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Regulation of starch granule-bound starch synthase I gene expression by circadian clock and sucrose in the source tissue of sweet potato

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Abstract

Studies on regulations of transitory starch synthesis and degradation in the leaf tissue are important for understanding how carbons could be distributed effectively from the source to sink tissues. Therefore, expressions of starch granule-bound starch synthase I (*GBSSI*) gene in leaves of sweet potato were studied under different photoperiodic conditions and various sugar treatments. Results indicated that accumulations of *GBSSI* mRNA and its protein were controlled by an endogenous biological clock. Starch accumulations in leaves also showed a pattern characteristic of circadian rhythm. In addition to circadian clock, sucrose also played an important role in regulating *GBSSI* mRNA accumulations. Although sucrose stimulated the transcription of *GBSSI*, it had no effect on the rhythmic pattern of *GBSSI* gene expressions. Protein phosphorylation/dephosphorylation were involved in the sucrose-related signal transduction for *GBSSI* gene expressions. However, the sugar sensing for regulation of *GBSSI* was independent of the hexokinase-mediated pathway. In conclusion, the *GBSSI* gene expression in leaves of sweet potato appears to be regulated by two independent pathways. First, light is responsible for setting up biological clock(s) that control the circadian expression of *GBSSI* gene; and second, light plays an indirect signal to enhance *GBSSI* mRNA accumulations mediated by the photosynthetic product, sucrose.

Keywords: Circadian rhythm; Granule-bound starch synthase I; Ipomoea batatas; Sink–source; Sucrose effect

1. Introduction

Starch granule-bound starch synthase I (GBSSI), also known as the WAXY protein, has been shown to be an important enzyme for the synthesis of amylose in the sink tissue [1–3]. However, expressions of this gene relative to starch metabolism in the source tissue remain unclear. We have isolated a *GBSSI* gene from sweet potato (*Ipomoea batatas*), and this gene was well expressed in tuberous roots, leaves, and stems. In tuberous roots, a sink tissue where accumulations of *GBSSI* mRNA increased along with developmental stages. On the other hand, mRNA levels in leaves fluctuated and exhibited a circadian rhythm during a 16-h light/8-h dark (16 L/8 D) photoperiod [4]. The circadian expression of *GBSSI* gene was also observed in snapdragon [5].

Circadian rhythm is defined as a phenomenon controlled by endogenous clock(s), and the free-running period of the clock is about 24 h [6]. Light is considered as an input signal that may set up circadian clocks, and the central clock regulates gene expressions through various signal transduction pathways [7]. Most of the circadian clock-controlled genes identified are involved in photosynthesis, carbon and nitrogen metabolisms. Periodic mRNA fluctuations of chlorophyll a/b binding protein (*Cab*) gene have been observed in various plant species [8,9]. In the area of carbon metabolism, expressions of ribulose-1,5-bisphosphate carboxylase/oxygenase (*RuBisCO*) gene have also been shown to exhibit circadian rhythms [10]. Nitrate and nitrite reductases are two major enzymes involved in the nitrogen metabolic pathway, and their expressions are controlled by biological clocks as well [11]. Furthermore, several

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genes that are not related to carbon and nitrogen metabolisms, e.g. catalase genes, were also observed to be under circadian controls [12].

In addition to the effect on gene expressions, endogenous circadian clocks have been shown to affect starch accumulation in several plant species. When sugar beet was grown under a 14-h photoperiod, accumulation of starch in leaves reached its maximum approximately 4 h after the beginning of illumination, and maintained steady until 2 h before the end of the light period. The pattern of starch accumulations was similar even though irradiance continued to sustain photosynthesis [13]. Although mechanisms controlling the circadian pattern of starch accumulation are still unclear, enzymes involved in the synthetic pathway were suggested as regulators [13]. A similar observation was made in *Snapdragon* [5].

While light may function as a signal to set up biological clocks, sugars also play an important role in controlling gene expressions related to photosynthesis and carbohydrate metabolism in plants [14]. For carbons metabolism, genes related to starch synthesis, such as ADP-glucose pyrophosphorylase, *GBSSI* and branching enzyme have been observed to be regulated by sugars [15–18]. Although sugar effects on gene regulations were observed in many plant systems, the definition of sugar signals is still not well established. Certain effects of sucrose could be substituted by glucose or fructose, suggesting that sucrose was not a direct signal [19]. However, sucrose could serve as a signal molecule to regulate gene expressions mediated by a specific sensor and signal transduction pathway [20].

Three different sugar-sensing systems have been postulated in plants [21,22]. First, hexokinase serves as an intracellular sensor in a way similar to that of the yeast system. Second, hexose-transporter functions as a sugar sensor located on plasma membrane. Third, sucrose-transporter plays a major role in sugar sensing. Several studies suggest that certain cascades of sugar-related signal transductions in higher plants might be similar to those of the yeast, and mediated by protein phosphorylation/dephosphorylation processes [22]. For example, protein phosphatase inhibitors strongly inhibited sucrose-inducible accumulations of the small subunit of ADP-glucose pyrophosphorylase mRNA in petioles of sweet potato [16].

