bradford, 1976).

### Measurement of photosynthetic activity

$CO_2$ fixation was measured using a portable photosynthesis system, LI-6400 (LI-COR, Lincoln, NE, USA). Net $CO_2$ assimilation rates were determined under 0–1,200 μmol photons $m^{-2}$ $s^{-1}$, 370 or 700 ppm $CO_2$, 25 °C, and 60% relative humidity after incubation of tobacco plants in the dark for more than 60 min.

### Enzyme assays

Leaf tissue was homogenized in an extraction buffer composed of 50 mM TRIS-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 12,000 *g* for 5 min at 4 °C. The supernatant was desalted with a PD10 column (GE Healthcare) and used for enzyme assays. The assays were performed as reported previously: aldolase and TK (Haake *et al.*, 1998); Rubisco (Tamoi *et al.*, 2006); 3-phosphoglycerate kinase (PGK) Macioszek *et al.*, 1990); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Stitt *et al.*, 1989); and fructose 1,6-bisphosphatase (FBPase) and phosphoribulokinase (PRK) (Koßmann *et al.*, 1994).

### Quantification of metabolites and carbohydrates

Samples were harvested after a 12 h light period. They were plunged immediately into liquid $N_2$ and stored at –80 °C until use. For quantification of intermediates of the Calvin cycle, samples were prepared as described in a previous report (Hasunuma *et al.*, 2010) and analysed by LC-MS/MS.

All analyses were performed using 4000 QTRAP LC-MS/MS system (AB SCIEX, Tokyo, Japan) equipped with a Prominence HPLC system (Shimadzu, Kyoto, Japan) and Turbo V ion source. Data acquisition and evaluation were done using Analyst software 1.4.2 (AB SCIEX). Separation was performed by reversed-phase ion-pair liquid chromatography with a Shim-pack VP-ODS column (5 μm, 150 mm×4.6 mm, Shimadzu). In this method di-*n*-butylammonium acetate (DBAA, Tokyo Kasei Kogyo, Tokyo, Japan) was used as the volatile ion-pair reagent. The gradient program consisted of initially 100% solvent A (water containing 5 mmol DBAA $l^{-1}$) for 1 min, 0–10% solvent B (methanol containing 5 mmol DBAA $l^{-1}$; in solvent A) for 30 min, 10–60% solvent B for 30 min, and 100% solvent B for 5 min. The initial condition was held for 10 min before the injection of the next sample. A flow rate of 0.2 ml $min^{-1}$ and a column temperature of 40 °C were used throughout.

Electrospray ionization-MS/MS was conducted in the negative ion mode. The setting for MS parameters were as follows: curtain gas, 30.0 psi; collision gas, 5.0 psi; ion spray voltage, –4.5 kV, temperature, 550 °C, ion source gas 1, 60.0 psi; ion source gas 2, 60.0 psi; entrance potential, –10.0 V. The Q1 and Q3 resolutions were set as unit. All intermediates were monitored by MRM. The MRM parameters, Q1 (m/z of deprotonated precursor ion), Q3 (m/z of product ion), declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) are listed in Supplementary Table S1. Concentration of all sugar phosphates was determined by comparing the areas of the MRM ion chromatograms with those for samples diluted with water or standard solutions. ribulose 5-phosphate and xylulose 5-phosphate, which are epimers, were not separated on HPLC and thus the summed concentration was calculated.

The levels of glucose, fructose, and sucrose were measured enzymically as described in a previous report (Stitt *et al.*, 1989). Starch level was measured using the method reported previously (Smith and Zeeman, 2006).

### Statistical analysis

All tests were performed with SPSS 15.0J software (SPSS Japan, Tokyo, Japan). Significant differences were determined based on two-tailed *t*-tests or one-way ANOVA followed by Tukey's post-hoc test. $P < 0.05$ was considered significant.

## Results

### Generation of transgenic tobacco expressing A. thaliana plastid aldolase

The *A. thaliana* plastid aldolase cDNA was modified by replacing its plastid targeting signal sequence with that of the tobacco Rubisco small subunit. The modified cDNA was introduced downstream of the cauliflower mosaic virus 35S promoter in the plant expression vector pIG121-Hm (Ohta *et al.*, 1990) (Fig. 1A). Following *Agrobacterium*-mediated transformation using tobacco leaf discs, primary transgenic plants were rooted on hormone-free selection medium, transferred to soil, and grown until maturity. By using reverse-transcription PCR, six transgenic lines of homozygous $T_2$ progeny expressing *Arabidopsis* aldolase were found (designated AtptAL 3, 4, 5, 7, 9, and 12). mRNA transcription of *Arabidopsis* aldolase was confirmed by Northern gel blot analysis (Fig. 1B). Localization of *Arabidopsis* aldolase in chloroplasts was confirmed by Western blot analysis of proteins extracted from chloroplasts isolated from mature leaves (Fig. 1C). Aldolase activities of transgenic plants were 1.4–1.9-fold higher than those of wild-type plants (Fig. 1D). Three lines showing different aldolase activities (AtptAL 3, 4 and 12) were selected and used to further analysis.

