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Analysis of Carbon Metabolism in Transgenic *Arabidopsis thaliana* Transformed with the Cyanobacterial Sucrose Phosphate Synthase Gene

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To investigate the effects of sucrose-phosphate synthase (SPS) on carbon partitioning, transgenic *Arabidopsis* plants transformed with *Synechocystis* SPS were constructed. The integration, copy number and expression level were confirmed by Southern and Northern blot analyses. SPS activity in leaves from the transgenic and wild type plants was not significantly different. The level of sucrose and starch in the leaves of transgenic plant were slightly decreased compared to wild type. The glucose and fructose contents were increased up to two-fold compared to wild type during the light period. It is our speculation that the decreased sucrose level of the transgenic plant might be caused by the high acid invertase.

Keywords: sucrose phosphate synthase, carbon metabolism, *Arabidopsis*, carbon metabolite, SPS activity

In most crop plants, e.g. cereals, potato, and sugar beet, carbohydrates are stored in the leaves as starch and exported as sucrose to other parts of the plants such as roots or growing seeds. Sucrose biosynthesis is an important factor for the photosynthesis, growth, and development of plants. Sucrose-phosphate synthase (SPS; EC 2.4.1.14; UDP-Glucose: fructose-phosphate glucosyltransferase) plays an important role in the regulation of photosynthetic sucrose synthesis in plants. SPS catalyses the synthesis of sucrose-6-phosphate (Suc-6-P) from uridine 5-diphosphoglucose (UDPGlc) and fructose-6-phosphate (Fru-6-P). Then, sucrose phosphate phosphatase irreversibly hydrolyzes the Suc-6-P into a sucrose and inorganic phosphate. SPS is an allosteric enzyme that is activated by a glucose-6-phosphate (Glc-6-P) and inhibited by an inorganic phosphate. In addition, the activity of SPS is regulated by covalent modification. In particular, SPS is phosphorylated or inactivated in the dark by several Ser/Thr kinases, and dephosphorylated or activated in the light by a type 2A protein phosphatase (Huber and Huber, 1996). In the light/dark modulation, spinach and maize leaf SPSs show alteration on their affinity for substrates and allosteric effectors. Over-expression of SPS in *A. thaliana* altered carbon partitioning (Signora et al., 1998) and has been reported to improve photosynthetic performance at low temperature as well as to increase freezing tolerance (Strand et al., 2001).

Plant cDNA clones encoding SPS have been isolated from maize (Worrell et al., 1991), spinach (Klein et al., 1993; Sonnewald et al., 1993), potato (EMBL accession number S34172), sugar beet (Hesse et al., 1995), *Citrus unshiu* (Komatsu et al., 1996), faba bean (Heim et al.,1996), banana (do Nascimento et al., 1997), *Craterostigma plantagineum* (Ingram et al., 1997), sugarcane (Sugiharto et al., 1997), and kiwi fruit (Langenkamper et al., 1998). In contrast to higher plants, little is known about the synthesis of sucrose in lower organisms. SPS activity has been detected in a number of unicellular, eukaryotic algae including *Chlorella vulgaris*, *Scenedesmus obliquus*, *Dunaliella tertiolecta* and *Prototheca zopfii* while appearing to resemble the higher plant enzymes in its properties (Duran and Pontis, 1977; Muller and Wegmann, 1978). The complete genome of the cyanobacterium *Synechocystis* sp. strain PCC6803 has been sequenced and found to contain a 2163 bp ORF that is very similar to the cDNA clones encoding plants SPS genes (Kaneko et al., 1996; Curatti et al., 1998; Lunn et al., 1999). The *Synechocystis* SPS has a molecular mass of 81.5 kDa, which is smaller than the typical higher plant SPS subunit, and lacks the consensus sequence of a phosphorylation site. Unlike higher plant enzymes, *Synechocystis* SPS is able to

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substitute ADP-glucose, CDP-glucose and GDP-glucose for UDP-glucose, as a glucosyl donor. In this study, *Synechocystis* SPS expressing transgenic *Arabidopsis* plants were constructed to study the roles of SPS in carbon partitioning. It should be noted that *Synecocystis* SPS is N-terminal truncated and lacks a phosphorylation site. Thus, it is meritorious to analyze the physiological function of carbon partitioning in *Arabidopsis* model system.