Because sucrose is a major photosynthetic product and a precursor for starch synthesis, effects of light and sucrose on *GBSSI* gene expressions were studied in leaves of sweet potato. Furthermore, the accumulation patterns of *GBSSI* mRNA and protein were compared with the profile of starch accumulation. It is anticipated that these studies should provide information for a better understanding on regulatory mechanisms of *GBSSI* expression in the source tissue as well as carbon distribution from the source to sink tissues.

2. Materials and methods

2.1. Plant materials and treatments

Sweet potato (*Pomoea batatas* Lam. cv. Tainong 57) was grown at 28 °C in a growth chamber under 16 L (06:00–22:00 h)/8 D (22:00–06:00 h). For treatments with different photoperiods, the 16 L/8 D entrained plants were moved to separate growth chambers with 24-h light (LL), 24-h dark (DD) or other photoperiods. Leaf samples were harvested at time intervals indicated. Before treatments with various sugars and chemicals, leaf-petioles (10 cm) were cut from plants (about 20 cm in height) and cultured in H_2O for 1 day under a dark condition (DD) to deplete endogenous sugars. These cuttings were then transferred and incubated in 175 mM of sugar solutions (or 6% of sucrose solution) [16] while maintaining in a dark growth chamber at 28 °C. Since the maximum level of *GBSSI* gene expressions resulted from sucrose treatment was observed at 8 h (unpublished data), leaf samples were harvested at 8 h after sugar treatments. In order to evaluate effects of various inhibitors of photosynthesis, protein kinases and phosphatases on the sucrose-stimulated *GBSSI* expressions, leaf-petiole cuttings were pretreated with various inhibitors for 1 h before the sucrose treatment. Again, leaf samples were harvested at 8 h after sucrose treatment. Inhibitors were not removed during the 8-h period of sucrose treatment. All experiments were repeated at least twice using H_2O-cultured leaf-petiole cuttings as controls.

2.2. RNA extraction from sweet potato leaves

RNA was isolated from sweet potato leaves according to the procedure described by Yeh et al. [23]. Leaf sample (0.2 g) was ground in liquid nitrogen and the powder was mixed with 2 ml of extraction buffer (7.5 M guanidine hydrochloride, 25 mM sodium citrate, pH5.2, 0.5% [w/v] lauroyl sarcosine and 0.1 M β-mercaptoethanol). The mixture was then incubated at room temperature for 10 min before centrifugation at $12{,}000 \times g$ for 10 min. The supernatant was treated in succession with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and chloroform:isoamyl alcohol (24:1, v/v). RNA was precipitated with 5 ml of 100% ethanol and 0.2 ml of 3 M sodium acetate, pH 5.2, at −70 °C for 30 min. After centrifugation, the pellet was dissolved in 0.5 ml H_2O, and treated again with phenol:chloroform: isoamyl alcohol (25:24:1, v/v/v) and chloroform: isoamyl alcohol (24:1, v/v) before precipitation. Finally, the RNA pellet was dissolved in 100 μl H_2O.

2.3. Analysis by northern blotting

Total RNAs (10 μg) were separated on 1% formaldehyde-agarose gels [24]. The *GBSSI* cDNA probe [4] was radioactively labeled with α-^{32}P-dCTP using a random primer labeling kit (Amersham, UK). After hybridization, the membranes were washed twice with 2 × SSC (1 l of 20 × SSC stock solution contained 175.3 g of NaCl and 88.2 g of sodium citrate, pH 7.0) containing 0.1% (w/v) SDS at room temperature for 30 min and twice with 0.1 × SSC containing 0.1% (w/v) SDS at 55 °C for 30 min [24]. EtBr-stained 28S rRNA patterns were used as internal standards in all northern blot analysis data. Accumulation of *GBSSI* mRNA were quantified from the northern blot using Nobel ABC-Tiger Gel Documentation and Analysis System version 2.0 (Taigen, Taiwan). Relative levels were based on the measurement determined for the sample of leaf-petiole cuttings that were cultured in H_2O (100%).

2.4. Isolation and immunoblotting of starch granule-bound proteins

Leaves (5 g) were powdered in liquid nitrogen and subsequently ground with 25 ml of extraction buffer (50 mM Tris–HCl, pH 7.4, 2 mM EDTA and 1 mM DTT). The sample mixture was then filtered through two layers of Micracloth (Calbiochem, La Jolla, CA) before centrifuging at 5000 × *g* for 5 min. The pellet was resuspended with 0.5 ml of gradient buffer (50 mM Tris–HCl, pH 7.4, 2 mM EDTA, 1 mM DTT and 350 mM sorbitol), and loaded onto a discontinuous Percoll density gradient (80, 60 and 40%; 80% Percoll solution contained 80% [v/v] Percoll, 0.6 mM glutathione [reduced form, GSH], 2.4% [w/v] polyethylene glycol [M_W 4000], 0.8% [w/v] BSA, 0.8% [w/v] Ficoll, 20% [v/v] 5 × gradient buffer). After centrifuging at 7000 × *g* for 15 min, the starch granule pellet was washed by cold acetone and then air-dried. Starch granule-bound proteins were extracted from starch granules by SDS-sample buffer [25]. Proteins were separated on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK) for the western blot analysis using an antibody raised against the potato GBSSI (kindly provided by Dr R. Visser).