### Growth under ambient or high $CO_2$ concentration

To assess the impact of overexpression of aldolase, transgenic plant growth in controlled environmental chambers was analysed. Plants were first grown for 4 weeks at 25 °C,

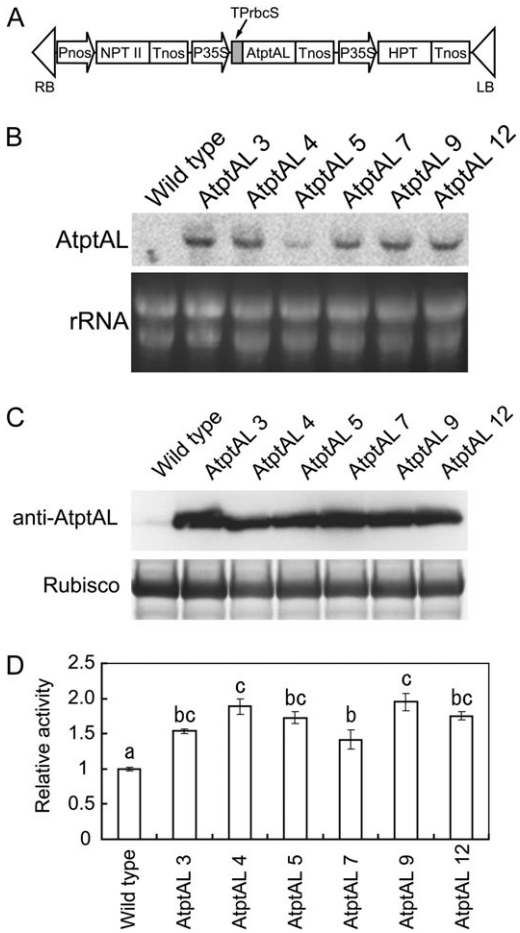


**Fig. 1.** Generation of transgenic tobacco with enhanced plastid aldolase activity. (A) Schematic representation of *A. thaliana* aldolase expression vector. RB, T-DNA right border; Pnos, nopaline synthase promoter; NTP II, neomycin phosphotransferase gene; Tnos, nopaline synthase terminator; P35S, cauliflower mosaic virus 35S promoter; TPrbcS, plastid targeting signal sequence of tobacco Rubisco small subunit gene; AtptAL, *A. thaliana* plastid aldolase gene; HPT, hygromycin phosphotransferase gene; LB, T-DNA left border. (B) Northern blot analysis. Total RNA was extracted from mature leaves and blotted and the signal was detected with AtptAL probes. rRNA bands were used as a loading control. (C) Western blot analysis of total soluble protein extracted from isolated chloroplasts. Soluble protein (5 μg) was prepared from isolated chloroplasts, separated by SDS-PAGE, and blotted with anti-AtptAL antibody (anti-AtptAL) or visualized by staining with SYPRO Ruby gel stain (Rubisco). (D) Aldolase activity in leaves of transformants. Enzyme activity was determined in samples prepared from leaves of 10-week-old plants and expressed as relative activity to that of wild-type plants. The data are mean ± SD from five individual plants per line. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test. Values followed by the same letter are not significantly different ($P > 0.05$).

16/8 light/dark regime, 400 μmol $m^{-2}$ $s^{-1}$ photon flux density, 370 ppm $CO_2$, and 60% relative humidity and thereafter subjected to ambient (370 ppm) or high (700 ppm) $CO_2$ concentration. There were no significant differences in the seed germination and early growth between transgenic plants and their wild-type counterparts (data not shown). Although transgenic plants proceeded to grow only slightly faster than their wild-type counterparts under ambient $CO_2$ in the late growth stages (Fig. 2A, B and Supplementary Fig. S2), they grew significantly faster than wild-type plants under high $CO_2$ (Fig. 2A, B). The stems and leaves of transgenic plants were larger than those of wild-type plants (Fig. 2A, B). At 9 weeks after sowing, the dry biomass of the above-ground parts of transgenic plants was 1.1–1.3-fold and 1.7–2.2-fold more than that of wild-type plants under ambient and high $CO_2$ concentrations, respectively (Fig. 2C). When plants were cultivated hydroponically under the same conditions as soil culture (Fig. 2D), the enhancement of plant growth and biomass production by aldolase overexpression was also observed.