MATERIALS AND METHODS

Chemicals

Biochemical reagents were purchased from Sigma Chemical (USA), Promega (USA), Quiagen (UK) and Bioneer (Korea) Radio-labeled [α-^{32}P] dCTP was purchased from Amersham Biosciences (Sweden). Medium for plant growing was purchased from Duchefa (Netherland). Restriction endonucleases were purchased from KOSCHEM (Korea). Ex Taq polymerase for PCR was purchased from TaKaRa (Japan).

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia was used for all the experiments. *Arabidopsis* seeds were purchased from LEHLE SEEDS (USA). Plants were grown in a mixture of top soil, perlite and vermiculite (w/w, 2:1:1) in a growth chamber at a temperature of 23°C and 50% relative humidity.

Cell Lines and Media Composition

Escherichia coli strain JM109 was used for cloning and *Agrobacterium tumefaciens* strain GV3101 was used for *Arabidopsis* transformaion. *E. coli* and *A. tumefaciens* strain GV3101 were grown in LB medium (1% trypton, 0.5% yeast extract, 1% sodium chloride, pH 7.0) at 37°C and 28°C, respectively.

Construction of Over-Expression Vectors

Genomic DNA of *Synechocystis* sp. PCC 6803 was provided by Korea Basic Science Institute (Korea). The sll0045 (*sps*) ORF (Kaneko et al., 1996) was amplified from genomic DNA by PCR. Cyanobacterial SPS gene was cloned into pGEM™-T Easy vector (Promega). For the cloning of the cyanobacterial SPS gene, the primers of 5′-GAGCTCATGAGCTATTCATCAAAATACATTTTAC-3′ and 5′-GTCGACTTAAACGGGGTCTAACAACTCAAAGAAGC-3′ were used. The underlined SacI and SalI sites were generated to facilitate the subcloning work. The clones were confirmed by sequence analysis, and subcloned into the plant expression vector pPZP211-Ex vector. The vector pPZP211-Ex was slightly modified from the binary vector pPZP211 (Hajdukiewicz et al., 1994). The pPZP211-Ex vector was constructed by introducing the 35S promoter and NOS terminator into the pPZP211 vector. The resulting plasmid was named as pSY216 (Fig. 1).

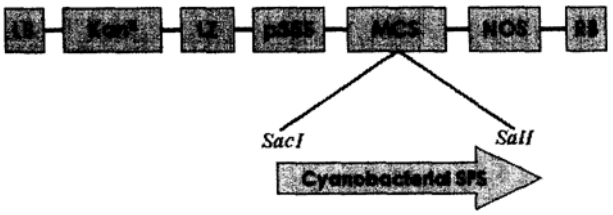

Figure 1. Construction of the cyanobacterial sucrose phosphate synthase expression vector pSY216.

Agrobacterium-Mediated Transformation

A. tumefaciens strain GV3101 carrying the constructed plasmids were used in all experiments (Clough and Bent, 1998; Lim et al., 1999). *Agrobacterium* was grown to stationary phase in LB liquid culture with streptomycin at 28°C, 250 rpm. Cultures were typically started from a 1:100 dilution of overnight cultures and grown for 18-24 h. Cells were harvested by centrifugation at 6,000 rpm for 10 min at room temperature, then resuspended in 5% sucrose and 0.05% Silwet L-77. Plants were inverted into the inoculum so that all above ground level tissues were submerged, and incubated for several seconds with gentle agitation. The dipped plants were removed from the beaker, placed in a plastic tray and covered with a tall clear-plastic dome to maintain a high humidity. Plants were under a low light intensity or darkness overnight and returned to the chamber next day.

Genomic DNA Extraction and Southern Blot Analysis

Southern blot was conducted to confirm a stable integration of the foreign DNA into chromosome of the recipient plants. Genomic DNA from *Arabidopsis* leaf tissue was isolated, digested for 8 hr with EcoRI and electrophoretically separated on 1% agarose gels. The DNA was subsequently blotted onto a Hybond-N$^+$ membrane (Amersham Biosciences) using 0.4 N NaOH as a transfer buffer. Specific probe was labeled with [α-^{32}P] dCTP using random priming (Rediprime II Ran-

dom Prime Labeling System, Amersham Biosciences) and hybridized for 16 hr in a hybridization solution containing 1% (w/v) BSA, 7% (w/v) SDS, 1M sodium phosphate (pH 7.2) and 0.5 M EDTA at 65°C. The membrane was washed three times with 0.2×SSC and 0.1% (w/v) SDS for 15 min each at 65°C and exposed onto a phosphorimage plate.

