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Review

Carbon metabolism in the Calvin cycle

Masahiro Tamoi, Miki Nagaoka, Yukinori Yabuta, Shigeru Shigeoka*

Department of Advanced Bioscience, Faculty of Agriculture, Kinki University
*E-mail: shigeoka@nara.kindai.ac.jp Tel: +81-742-43-8083 Fax: +81-742-43-8976

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Abstract In order to study the regulation of photosynthetic carbon flow in higher plants, many researchers have created and analyzed transgenic plants that had reduced or increased respective enzyme activity involved in the Calvin cycle, sucrose synthesis and starch metabolism. We have succeeded in enhancing the photosynthetic carbon fixation capacity by introducing certain enzymes, fructose-1,6-bisphosphatase and/or sedoheptulose-1,7-bisphosphatase, involved in the Calvin cycle. In this review, we discuss that the contribution of some enzymes and processes to controlling the metabolic flux and storage of carbohydrates and plant growth using transgenic plants. These results lead to a reassessment of ideas about the regulation of carbon metabolism and have consequences for design of bioengineering strategies to increase crop productivity in plants.

Key words: Calvin cycle, fructose-1,6-bisphosphatase, Rubisco, sedoheptulose-1,7-bisphosphatase.

The Calvin cycle is the primary pathway for carbon fixation and this cycle has 13 reaction steps catalyzed by 11 enzymes in the chloroplasts of C3 plants. It is considered to have three stages, the first of these being carboxylation of the CO_2 accepter molecule, ribulose-1,5-bisphosphate (RuBP), by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), resulting in the formation of 3-phosphoglycerate (PGA). The second stage is the reduction phase, which produces the triose phosphate by consuming ATP and NADPH. The final stage of the cycle is the regenerative phase, in which triose phosphates are used to produce RuBP. In the cycle, the triose phosphates are key intermediates, and they are also available for allocation to either the starch or sucrose biosynthetic pathway (Woodrow and Berry 1988; Geiger and Servaites 1994; Quick and Neuhaus 1997). It is extremely important to maintain a balance between export and regeneration in order that the cycle does not become depleted of intermediates. To achieve this balance, the catalytic activities of certain enzymes within the cycle are highly regulated (Fridlyand et al. 1999; Raines et al. 1999). In particular, the activities of certain enzymes, including sedoheptulose-1,7-bisphosphatase (SBPase) and fructose-1,6-bisphosphatase (FBPase), are regulated by the redox potential via the ferredoxin/thioredoxin system, which modulates the enzyme activities in response to light/dark conditions (Scheibe 1990; Buchanan 1991). The product of the reaction catalyzed by FBPase, i.e. fructose 6-phosophate, is the branch point for metabolites leaving the Calvin cycle and moving into starch biosynthesis. Generally, the flux-limiting step is the first step of a pathway branching from another pathway or the virtually irreversible step with a large free-energy change. The levels of FBPase and SBPase in the chloroplasts are extremely low compared to those of the other enzymes in the Calvin cycle (Woodrow and Mott 1993). In addition, study of the computer simulation of the Calvin cycle reactions indicated that the flux control coefficient (C_E^J) of SBPase was high compared with those of other enzymes in the Calvin cycle (Poolman et al. 2001). 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.

Antisense inhibition of endogenous Calvin cycle genes

In order to determine the limiting steps of the Calvin cycle and factors that influence the carbon allocation, a considerable number of studies have been undertaken on the regulation of carbohydrate metabolism in the photosynthetic CO_2 fixation in plant leaves (Table 1). Transgenic plants with reduced levels of

Abbreviations: F6P, fructose 6-phosphtate; FBP, fructose 1,6-bisphosphate; FBPase, fructose-1,6-bisphosphatase; FBP/SBPase, fructose-1,6-/sedoheptulose-1,7-bisphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGA, 3-phosphoglycerate; PRK, phosphoribulokinase; RuBP, ribulose-1,5-bisphosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SBPase, sedoheptulose-1,7-bisphosphatase; SPS, sucrose-phosphate synthase.