### *Photosynthetic characterization of transgenic plants*

After 4 weeks of cultivation under 400 μmol $m^{-2}$ $s^{-1}$ photon flux density and 370 ppm $CO_2$, the photosynthetic performance of the leaves measured at various light intensities (0–1200 μmol $m^{-2}$ $s^{-1}$) did not reveal any significant $CO_2$ fixation rate differences between transgenic plants and their wild-type counterparts (Fig. 3A). However, the $CO_2$ fixation rate apparently increased at irradiances above 800 μmol $m^{-2}$ $s^{-1}$ under high $CO_2$ concentration (Fig. 3B). At 1000 μmol $m^{-2}$ $s^{-1}$ irradiance, rates of 15.23 ± 0.98, 19.03 ± 0.55, 20.47 ± 1.23 and 20.00 ± 1.10 μmol $CO_2$ $m^{-2}$ $s^{-1}$ were observed for wild-type and AtptAL 3, 4, and 12 plants, respectively. Notably, the AtptAL 4 rate was 1.34 times higher than the corresponding wild-type $CO_2$ fixation rate. The maximum quantum yield of photosystem II was not significantly different between transgenic and wild-type plants (Supplementary Fig. S3). Furthermore, the increase in photosynthesis measured at high $CO_2$ concentration was also observed when plants were grown under high $CO_2$ concentration (Fig. 3C). The $CO_2$ fixation rate of transgenic plants was significantly higher than that of wild-type plants at irradiances above 400 μmol $m^{-2}$ $s^{-1}$, with a maximum of 1.5 times the corresponding wild-type $CO_2$ fixation rate. Therefore, the elevated plastid aldolase activity of transgenic plants resulted in increased photosynthetic $CO_2$ fixation rate and, consequently, improved plant growth and biomass yield under high $CO_2$ concentration.

### *Assessment of effects of plastid aldolase overexpression on the Calvin cycle*

The enzyme activities of Rubisco (initial and total activity), PGK, GAPDH, FBPase, TK, and PRK showed no significant differences between the transgenic lines (Fig. 4). Thus, only the plastid aldolase activity was enhanced in transgenic plants, with the gene overexpression not affecting the activities of any of the remaining Calvin cycle enzymes. There were no differences in Rubisco activity between transgenic and wild-type plants, suggesting that the carboxylation step was not inhibited by aldolase overexpression.