RNA Extraction and Northern Blot Analysis

Total RNA was prepared from *Arabidopsis* leaves by extraction with TRIzol reagent (Invitrogen, Netherlands). Total RNA (40 μg per lane) was denatured, electrophoresed on an agarose/formaldehyde gel and transferred onto a Hybond-N^+ membrane (Amersham Biosciences) using 25 mM sodium phosphate as transfer buffer. Cyanobacterial specific probe was prepared by random priming and hybridized for 16 hr with a ^{32}P-labelled probe in a hybridization solution containing 1% (w/v) BSA, 7% (w/v) SDS, 1 M sodium phosphate (pH 7.2) and 0.5 M EDTA at 65°C. The membrane was washed three times with 0.2× SSC and 0.1% (w/v) SDS for 15 min each at 65°C and exposed onto a phosphorimage plate.

SPS Activity Assay

SPS activity was assayed as described by Ono et al. (1999). One hundred mg of samples were ground with a mortar and pestle and extracted with 800 μl of ice-cold buffer [50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid, 5 mM dithiothreitol, 1 mM phenylmethylslufonylfluoride, 5 mM ε-aminocaproic acid, 0.5% (v/v) bovine serum albumin, 0.1% (v/v) Triton X-100, 2% (w/v) polyvinylpyrrolidone]. After centrifugation at 10,000*g* for 5 min, the supernatant was desalted by passage through a column of Sephadex G-25. SPS activity was assayed by measuring the Fru-6-P dependent production of UDP from UDP-glucose (Lunn and ap Rees, 1990).

Metabolite Measurements

Starch and soluble sugar contents of *Arabidopsis* leaves were assayed according to the enzymatic method described by Stitt et al. (1989). *Arabidopsis* leaves (0.1-0.2 g) were ground with liquid nitrogen to fine powder and then extracted with 10% perchloric acid. Insoluble solids in neutralized perchloric acid extracts were separated by centrifugation at 20000*g* for 5 min. The sediment was used for determination of starch contents and the aqueous phase for soluble sugars and metabolic intermediates.

RESULTS

Southern Blot Analysis of Transgenic *Arabidopsis*

The genomic DNAs were isolated from the transgenic and wild type *Arabidopsis*. To determine the transgene copy number, Southern hybridization was performed on homozygous T3 lines. Genomic DNAs were digested with EcoRI and hybridized with 780 bp of cyanobacterial SPS as specific probes. Two single copy transgenic lines were confirmed in 2 and 3 lanes (SPS-2 and SPS-3) (Fig. 2).

Analysis of Cyanobacterial SPS Expression in Transgenic *Arabidopsis*

Northern hybridization was conducted to analyze the target protein expression at transcription level. Total RNA was isolated from leaves of transgenic and wild type

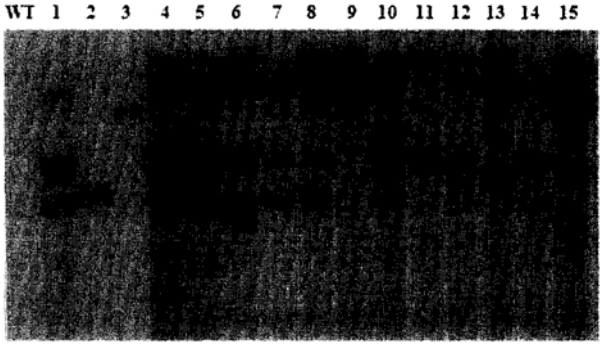

Figure 2. Southern blot analysis of transgenic plants expressing cyanobacterial SPS.

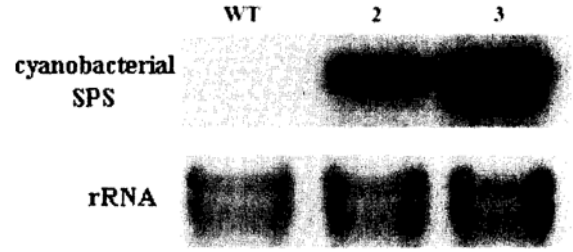

Figure 3. Northern blot analysis of transgenic plants expressing cyanobacterial SPS. Forty μg of total RNA isolated from leaves was blotted onto a nylon membrane and hybridized to the [α-^{32}P] labeled cyanobacterial SPS probe. An equal amount of total RNA was electrophoresed and stained with ethidium bromide as a reference.