2.5. Starch measurements

A leaf sample (0.5 g) was ground in liquid nitrogen, then the powdered sample was washed by shaking with 70% ethanol at room temperature. After washing, the sample was centrifuged at 13,000 × *g*, and the pellet was analyzed for starch by the test-combination for carbohydrate (Boehringer Mannheim, Germany) according to the instruction. All measurements were repeated three times using different leaf samples, and their mean values were presented.

3. Results

3.1. Effect of light on circadian rhythm of GBSSI expressions

In order to evaluate whether or not the biological clock was set by light, some plants maintained under 16 L/8 D with light turning on at 06:00 h were moved to a separate growth chamber under the same photoperiod but with the light turning on at 18:00 h for a period of 14-days adaptation. Northern blot analysis showed that the resetting of time for light–dark transition did cause a shift in the accumulation peak of *GBSSI* mRNA. For the control, when light was turned on at 06:00 h, the peak of *GBSSI* mRNA accumulation was observed at 10:00 h, then the level of mRNA decreased gradually until almost completely disappeared at 02:00 h. However, the transcript reappeared at end of the photoperiod (Fig. 1A). When the time of light–dark transition was reset with light turning on at 18:00 h, the rhythmic pattern of *GBSSI* mRNA accumulations was similar to that of the control, but the peak of accumulation appeared at 22:00 h (Fig. 1B). However, in either case, the peak of

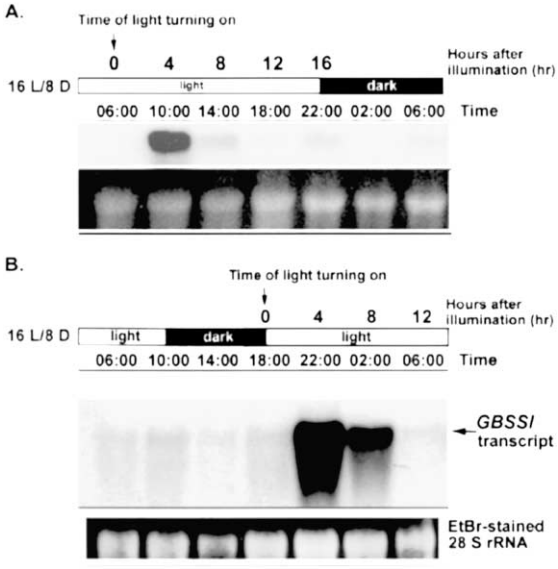

Fig. 1. Effects of resetting the time of light-dark transition on *GBSSI* gene expressions during a diurnal cycle. (A) Plants were maintained in a growth chamber under 16 L/8 D with light turning on at 06:00 h. Leaves were harvested at 4-h intervals. (B) Some plants originally maintained in the condition A were moved to a separate chamber under 16 L/8 D, but the time of light turning on was shifted from the regular 06:00–18:00 h and these plants were entrained for 14 days before leaves were harvested at 4-h intervals. Total RNAs (15 μg per lane) were probed with the α-^{32}P labeled *GBSSI* cDNA.

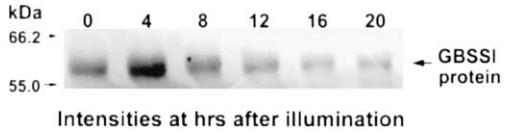

Fig. 2. Accumulation patterns of GBSSI protein during a diurnal cycle in leaves under 16 L/8 D. Granule-bound proteins were isolated from starch granules (5 mg) of leaves that were harvested at 4-h intervals. Proteins were separated on a 7.5% SDS-PAGE gel and immunoblotted with antiserum against the GBSSI of potato.

GBSSI mRNA accumulations always appeared in 4 h after the light was turned on.

3.2. Changes in accumulation patterns of GBSSI protein and starch in leaves during diurnal cycles

Under the 16 L/8 D photoperiod (light was turned on at 06:00 h), changes in GBSSI protein levels were observed clearly in the leaf tissue (Fig. 2). The peak of GBSSI protein accumulation was observed in 4 h after the light was turned on, then the level of protein decreased gradually. Similarly, the amount of starch accumulated rapidly after illumination and decreased during the dark period (Fig. 3A). When the light period was prolonged to 24 h in a separated experiment, the pattern of starch accumulation was also similar to that of the control (Fig. 3B). The amount of starch still declined at the usual dark period, even though the illumination was continued. Under the DD environment, starch content also increased in 4 h and decreased quickly until the end of the cycle, and the level of starch in this condition was lower than that of the 16 L/8 D or LL (Fig. 3C).