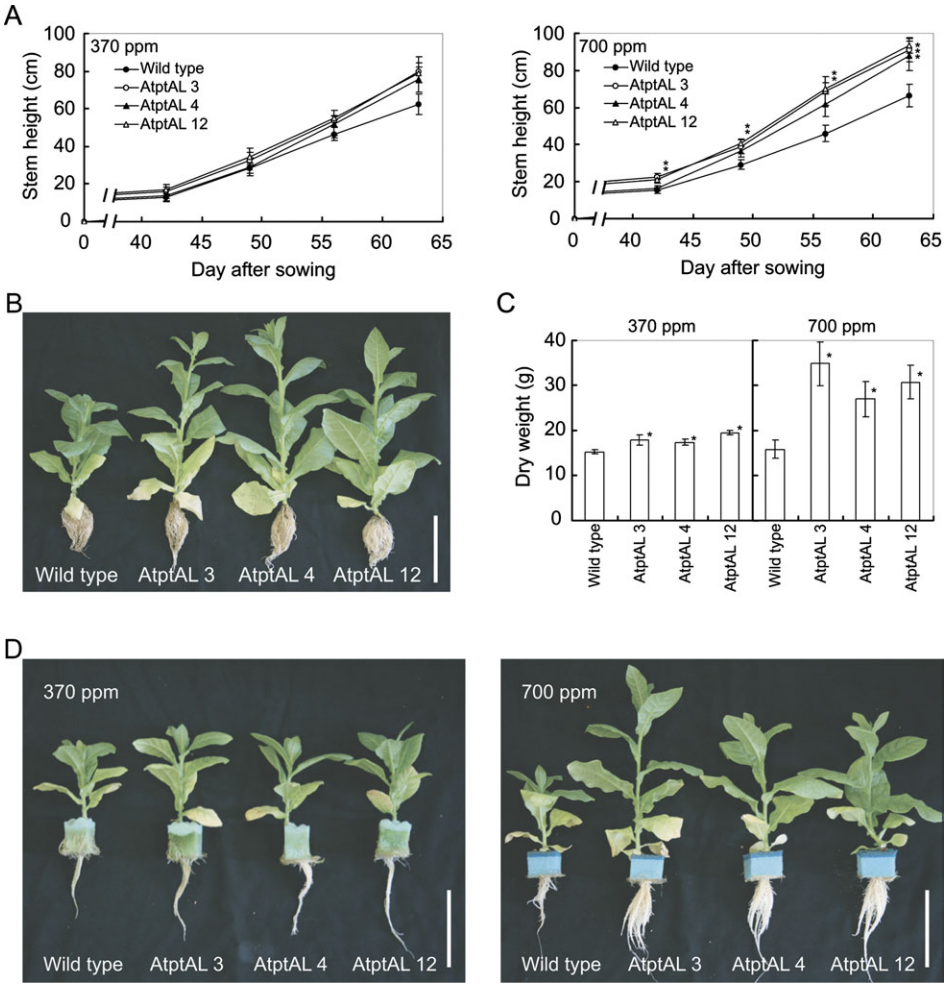


**Fig. 2.** Effects of increased plastid aldolase activity on plant growth and biomass yield. (A) Growth rates of transformants and wild-type plants. Plants were grown under 370 ppm $CO_2$ for 4 weeks and were then transferred to ambient (370 ppm) or high (700 ppm) $CO_2$ concentration. Plant height was measured from 6 to 9 weeks after sowing. (B) Aerial phenotypes of transformants and wild-type plants grown under high $CO_2$ concentration, described in (A), 7 weeks after sowing. Bar, 10 cm. (C) Comparison of dry weight of the above-ground parts of 9-week-old plants grown under conditions described in (A). (D) Hydroponic cultivation of transformants and wild-type plants. Plants were grown hydroponically under 370 ppm $CO_2$ for 4 weeks and then transferred to ambient (370 ppm) or high (700 ppm) $CO_2$ concentration. Photographs were taken at 6 weeks after sowing. Bars, 10 cm. The data are mean ± SEM from 12 individual plants per line. Asterisks indicate that differences between transformants and wild-type plants are significant (*t*-test, $P < 0.05$).

Intermediates of carbohydrate (sucrose or starch) synthesis pathways and the Calvin cycle were measured during the light period. The contents of erythrose 4-phosphate, ribose 5-phosphate, ribulose 5-phosphate/xylulose 5-phosphate, RuBP, and ADP-glucose in transgenic plant leaves to be significantly higher than those in wild-type plants (Fig. 5A). RuBP and ADP-glucose contents were particularly high in AtptAL 4 at 2.2-fold and 1.9-fold higher, respectively. The corresponding values for AtptAL 3 and 12 were 1.5-fold and 1.7-fold, and 1.4-fold and 1.4-fold, respectively. Sedoheptulose 7-phosphate (S7P) was significantly elevated in AtptAL 3 and 4, but not in AtptAL 12. Dihydroxyacetone phosphate was most elevated in AtptAL 4 and 12, but not AtptAL 3. Significant elevation of glyceraldehyde 3-phosphate was observed only in AtptAL 4. In contrast, 3-phosphoglycerate contents of AtptAL 3, 4, and 12 were suppressed to 77, 71, and 62% of wild-type levels, respectively. Concentrations of carbohydrates in leaves of transgenic and wild-type plants were measured under the same conditions (Fig. 5B), but there were no differences in the contents of glucose, fructose, sucrose, and starch. Since erythrose 4-phosphate, ribose 5-phosphate, ribulose 5-phosphate, xylulose 5-phosphate, and S7P are precursors of RuBP regeneration in chloroplasts, these results indicate that the elevation of plastid aldolase activity caused promotion of RuBP regeneration.

## Discussion

Antisense analysis has shown that relatively small decreases in plastid aldolase lead to decreased RuBP content, inhibition of photosynthesis, and decreased growth, suggesting that plastid aldolase is never expressed in excess amounts and that it exerts flux control of photosynthesis by limiting

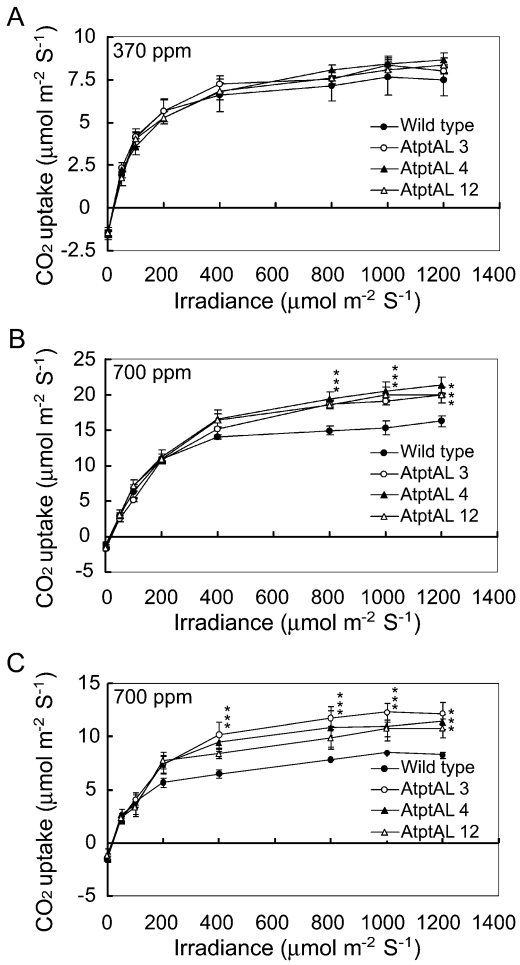


**Fig. 3.** Comparison of photosynthetic activity between transformants and wild-type plants. Plants were grown at 25 °C with photon flux density of 400 μmol $m^{-2}$ $s^{-1}$ and 370 ppm $CO_2$ for 4 weeks. $CO_2$ assimilation rates at 370 (A) or 700 (B) ppm $CO_2$ were measured in the fourth leaves from the top under various irradiance conditions. (C) After 4 weeks under ambient $CO_2$ concentration, transformants and wild-type plants were transferred to high (700 ppm) $CO_2$ concentration. After 2 weeks cultivation under high $CO_2$ concentration, $CO_2$ assimilation rates at 700 ppm $CO_2$ of transformants and wild-type plants were measured in the fourth leaves from the top under various irradiance conditions. The data are mean ± SEM from 12 individual plants per line. Asterisks indicate differences between transformants and wild-type plants are significant (*t*-test, $P < 0.05$).