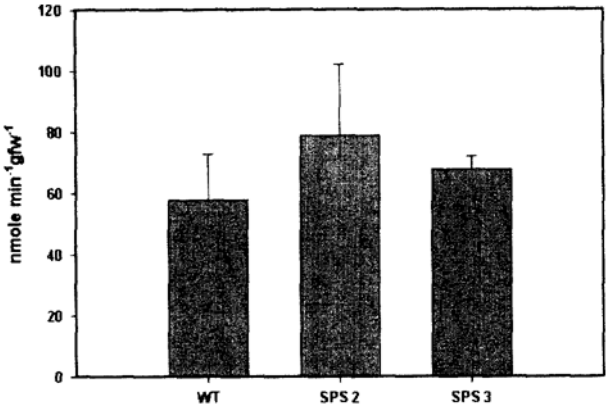

Figure 4. SPS activity in transgenic plants expressing cyanobactrial SPS. The results are given as means ± SE (n = 3 separate plants). WT is wild type, SPS 2 and SPS 3 are the transgenic plants of cyanobacterial SPS.

Arabidopsis at the same developmental stages. Forty µg of the RNA was electrophoresed through RNA running gel and hybridized against a random primed radiolabeled probe generated from cyanobacterial SPS gene. Expression of cyanobacterial SPS was confirmed in two single copy lines, SPS-2 and SPS-3 (Fig. 3).

Activity of Cyanobacterial SPS

SPS activity of the transgenic plant was assayed. For the SPS activity assay, growth conditions were maintained equal for the wild type and transgenic plants. The wild type plant had SPS activity of 57 nmoles·min^{-1}·gFW^{-1}. The SPS activities of cyanobacterial SPS-2 and SPS-3 transgenic plants were 79 and 66 nmoles·min^{-1}·gFW^{-1}, respectively. SPS activity of the transformed plant was slightly higher compared to the wild type (Fig. 4).

Carbon Metabolite Measurements of Cyanobacterial SPS Transgenic *Arabidopsis*

In case of the transgenic *Arabidopsis* line SPS-2, the levels of glucose and fructose were similar compared to the wild type at the end of the day (Fig. 5A and B). However, there were unexpected alterations of glucose and fructose contents during the diurnal change. At the end of the night, the levels of glucose and fructose drastically decreased in the transgenic *Arabidopsis*. Then, after a few hours of photoperiod, the levels of glucose and fructose increased up to two-fold compared to the wild type. However, the levels of sucrose and starch of the transgenic *Arabidopsis* were slightly lower than that of the wild type. The patterns of sucrose and

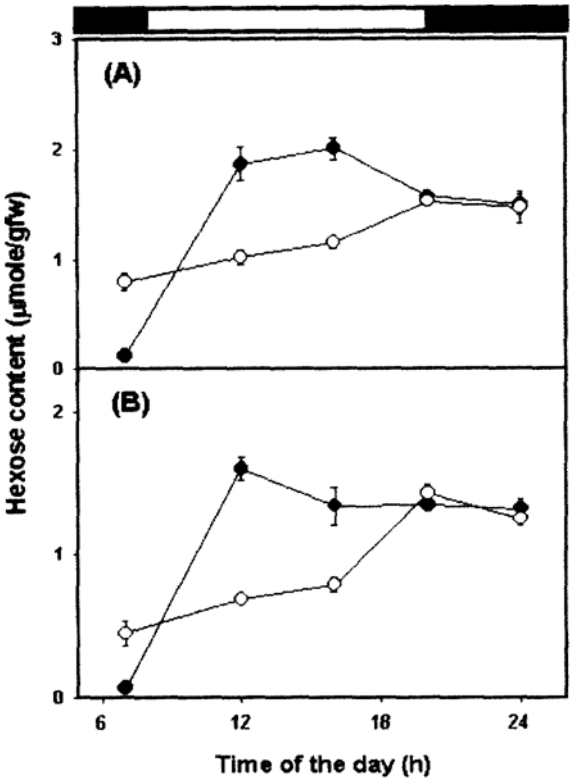

Figure 5. Determination of glucose and fructose contents in the wild type plant and the transgenic plant of cyanobacterial SPS during diurnal light/dark cycle (12 h light/12 h dark). Black bars on the top indicate the dark period. The results are given as means ± SE (n = 5 separate plants). (A) Glucose, (B) Fructose, (●) transgenic plant of cyanobacterial SPS, (○) wild type.

starch contents between the wild type and the transgenic *Arabidopsis* were very similar during diurnal change (Fig. 6A and B).