3.3. Effects of sugars on GBSSI gene expressions

When leaf-petiole cuttings were treated with 175 mM sorbitol or sucrose, only sucrose produced a significant stimulation in the accumulation of *GBSSI* transcripts based on the northern blot analysis (Fig. 4A, D). Since 8 h-treatments with sorbitol could not cause an increase in *GBSSI* mRNA accumulation, the sucrose-stimulated expression of *GBSSI* transcripts was not a result of osmotic effect. In order to study whether hydrolysis of sucrose is required for the stimulation of *GBSSI* expression, leaf-petiole cuttings were cultured in 175 mM glucose, fructose, or their combination. The result showed that glucose or fructose alone was ineffective in stimulating the accumulation of *GBSSI* mRNA (Fig. 4B, D). However, the accumulation was enhanced by a combination of glucose and fructose at a concentration of 87.5 mM for each hexose (Fig. 4B, D). A treatment of cuttings with glucosamine, an inhibitor for hexokinase, did not reduce the accumulation of *GBSSI* transcripts (Fig. 4C, D). On the contrary, glucosamine produced an effect stronger then that of the sucrose treatment, and both sucrose and glucosamine might have an additive effect in stimulating the expression of *GBSSI* gene (Fig. 4C, D).

3.4. Effects of inhibitors for protein kinases and phosphatases on the sucrose-stimulated GBSSI expressions

Fig. 5A and C shows that a pretreatment for 1 h with 50 μM or 100 μM cycloheximide (CHX) had no effect on the expression of *GBSSI* gene stimulated by sucrose. Previous experiments indicated that leaf-petiole cuttings pretreated with 50 μM CHX for 1 h was effective to inhibit de novo synthesis of proteins (unpublished

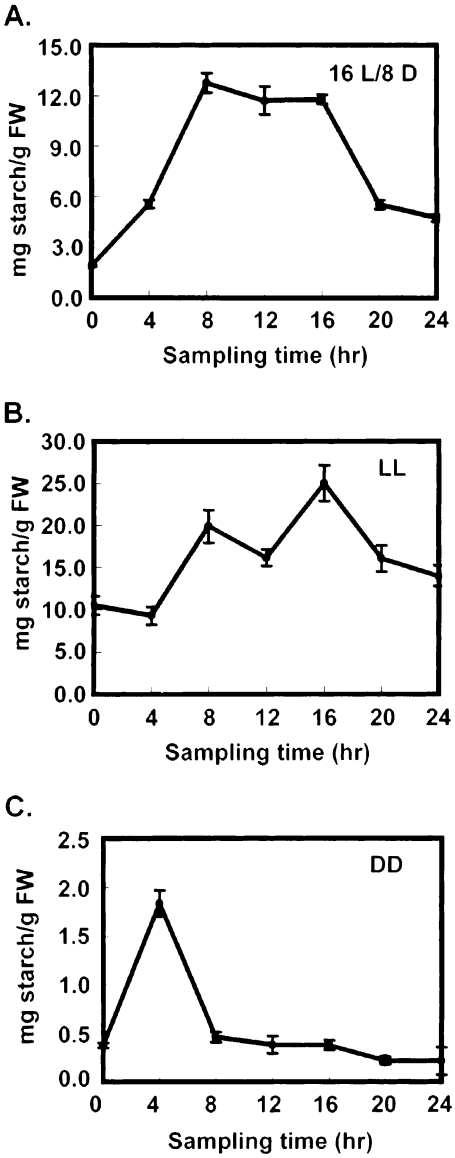

Fig. 3. Patterns of starch accumulation in leaves treated with different photoperiods. Starch contents were determined under 16 L/8 D (A), LL (B) and DD (C) conditions.