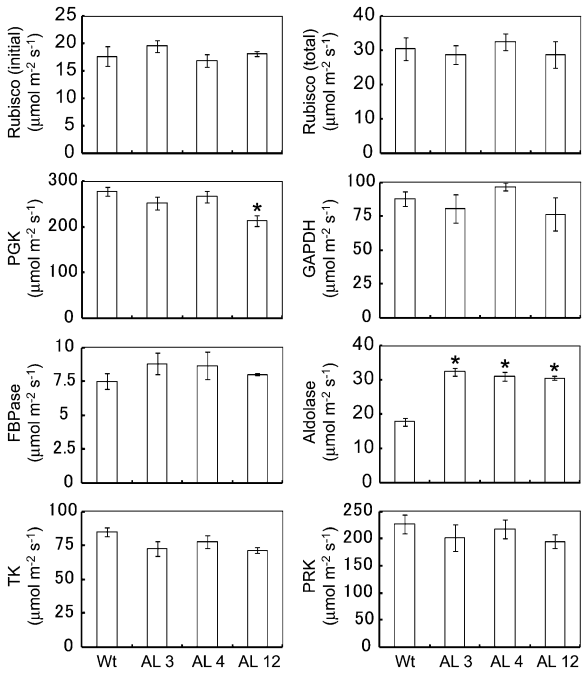


**Fig. 4.** Measurement of activities of Calvin cycle enzymes. Plants were grown in controlled environmental chambers at 25 °C with photon flux density of 400 μmol $m^{-2}$ $s^{-1}$ and 370 ppm $CO_2$. Samples were prepared from the fourth leaves from the top of 6–8-week-old plants after a 12-h light period and used for determination of enzyme activities. The data are mean ± SD from 12 individual plants per line. Asterisks indicate that differences between transformants and wild-type plants are significant (*t*-test, $P < 0.05$). AL, AtptAL; aldolase, fructose 1,6-bisphosphate aldolase; FBPase, fructose 1,6-bisphosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, 3-phosphoglycerate kinase; PRK, phosphoribulokinase; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; TK, transketolase; Wt, wild type.

the RuBP regeneration steps catalysed by it (Haake *et al.*, 1998, 1999). The consequent flux control coefficient of aldolase for photosynthesis was estimated at 0.18 under ambient $CO_2$ and 0.55 under high $CO_2$. This estimation is in agreement with the current observation that elevation of plastid aldolase activity stimulates photosynthesis under high $CO_2$, but not under ambient $CO_2$. In previous reports, alteration of aldolase activity in response to irradiance and/or $CO_2$ concentration was observed, inviting the conclusion that aldolase activity is involved in regulation of photosynthetic carbon flux (Haake *et al.*, 1999). The current results suggest that native plastid aldolase activity is sufficient for photosynthesis at ambient $CO_2$ at least under the experimental conditions tested.

Overexpression of plastid aldolase imposed constitutive elevation of aldolase activity in chloroplasts and cancelled the limitation of RuBP regeneration by increasing flux in two reactions catalysed by aldolase, resulting in accumulation of RuBP and its precursors and decrease of dihydroxyacetone phosphate. This idea is supported by the fact that the heterozygous lines with less aldolase activities showed less RuBP accumulation under ambient $CO_2$ concentration and slower growth under high $CO_2$ concentration compared to the homozygous lines (data not shown). Promotion of RuBP regeneration by overexpression of plant aldolase in cyanobacteria has previously been suggested (Kang *et al.*, 2005; Ma *et al.*, 2007, 2008). In these cyanobacteria, increased aldolase activity was shown to accelerate RuBP regeneration via stimulation of SBPase. It is unclear in the current study whether increased plastid aldolase activity promoted RuBP regeneration via regulation of SBPase or by other mechanism, since SBPase activity could not be determined because of the unavailability of sedoheptulose 1,7-bisphosphate standard for the enzyme assay. Further