DISCUSSION

The cyanobacterium *Synechocystis* SPS appears to have few regulatory properties, in particular, the allosteric effectors of the higher plant enzyme. Glc-6-P and Pi have little or no effect on activity and their binding sites are unknown. There are several regions in the higher plant SPS sequences that are missing from the *Synechocystis* SPS, including the N-terminus and the phosphorylation site motifs. They are associated with light/dark- and osmotic stress-induced regulation in most higher plant SPS. These differences are reflected in the lower molecular mass of the *Synechocystis* enzyme (81.5 kDa) as compared to the higher plant enzyme (117-119 kDa) (Lunn et al., 2003).

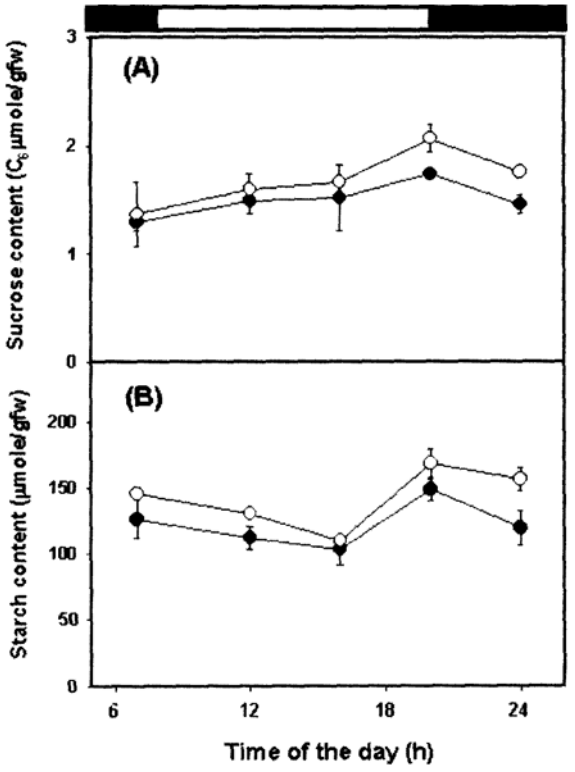

Figure 6. Determination of sucrose and starch contents in the wild type plant and the transgenic plant of cyanobacterial SPS during diurnal light/dark cycle (12 h light/12 h dark). Black bars on the top indicate the dark period. The results are given as means ± SE (n = 5 separate plants). (A) Sucose, (B) Starch, (●) transgenic plant of cyanobacterial SPS, (○) wild type.

The cyanobacterial SPS gene was cloned from the genome of cyanobacterium *Synechocystis* sp. strain PCC6803 to produce a transgenic *Arabidopsis* in order to study its effects on carbon partitioning. However, our study showed that SPS activity of the transformed *Arabidopsis* with cyanobacterial SPS was slightly higher than the wild type. It has been reported that when maize SPS was overexpressed in tomato and *Arabidopsis*, SPS activity increased more than two folds (Signora et al., 1998; Lunn et al., 2003; Laporte et al., 2001). However, in respect to carbon partitioning, soluble sugar and starch in the leaves of the transgenic *Arabidopsis* were similar or slightly increased compared to wild type (Signora et al., 1998). In the case of tomato, soluble sugar and starch in the leaves were decreased (Lunn et al., 2003). Laporte (2001) reported that the fruit, stem and leaf fresh mass of transgenic tomato increased. Increased expression or activity of SPS could be expected to alter carbon partitioning in source organs. Especially, sucrose synthesis or related photosynthetic performance could increase. Lunn (2003) reported that soluble sugar and starch content were reduced slightly in the leaves of transgenic tomato overexpressing cyanobacterial SPS from *Synechocystis* sp. PCC6803. In our case, sucrose and starch contents were slightly reduced but the levels of glucose and fructose increased two-fold in the transgenic plants. This could be explained by the fact that the high acid invertase activity present in tomato leaves (Gao et al., 1998) would probably limit the amount of sucrose accumulation, even if more sucrose were synthesized (Winter and Huber, 2000). In addition, even though active *Synechocystis* SPS was expressed in the transgenic plants, some putative inhibitory proteins or proteolytic cleavage of the enzyme could exist to give a truncated form.

In this study, the contents of glucose and fructose in the leaves of the transgenic *Arabidopsis* expressing cyanobacterial SPS showed two-fold increase compared to wild type during the day time. However, the levels of sucrose and starch, which are the final photosynthetic product in source organs, were similar between the wild type and the transgenic *Arabidopsis*. We speculate that the high concentrations of glucose and fructose in transgenic plants might be caused by the high acid invertase activity (Gao et al., 1998).

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