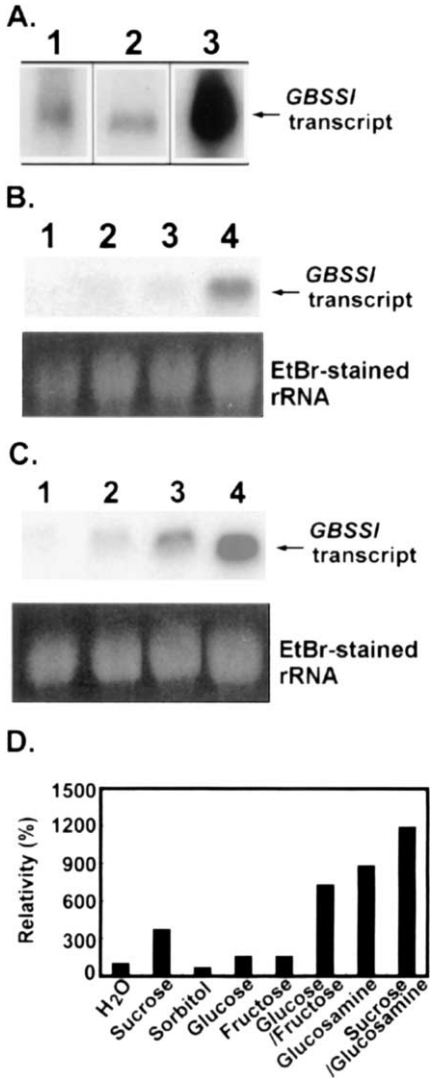

Fig. 4. Effects of various sugars and hexokinase inhibitor on *GBSSI* expressions. Leaf-petioles were cultured with H_2O in DD for 1 day before treating with 175 mM of sugar solutions. After treatments for 8 h, leaves were harvested and RNA extracted. *GBSSI* mRNA was determined by the northern blot hybridization. (A) Lane 1, H_2O; lane 2, 175 mM sorbitol; lane 3, 175 mM sucrose. (B) Lane 1, H_2O; lane 2, 175 mM glucose; lane 3, 175 mM fructose; lane 4, a combination of 87.5 mM glucose and 87.5 mM fructose. (C) Effect of hexokinase inhibitor on the sucrose-stimulated *GBSSI* expression. Lane 1, leaf-petiole cuttings were kept in H_2O as a control. Lane 2, cuttings were cultured in 175 mM sucrose solution. Lane 3, cuttings were treated with 175 mM glucosamine. Lane 4, leaf-petiole cuttings were pre-treated with 175 mM of glucosamine for 1 h before 175 mM of sucrose was added into the solution and cultured for 8 h. D. Data in (A), (B) and (C) were quantified by a densitometer. Relative levels were based on the measurement determined for the H_2O-cultured leaf-petiole cuttings (100%).

data). Therefore, the lack of effect on the sucrose-stimulated *GBSSI* expression was not the result of inability to take up CHX in the cutting. In order to analyze whether protein dephosphorylation was involved in the signal transduction pathway of sucrose-stimulated *GBSSI* expressions, effects of protein phosphatase 1 and 2A inhibitors were examined. Results showed that the sucrose-stimulated expression of *GBSSI* was not inhibited by cantharidin, but was completely inhibited by okadaic acid (Fig. 5B, C). This inhibitory effect of okadaic acid was not attributable to DMSO, the solvent used for cantharidin and okadaic acid, since

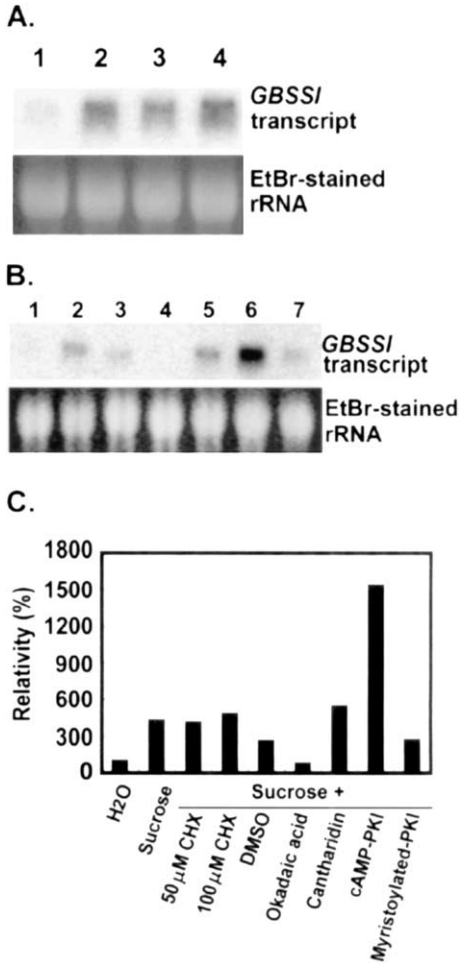

Fig. 5. Northern blot analysis of the sucrose-stimulated *GBSSI* expressions following treatments with cycloheximide (CHX), protein kinase or phosphatase inhibitors. (A) Effect of CHX on the sucrose-stimulated *GBSSI* gene expressions. Lane 1, leaf-petiole cuttings were maintained in H_2O as a control. Lane 2, cuttings were treated with 175 mM sucrose for 8 h. Lane 3, leaf-petiole cuttings were pretreated for 1 h with 50 μM of CHX before 175 mM of sucrose was added. Lane 4, leaf-petiole cuttings were pretreated for 1 h with 100 μM of CHX before 175 mM of sucrose was added. (B) Leaf-petiole cuttings were pretreated for 1 h with protein phosphatase inhibitors or protein kinase inhibitors (PKI) before 175 mM of sucrose was added into cultured solutions. Lane 1, H_2O; lane 2, 175 mM sucrose only; lane 3, 0.1% (v/v) DMSO/175 mM sucrose; lane 4, 1 μM okadaic acid/175 mM sucrose; lane 5, 1 μM cantharidin/175 mM sucrose; lane 6, 1 μM cAMP-dependent PKI/175 mM sucrose; lane 7, 1 μM myristoylated-dependent PKI/175 mM sucrose. RNAs were extracted from leaves after 8 h of treatments with sucrose and different chemicals. *GBSSI* mRNA was determined by the northern blot hybridization. (C) Data in (A) and (B) were quantified by a densitometer. Relative levels were based on the measurement determined for the H_2O-cultured leaf-petiole cuttings (100%).