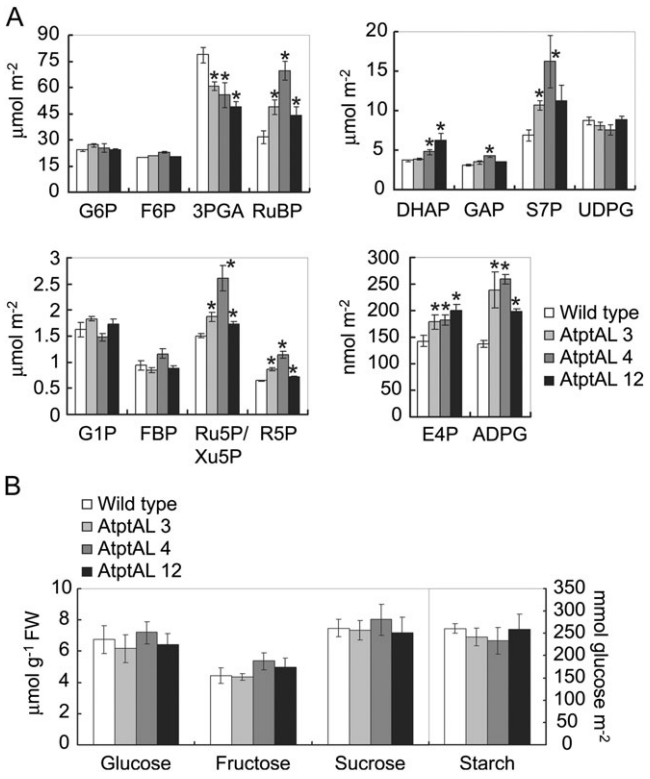


**Fig. 5.** Impact on carbon flow through the Calvin cycle. (A) Quantification of intermediates involved in Calvin cycle starch/sucrose synthesis. Intermediates were extracted from the fourth leaves from the top and analysed by LC-MS/MS. 3PGA, 3-phosphoglycerate; ADPG, ADP-glucose; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; R5P, ribose 5-phosphate; Ru5P/Xu5P, ribulose 5-phosphate/xylulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; S7P, sedoheptulose 7-phosphate; UDPG, UDP-glucose. (B) Measurement of carbohydrate contents in photosynthetic leaves. The levels of hexose (glucose and fructose), sucrose, and starch were measured in the fourth leaves from the top. All samples were harvested in the same way as for Fig. 4. The data are mean ± SD from 12 individual plants per line. Asterisks indicate that differences between transformants and wild-type plants are significant (*t*-test, $P < 0.05$).

analysis is therefore still required to reveal the mechanism underlying the observed promotion of RuBP regeneration in transgenic *N. tabacum*.

While overexpression of plastid aldolase promoted RuBP regeneration and slightly increased growth and biomass productivity in this study (Fig. 2A, C), it had no noticeable effect on photosynthetic $CO_2$ fixation rate under ambient $CO_2$ concentration (Fig. 3A). This is in contrast with cyanobacterial FBP/SBPase-overexpressing tobacco plants that exhibited stimulated RuBP regeneration in addition to increased photosynthetic activity and improved growth and productivity under the same conditions (Miyagawa *et al.*, 2001). These phenotypic differences may be explained by differences in Rubisco activity between the two groups of transgenic tobacco plants. Since photosynthetic $CO_2$ fixation rate is known to be limited by Rubisco activity at relatively low $CO_2$ concentrations (Farquhar *et al.*, 1980; Stitt and Schulze, 1994; Raines, 2006), FBP/SBPase-overexpressing plants with elevated Rubisco activity (Miyagawa *et al.*, 2001) are able to maintain high-enough photosynthetic $CO_2$ fixation rates that lead to enhanced growth under ambient $CO_2$ concentration. The higher RuBP and decreased dihydroxyacetone phosphate observed in aldolase-overexpressing plants (Fig. 5A) also support the dependence, at ambient $CO_2$ concentration, on carbon flow for Rubisco activity in the Calvin cycle.

Aldolase-overexpressing plants grew faster than wild-type plants and had higher biomass yield (Fig. 2A, C) and higher photosynthetic $CO_2$ fixation rate (Fig. 3B, C) under high $CO_2$ concentration. Rubisco activity under these conditions showed no differences between the transgenic and wild-type plants (data not shown). Since the dependence on carbon flow for Rubisco activity is relatively reduced under high $CO_2$, RuBP regeneration is considered to be the major contributor to the enhanced rate of photosynthesis under these conditions (Hudson *et al.*, 1992; Stitt and Schulze, 1994). Therefore, the enhancement of photosynthesis and growth of aldolase-overexpressing plants observed under high $CO_2$ could be due to the enhancement of RuBP regeneration and the transition of the rate-limiting step from Rubisco activity to RuBP regeneration.

FBP/SBPase-overexpressing plants have been reported to accumulate hexose, sucrose, and starch compared to wild-type plants (Miyagawa *et al.*, 2001). However, in this study, aldolase overexpression did not affect the carbohydrate content of leaves (Fig. 5B). Since increased plastid aldolase activity is known to stimulate sucrose loading into the phloem (Barry *et al.*, 1998), synthesized sucrose should be quickly transported to the phloem and not accumulated in photosynthetic leaves. Although remarkable accumulation of ADP-glucose, which is a precursor of starch synthesis, was detected in the current study (Fig. 5A), increased starch accumulation was not observed in aldolase-overexpressing plants (Fig. 5B). Similar results have been described in transgenic tobacco expressing bacterial aldolase in chloroplasts (Barry *et al.*, 1998). Starch is more rapidly accumulated in the early photoperiod in transgenic plants, but its maximum concentration in leaves remains the same as that in wild-type plants, suggesting that overexpression of plastid aldolase should stimulate starch synthesis but not affect starch accumulation capacity.