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Table 1. Transgenic plants with altered enzyme activities involved in the photosynthetic carbon metabolism

Enzyme	Host plant (localization)	Activity (% of WT)	Primary references
Antisense			
GAPDH	tobacco (Chl)	100–36	Price et al. 1995
PRK	tobacco (Chl)	95–5	Paul et al. 1995
Rubisco	tobacco (Chl)	76–41	Hudson et al. 1992
FBPase	potato (Chl)	36–14	Koßmann et al. 1994
SBPase	tobacco (Chl)	71–15	Harrison et al. 1998
aldolase	potato (Pt)	65–22	Haake et al. 1998
transketolase	tobacco (Pt)	60–40	Hankes et al. 2001
FBPase	potato (Cyt)	55–9	Zrenner et al. 1996
Sense			
FBP/SBPase	tobacco (Chl)	170–230	Miyagawa et al. 2001
SBPase	tobacco (Chl)	130–160	Lefebvre et al. 2005
FBPase	potato (Cyt)	1000–2000	Thorbjornsen et al. 2002
invertase	tobacco (Cyt, Apo, Vac)		Bussis et al. 1997
SPS	tomato (Cyt)	200–300	Galtier et al. 1993
hexokinase	tomato (Cyt)	600–700	Dai et al. 1999
AGPase (mutant)	potato (Pt)		Stark et al. 1992
	rice (Es)	270	Smidansky et al. 2003

Chl, chloroplast; Pt, plastid; Cyt, cytosol; Apo, apoplast;Vac, vacuole; Es, endosperm

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoribulokinase (PRK), Rubisco and aldolase showed little effects on photosynthesis (Price et al. 1995; Paul et al. 1995; Hudson et al. 1992; Haake et al. 1998). These data indicated that a number of enzymes involved in the Calvin cycle are present at protein levels well in excess of that required to sustain a continued rate of CO_2 fixation.

In contrast, especially in the antisense plants of chloroplastic FBPase or SBPase, the rate of photosynthesis was significantly diminished in proportion to the decrease in the respective enzyme activity due to the decrease in the RuBP regeneration capacity in the Calvin cycle. Antisense potato plants that displayed 36% of the wild-type level of FBPase activity showed photosynthetic activity similar to the wild-type plants, whereas the photosynthesis and growth rate were drastically inhibited when the FBPase activity was decreased to below 14% of the wild-type level (Koßmann et al. 1994). The antisense inhibition of SBPase activity had a stronger effect on photosynthesis (Harrison et al. 1998, 2001; Ölçer et al. 2001). In the SBPase antisense plants that retained 71% of the wild-type level of SBPase activity, the light-saturated photosynthetic activity was reduced by 36%. These reports indicate that the photosynthesis, carbon partitioning, carbon flow of the Calvin cycle and plant growth are remarkably sensitive to small decreases of SBPase activity.

Transgenic tobacco plants expressing FBPase and/or SBPase in chloroplasts

To clarify the contribution of the levels of FBPase and/or SBPase to the photosynthesis rate and the carbon flow in source and sink organs, we generated transgenic tobacco plants with enhanced activities of FBPase and/or SBPase in the chloroplasts.

We have previously isolated and characterized two FBPase isozymes, designated fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) and FBPase-II from cyanobacterium *Synechococcus* PCC7942 (*S.* 7942). FBP/SBPase can hydrolyze both FBP and SBP with almost equal specific activities (Tamoi et al. 1996, 1998). The deduced amino acid sequence of the FBPase-II gene has been found to be considerably similar to those of the cytosolic and chloroplastic forms from eukaryotic cells. The enzymatic properties of FBPase-II were more similar to those of chloroplastic FBPase than to the cytosolic form of FBPase in higher plants; AMP and fructose 2,6-bisphosphate had no effect on the FBPase-II activity. Furthermore, we have also isolated and characterized SBPase from halotolerant *Chlamydomonas* W80 (Tamoi et al. 2001).

We generated transgenic tobacco plants expressing cyanobacterial FBP/SBPase (TpFS), FBPase-II (TpF), or *Chlamydomonas* SBPase (TpS) in chloroplasts (Miyagawa et al. 2001; Tamoi et al. unpublished data). In TpFS-6 and TpFS-3, the total FBPase and SBPase activities derived from endogenoues plastidic enzymes and cyanobacterial FBP/SBPase was 1.7 ± 0.1- and 2.3 ± 0.4-fold higher than those in the wild-type plants, respectively. TpF-9 and TpF-11 showed 1.7- or 2.3-fold higher FBPase activities compared with those in the wild-type plants, respectively. In TpS-2, TpS-11 and TpS-10, SBPase activity was 1.3-, 1.6- and 4.3-fold higher than that in the wild-type plants.