DMSO alone had only a slight effect on the sucrose-stimulated expression of *GBSSI* (Fig. 5B, C). In addition, protein phosphorylation might also be involved in this signal transduction pathway. Although myristoylated protein kinase C inhibitor produced a slight inhibitory effect on the sucrose-stimulated *GBSSI* expression, cAMP-dependent protein kinase inhibitor enhanced the expression (Fig. 5B, C).

3.5. Effect of sucrose on the circadian expression of GBSSI gene

In order to determine whether or not sucrose was involved in the signal transduction pathway for the circadian expression of *GBSSI*, the following experiments with sucrose treatments were carried out under 16 L/8 D. First, all leaf-petiole cuttings were cultured in H_2O under 16 L/8 D for 7 days, then leaf-petiole cuttings were moved to 175 mM (6%) sucrose solution at the beginning of a dark cycle on the 7th day until all samples were harvested at indicated time points on the 8th day (see also Fig. 6B). Accumulations of *GBSSI* mRNA were enhanced at all sampling times and the circadian rhythm was also maintained (Fig. 6B). In a second experiment to further evaluate the sucrose effect, leaf-petiole cuttings were also transferred to sucrose solution, but only for 8 h. Subsequently, these cuttings were moved back to H_2O at the beginning of 8th day before leaves were harvested at indicated time points. Results indicated that the removal of sucrose produced no effect on the circadian rhythm of *GBSSI* mRNA accumulations; however, the sucrose-enhanced effect on transcription was reduced gradually (Fig. 6C).

3.6. Effects of photosynthesis on GBSSI gene expressions

In order to minimize the effect of endogenous sucrose, H_2O-cultured leaf-petiole cuttings were moved to DD on the 8th day to deplete endogenous sucrose after 16 L/8 D entraining for 7 days. Then, leaf-petiole cuttings were transferred to a sucrose solution at the beginning of 9th day while maintaining under DD condition. Leaf samples were harvested after sucrose treatment for 4 h (10:00 h) and 12 h (18:00 h), respectively (Fig. 7A). Northern blot analysis revealed a similar pattern to that was observed under 16 L/8 D, indicating that *GBSSI* mRNA accumulations were enhanced by sucrose, but sucrose had no effect on the rhythmic pattern. When leaf-petiole cuttings were kept under DD condition, the expression of *GBSSI* was very weak (Fig. 7B, lane 1); however, this dark effect could be reversed by illumination and resulted in a large accumulations of *GBSSI* transcripts (Fig. 7B, lane 2). When leaf-petiole cuttings were pretreated under DD for 1 h with the 3-(3,4-dichlorophenyl)-1,1-dimethy-