Nonregulated enzymes which catalyse reversible reactions, e.g. aldolase and TK, have been thought to exert less influence on photosynthetic carbon flux and therefore have not received much attention (Raines, 2003). However, recent antisense studies suggest that such enzymes could contribute to carbon flux through the Calvin cycle *in vivo* (Haake *et al.*, 1998, 1999; Henkes *et al.*, 2001). Recent mathematical modelling, moreover, implicates plastid aldolase in the control of photosynthetic carbon metabolic flux (Zhu *et al.*, 2007). The current results support these conclusions and

demonstrate that an increase of activity of a nonregulated Calvin cycle enzyme stimulates carbon fixation and productivity.

This study generated transgenic tobacco overexpressing plastid aldolase and assessed the effect of elevated plastid aldolase activity on physiological and biochemical characteristics. Overexpression of a single native plant enzyme, aldolase, which is known as a nonregulated enzyme, accelerated Calvin cycle turnover and improved plant growth and biomass yield. Under a constant light intensity and a high $CO_2$ concentration, photosynthesis and plant growth in transgenic plants was enhanced (Fig. 2A, C). In the field, light intensity is altered diurnally or by meteorological factors, which results in changes in the limiting factors of the Calvin cycle from those associated with Rubisco activity to those associated with RuBP regeneration. Therefore, further analysis is required to reveal whether the promoting effects of aldolase overexpression seen in this study are observed under field conditions.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Table S1.** MRM settings for quantification of intermediates on the Calvin cycle.

**Supplementary Fig. S1.** Simplified scheme of the Calvin cycle.

**Supplementary Fig. S2.** Aerial phenotypes of transformants and wild-type plants grown under ambient $CO_2$ conditions.

**Supplementary Fig. S3.** Maximal quantum yield of photosystem II of transformants and wild-type plants.

## Acknowledgements

The authors thank CA Omumasaba (RITE) for critical reading of the manuscript. This work supported a grant from the New Energy and Industrial Technology Department Organization (NEDO), Japan.

## References

**Barry GF, Cheikh N, Kishore GM.** 1998. Expression of fructose 1,6 bisphosphate aldolase in transgenic plants. Patent Application WO/1998/058069.

**Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248–254.

**Caemmerer S, Farquhar GD.** 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153,** 376–387.

**Farquhar GD, Caemmerer S, Berry JA.** 1980. A biochemical model of photosynthetic $CO_2$ assimilation in leaves of $C_3$ species. *Planta* **149,** 78–90.

**Feng L, Wang K, Li Y, Tan Y, Kong J, Li H, Li Y, Zhu Y.** 2007. Overexpression of SBPase enhances photosynthesis against high temperature stress in transgenic rice plants. *Plant Cell Reports* **26,** 1635–1646.

**Furbank RT, Taylor WC.** 1995. Regulation of photosynthesis in C3 and C4 plants: a molecular approach. *The Plant Cell* **7,** 797–807.

**Graciet E, Lebreton S, Gontero B.** 2004. Emergence of new regulatory mechanisms in the Benson-Calvin pathway via protein-protein interactions: a glyceraldehyde-3-phosphate dehydrogenase/CP12/phosphoribulokinase complex. *Journal of Experimental Botany* **55,** 1245–1254.

**Haake V, Geiger M, Walch-Liu P, Engels C, Zrenner R, Stitt M.** 1999. Changes in aldolase activity in wild-type potato plants are important for acclimation to growth irradiance and carbon dioxide concentration, because plastid aldolase exerts control over the ambient rate of photosynthesis across a range of growth conditions. *The Plant Journal* **17,** 479–489.

**Haake V, Zrenner R, Sonnewald U, Stitt M.** 1998. A moderate decrease of plastid aldolase activity inhibits photosynthesis, alters the levels of sugars and starch, and inhibits growth of potato plants. *The Plant Journal* **14,** 147–157.

**Harrison EP, Willingham NM, Lloyd JC, Raines CA.** 1997. Reduced sedoheptulose-1,7-bisphosphatase levels in transgenic tobacco lead to decreased photosynthetic capacity and altered carbohydrate accumulation. *Planta* **204,** 27–36.

**Hartman FC, Harpel MR.** 1994. Structure, function, regulation, and assembly of D-ribulose-1,5-bisphosphate carboxylase/oxygenase. *Annual Review of Biochemistry* **63,** 197–234.