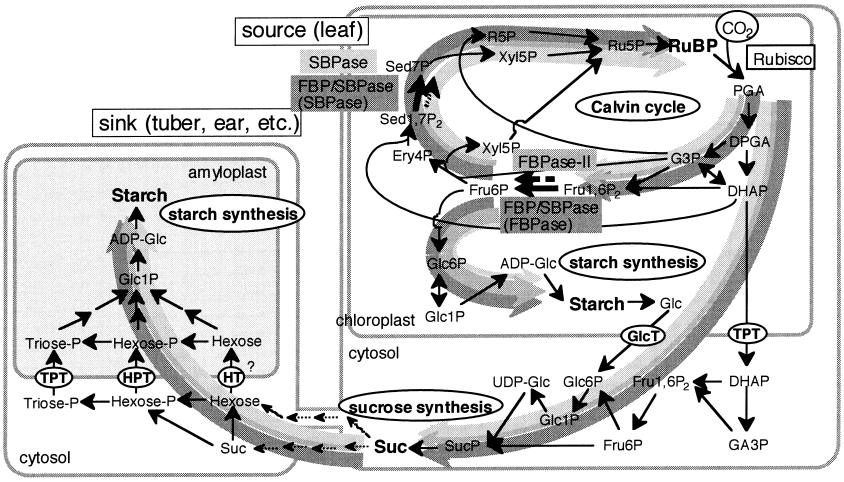

Figure 1. Carbon metabolism of source and sink organs in the wild-type and transgenic tobacco plants. The gray arrows indicated carbon flow enhanced by the introductions of FBPase-II, SBPase, and FBP/SBPase in the chloroplast.

The transgenic plants having more than 2.3-fold higher FBPase activity and/or 1.6-fold SBPase activity (TpFS-6, TpFS-3, TpF-11, TpS-11, and TpS-10) showed significantly larger body sizes (height and dry weight) and faster growth rates compared with wild-type plants grown hydroponically under atmospheric conditions (360 ppm CO_2, 400 μmol photons m^{-2} s^{-1}). Similar differences were also observed between transgenic plants and wild-type plants that were cultivated in soil. It is worth noting that the leaf size, stem thickness and root size of transformants were larger than those of the wild-type plants. In particular, the fresh weight of roots in the transformants was approximately 3-fold larger that that in wild-type plants. However, thickening of the leaf, size of cell, and structural changes of the chloroplast in the transgenic plants were not observed by either photomicroscopy or electron microscopy, respectively. These data indicated that the increased plant size (leaf area and root number) resulted from increased cell number of the transformants.

These transformants showed enhanced phothosynthetic activity (1.20- and 1.24-fold) under saturated light conditions. The *in vivo* activation state of Rubisco in the transformants was approximately 1.1–1.2-fold higher than that in wild-type plants. However, there were no differences in the total activities of Rubisco between wild-type and transgenic plants. The content of RuBP in transformants was 1.4–1.8-fold larger than that in the wild-type plants, respectively. These findings clearly indicated that the enhancement of either more than a 2.3-fold increase of FBPase and 1.6-fold increase of SBPase in the chloroplasts had a marked positive effect on the process of RuBP regeneration, resulting in an enhancement of the level of RuBP, an increase in the initial activity of Rubisco and thus an increase of the photosynthetic rate in the chloroplasts of the transgenic plants. On the other hand, no differences were observed in the photosynthetic activities among TpF-9, TpS-2 and wild-type plants. Enhancement of the 1.7-fold FBPase activity or 1.3-fold SBPase activity has no effect on RuBP regeneration and thus on the photosynthetic activity. In these transformants, the carbon flow of the Calvin cycle may be controlled by another limiting factor.

The data from metabolite analysis of transformants suggests that an increase in the chloroplastic FBPase or SBPase level correlates with an increase of the RuBP level through the regeneration of RuBP, and thus affects the photosynthetic capacity and the growth in transgenic plants, while a slight increase in the FBPase activity seems to contribute to starch synthesis rather than to RuBP regeneration in chloroplasts (Figure 1).

Raines (2003) and Lefebvre et al. (2005) have reported that in the *Arabidopsis* SBPase overexpressing tobacco plants with 10–65% increased activity, the photosynthetic rate in the young expanding leaves was 12% higher than that in the equivalent leaves on the wild-type plants, and shoot biomass was increased by 40% compared with the wild-type. Judging from our data, together with the findings reported so far, it seems likely that in the chloroplasts of higher plants, SBPase is the most important factor for RuBP regeneration in the Calvin cycle and the level of chloroplastic FBPase strictly controls the regeneration of RuBP in the Calvin cycle and the starch synthesis.