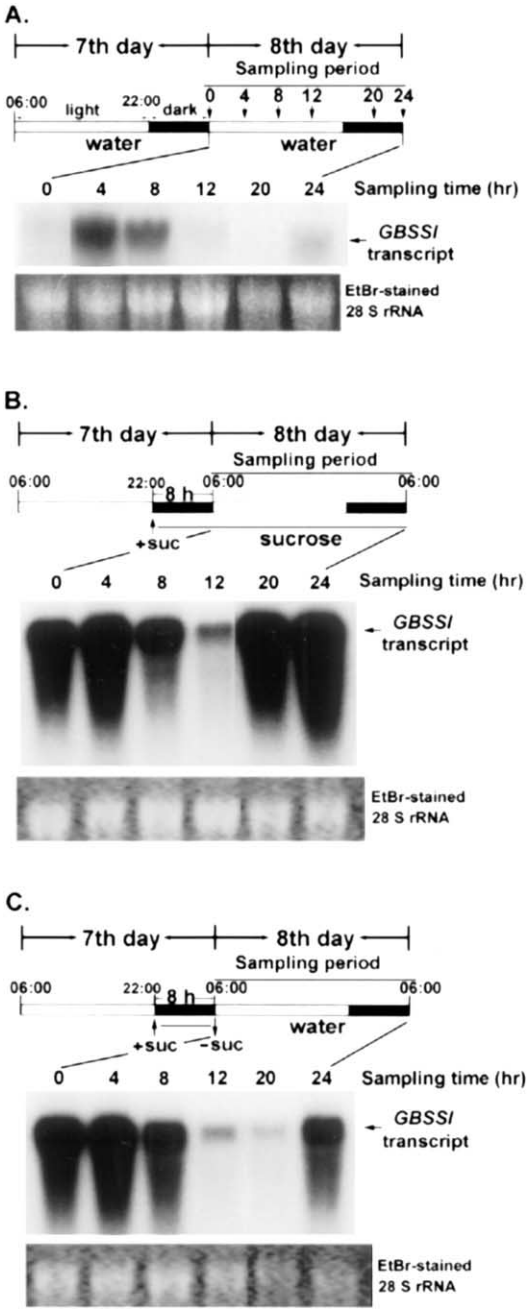

Fig. 6. Effects of sucrose on *GBSSI* gene circadian expressions under 16 L/8 D condition. (A) 16 L/8 D-entrained leaf-petiole cuttings were incubated in H_2O for 8 days as a control, and leaves were harvested at the indicated time points on the 8th day. (B) 16 L/8 D-entrained leaf-petiole cuttings were incubated in H_2O for 7 days and then transferred to 175 mM (6%) sucrose solution at the beginning of dark period on the 7th day before leaf samples were harvested on the 8th day. During the entire harvesting period, leaf-petiole cuttings were kept in the sucrose solution. (C). Leaf-petiole cuttings were treated in a fashion identical to the condition described under (B), except that these cuttings were transferred back to H_2O on the 8th day at 06:00 h, i.e. after 8 h of sucrose-treatment during the night of 7th day. Total RNAs (5 μg for each sample) were separated on a 1% formaldehyde-agarose gel, and *GBSSI* mRNA was detected using the α-^{32}P labeled *GBSSI* cDNA probe.

lurea (DCMU), an inhibitor for the transport of photosynthetic electrons, before these cuttings were illuminated for 8 h, accumulations of *GBSSI* mRNA were reduced (Fig. 7B, lane 3). When the DCMU treatment was kept continuously for 3 days under a continuous light condition, the expression of *GBSSI* was similar to that observed in leaves treated under the DD condition (Data not shown).

When leaf-petiole cuttings were entrained in 16 L/8 D condition (light was turned on at 06:00 h and turned off at 22:00 h) for 7 days, the expression of *GBSSI* showed a pattern typical of circadian fluctuation. The peak of *GBSSI* mRNA accumulation was observed at 4 h (10:00 h) after the light was turned on, and the level of *GBSSI* transcripts was greatly decreased at 12 h (18:00 h) (Fig. 7C, lanes 1–3). In a similar experiment to demonstrate that DCMU had no effect on the circadian rhythm, some leaf-petiole cuttings were moved to DD for 1 day to deplete endogenous sugars, and then these cutting were pretreated with DCMU for 1 h before transferring back to the 16 L/8 D condition.

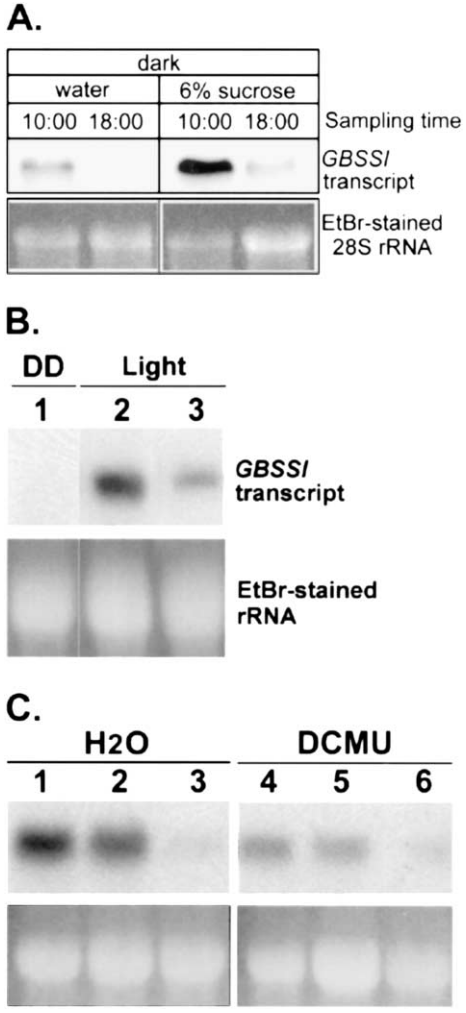

Fig. 7. (*Continued*)

As expected, the northern blot analysis showed that DCMU reduced the amount of *GBSSI* transcript but had no effect on the pattern of circadian expression (Fig. 7C, lanes 4–6).