**Hasunuma T, Harada K, Miyazawa S, Kondo A, Fukusaki E, Miyake C.** 2010. Metabolic turnover analysis by a combination of in vivo $^{13}C$-labelling from $^{13}CO_2$ and metabolic profiling with CE-MS/MS reveals rate-limiting steps of the $C_3$ photosynthetic pathway in *Nicotiana tabacum* leaves. *Journal of Experimental Botany* **61,** 1041–1051.

**Hasunuma T, Takeno S, Hayashi S, Sendai M, Bamba T, Yoshimura S, Tomizawa K, Fukusaki E, Miyake C.** 2008. Overexpression of 1-deoxy-d-xylulose-5-phosphate reductoisomerase gene in chloroplast contributes to increment of isoprenoid production. *Journal of Bioscience and Bioengineering* **105,** 518–526.

**Henkes S, Sonnewald U, Badur R, Flachmann R, Stitt M.** 2001. A small decrease of plastid transketolase activity in antisense tobacco transformants has dramatic effects on photosynthesis and phenylpropanoid metabolism. *The Plant Journal* **13,** 535–551.

**Horsch RB, Fry J, Hoffman NL, Eichholtz D, Rogers SG, Fraley R.** 1985. A simple and general method for transferring genes into plants. *Science* **227,** 1229–1231.

**Hudson GS, Evans JR, von Caemmerer S, Arvidsson YB, Andrews TJ.** 1992. Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. *Plant Physiology* **98,** 294–302.

**Kang R, Shi D, Cong W, Ma W, Cai Z, Ouyang F.** 2005. Effects of co-expression of two higher plants genes ALD and TPI in *Anabaena* sp. PCC7120 on photosynthetic $CO_2$ fixation. *Enzyme and Microbial Technology* **36,** 600–604.

**Koßmann J, Sonnewald U, Willmitzer L.** 1994. Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants

impairs photosynthesis and plant growth. *The Plant Journal* **6,** 637–650.

**Lefebvre S, Lawson T, Zakhleniuk OV, Lloyd JC, Raines CA, Fryer M.** 2005. Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant Physiology* **138,** 451–460.

**Ma W, Wei L, Wang Q, Shi D, Chen H.** 2007. Increased activity of the non-regulated enzymes fructose-1,6-bisphosphate aldolase and triosephosphate isomerase in *Anabaena* sp. strain PCC 7120 increases photosynthetic yield. *Journal of Applied Phycology* **19,** 207–213.

**Ma W, Wei L, Wang Q, Shi D, Chen H.** 2008. Increased activity of the tandem fructose-1,6-bisphosphate aldolase, triosephosphate isomerase and fructose-1,6-bisphosphatase enzymes in *Anabaena* sp. strain PCC 7120 stimulates photosynthetic yield. *Journal of Applied Phycology* **20,** 437–443.

**Macioszek J, Anderson JB, Anderson LE.** 1990. Isolation of chloroplastic phosphoglycerate kinase: kinetics of the two-enzyme phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase couple. *Plant Physiology* **94,** 291–296.

**Miyagawa Y, Tamoi M, Shigeoka S.** 2001. Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nature Biotechnology* **19,** 965–969.

**Murashige T, Skoog F.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

**Ohta S, Mita S, Hattori T, Nakamura K.** 1990. Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant and Cell Physiology* **31,** 805–813.

**Paul MJ, Knight JS, Habash D, Parry MAJ, Lawlor DW, Barnes SA, Loynes A, Gray JC.** 1995. Reduction in phosphoribulokinase activity by antisense RNA in transgenic tobacco: effect on $CO_2$ assimilation and growth in low irradiance. *The Plant Journal* **7,** 535–542.

**Price GD, Evans JR, Caemmerer S, Yu J, Badger MR.** 1995. Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces $CO_2$ assimilation via a reduction in ribulose bisphosphate regeneration in transgenic tobacco plants. *Planta* **195,** 369–378.

**Raines CA.** 2003. The Calvin cycle revisited. *Photosynthesis Research* **75,** 1–10.

**Raines CA.** 2006. Transgenic approaches to manipulate the environmental responses of the $C_3$ carbon fixation cycle. *Plant, Cell and Environment* **29,** 331–339.

**Smith AM, Zeeman SC.** 2006. Quantification of starch in plant tissues. *Nature Protocols* **1,** 1342–1345.

**Stitt M, Lilley RMC, Gerhardt R, Heldt HW.** 1989. Metabolite levels in specific cells and subcellular compartments of plant leaves. *Methods in Enzymology* **174,** 518–552.

**Stitt M, Schulze D.** 1994. Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant, Cell and Environment* **17,** 465–487.

**Tamoi M, Nagaoka M, Miyagawa Y, Shigeoka S.** 2006. Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants. *Plant and Cell Physiology* **47,** 380–390.

**United Nations, Department of Economic and Social Affairs, Population Division.** 2009. *World Population Prospects: the 2008 revision*, highlights, working paper no. ESA/P/WP.210. New York: United Nations.

**Zhu XG, de Sturler E, Long SP.** 2007. Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. *Plant Physiology* **145,** 513–526.