Effect of overexpression of cytosolic FBPase on photosynthetic carbon metabolisms

In order to clarify the effect of increased cytosolic FBPase activity on photosynthetic carbon metabolism, we have generated transgenic plants expressing cyanobacterial FBP/SBPase driven by the CaMV35S

promoter. Increased FBPase activity in the cytosol led to an increase in the levels of sucrose in the leaves, stems and roots during the light period, whereas the levels of starch were decreased in the leaves of transgenic plants. These effects were correlated with the increased levels of FBPase in cytosol. Consequently, the transgenic plants showed high sucrose/starch ratios compared to the wild-type plants. However, there were no differences in plant growth, dry weight and photosynthetic activity between the transgenic and wild-type plants. These results indicated that the FBPase in cytosol has a large degree on the sucrose biosynthesis pathway in higher plants. The key regulatory steps of sucrose biosynthesis are thought to be the interconversion of fructose 1,6-bisphosphate (FBP) and fructose 6-phosphtate (F6P) and the formation of sucrose-6-phosphate from UDP-glucose and F6P (Daie 1993; Huber et al. 1985; Stitt and Quick 1989). It has been reported that the overexpression of sucrose-phosphate synthase (SPS), which catalyzes the formation of sucrose-6-phosphate, caused an accumulation of sucrose and a decrease in the starch content in leaves. The overexpression of SPS in tomatoes resulted in 2- to 3-fold higher levels of sucrose in the leaves and a decrease in the starch content (Galtier et al. 1993, 1995). Similar studies with *Arabidopsis* plants showed that increasing SPS activity changes the ratio of sucrose/starch in leaves and prevents starch accumulation in leaves that had been grown with CO_2 enrichment (700 ppm) throughout their lives (Signora et al. 1998).

The impact of reduced cytosolic FBPase activity has been studied in transgenic potato plants, which showed starch accumulation in leaves during the day (Zrennner et al. 1996). A decreased expression of cytosolic FBPase in *Arabidopsis* plants led to the accumulation of phosphorylated intermediates, Pi-limitation of photosynthesis and higher rates of starch synthesis (Strand et al. 2000). From these facts, including those of our study, it is conceivable that the flux direction of carbon partitioning away from starch accumulation towards sucrose is strongly regulated by the capacity of sucrose biosynthesis in the cytosol.

Future directions

The data reported here, together with the findings reported so far, suggest that the increase in respective enzyme levels involved in the Calvin cycle and sucrose biosynthetic pathway correlates with photosynthetic capacity and carbon partitioning of source and sink organs in higher plants. It is still unclear how the balance of intermediate contents of carbon metabolisms including the Calvin cycle is regulated in higher plants. In order to answer this question, we have generated the transgenic plants with significantly increased levels of FBPase and SBPase in the Calvin cycle by a chloroplast transformation technique. The transplastomic tobacco plants with 20–30-fold higher FBPase and SBPase activities in the chloroplasts showed enhanced photosynthetic capacity and growth compared with the wild-type (unpublished data). Now we are trying to analyze the metabolic profiling of transgenic tobacco plants having significantly enhanced activities of enzymes involved in the Calvin cycle to discuss the regulation of carbon flux among various metabolic pathways, including the Calvin cycle.

The transgenic plants with improved photosynthetic capacity and growth seem to be controlled by various enzymes and metabolites involved in carbon metabolism. Accordingly, in order to identify the kinds of genes that participate in increased Rubisco activation, photosynthesis and growth of the transgenic plants, we generated FBP/SBPase-introduced transgenic *Arabidopsis*, in which the complete genome sequence has been analyzed and the microarray technology has been established. We have found that FBP/SBPase-introduced *Arabidopsis* plants showed enhanced photosynthesis and growth, in agreement with those of transgenic tobacco plants. Now, we are analyzing the upregulated and downregulated genes in the transgenic plants by comprehensive analysis of transcriptional levels by DNA microarray analysis.

In the near future, we may be faced with a food shortage as a result of the explosive increase in world population and environmental deterioration. The findings reported here suggest that it may be possible to use a cyanobacterial gene to manipulate photosynthetic carbon metabolism and improve the crop yield of various plant species. Plant productivity is also limited by various environmental stresses. Much of the injury to plants imposed by environmental stresses is associated with oxidative damage at the cellular level, including lipid hydroperoxidation leading to membrane damage and DNA damage (Shigeoka et al. 2002). Recently, we have generated the transgenic plants expressing the antioxidative enzymes, such as catalase, ascorbate peroxidase or glutathione peroxidase (Miyagawa et al. 2000; Yabuta et al. 2002; Yoshimura et al. 2004). These transgenic plants have an increased tolerance to photooxidative stress imposed by various types of environmental stress. Accordingly, it seems likely that attempting multigene transfer for the simultaneous increase in several components involved in the Calvin cycle and the active oxygen species-scavenging systems seems to be necessary to obtain a substantial increase in plant productivity.

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