4. Discussion

Our previous study showed that levels of *GBSSI* mRNA accumulation in sweet potato leaves exhibited a circadian-regulated pattern [4]. This experiment was repeated here for the purpose of comparisons with effects of time resetting for light-dark transition. One of the features of circadian rhythms is entrainability, and factors involved in entraining are light and temperature [26]. In an effort to demonstrate that light is responsible for setting the rhythm of *GBSSI* expressions, the time of light–dark transition was reset and plants were entrained under the new condition for 14 days. The result showed that the peak of *GBSSI* gene expression was indeed shifted. However, the accumulation peak of *GBSSI* transcript still occurred in 4 h after illumination regardless of the time when the light was turned on (Fig. 1). These results clearly demonstrate that the clock, which controlled the expression of *GBSSI* gene, was set by light. This hypothesis was also supported by a study on suspension cells of sweet potato [27]. In a dark-adapted suspension cell culture, expressions of *GBSSI* gene did not show circadian rhythm, but the situation could be reversed by light-treatment and pho-

Fig. 7. Effects of sucrose and photosynthesis on *GBSSI* gene expressions. (A) Effects of sucrose on *GBSSI* gene expressions under a DD condition. Leaf-petiole cuttings were moved to DD on the 8th day after 7 days of entraining under 16 L/8 D in H_2O with light turning on at 06:00 h. At 06:00 h on the 9th days (1 day after DD treatment), some cuttings were transferred to 175 mM (6%) sucrose solution while other cuttings were maintained in H_2O as a control. RNAs were prepared from leaves harvested at 10:00 h (4 h after sucrose treatment) and 18:00 h (12 h after sucrose treatment), respectively. (B) Effect of DCMU on the accumulation level of *GBSSI* mRNA in light. All leaf-petiole cuttings were cultured with H_2O in DD for 1 day before various treatments. Lane 1, cuttings were continuously kept in DD for 8 h before leaves were harvested. Lane 2, cuttings were illuminated for 8 h before leaves were harvested. Lane 3, cuttings were pretreated with 500 μM of DCMU for 1 h before illumination for 8 h, and DCMU was not removed during the period of illumination. (C) Effect of DCMU on the circadian expression of *GBSSI* gene under 16 L/8 D. Leaf-petiole cuttings were entrained under 16 L/8 D (light was turned on at 06:00 h and turned off at 22:00 h) for 7 days. Samples in lanes 1–3 were maintained under 16 L/8 D, and leaves were harvested at times indicated right after the entrained period. Sampling time were 10:00 h (lane 1), 14:00 h (lane 2) and 18:00 h (lane 3), respectively. Some of leaf-petiole cuttings were moved to DD for 1 day to deplete endogenous sucrose after the entrained period, and then the cuttings were pretreated with DCMU for 1 h before transferring back to the 16 L/8 D condition. Leaves were then harvested at the indicated times (lane 4, 10:00 h; lane 5, 14:00 h; lane 6, 18:00 h). Expressions of *GBSSI* gene were determined by the northern blot hybridization.

toperiod entraining [27]. Like *GBSSI* mRNA, the accumulation of GBSSI protein also exhibited a circadian pattern (Fig. 2). Since the GBSSI was not detected in soluble fractions, the fluctuation of GBSSI protein in starch granules was not the result of GBSSI re-distribution between granule-bound and soluble fractions. As expected, starch accumulation pattern was also circadian-fluctuated (Fig. 3). The circadian regulations of starch synthetic rate or starch accumulation were also observed in sugar beet and snapdragon [5,13], and the fluctuation in starch accumulation was suggested as a result of changes in synthetic activities [13].

In addition to regulation by a circadian clock, the expression of *GBSSI* gene in sweet potato was also regulated by sucrose. Levels of sucrose in sweet potato leaves were significantly lower under DD than 16 L/8 D conditions, and the accumulation of *GBSSI* mRNA was also reduced under this condition (data not shown). Furthermore, the level of *GBSSI* mRNA could be enhanced by supplementing with sucrose (Fig. 4). These observations clearly suggest that sucrose function as a stimulator for the transcription of *GBSSI* gene. This result is in agreement with a previous study using promoter assay, which indicated that expression of *GBSSI* gene could be enhanced by sugars in potato [17]. The stimulation of *GBSSI* gene expressions in leaves of sweet potato by sucrose was not the result of an osmotic effect because treatments with sorbitol and hexose were ineffective (Fig. 4A, B and D).

Sucrose has been shown to function as an indirect signal to regulate gene expressions since it could be replaced by lower concentrations of glucose or fructose [19]. However, present studies indicate that, in 8-h of treatments, glucose or fructose alone could not enhance the accumulation of *GBSSI* transcripts (Fig. 4B, D). This observation suggests that sucrose may function as a specific regulator for *GBSSI* expressions in sweet potato. This hypothesis was supported by the observation that a treatment with combination of glucose and fructose stimulated the expression of *GBSSI* gene to a degree much higher than with glucose or fructose alone (Fig. 4B, D), presumably, the combination facilitated the synthesis of sucrose inside the leaf tissue. When glucose treatment was prolonged to 24 h, accumulation of *GBSSI* transcripts in sweet potato leaves was also enhanced but still less than that of the sucrose-treated leaves (data not shown). It is not clear whether this 'glucose effect' due to a prolonged treatment is the result of isomerization of the hexose sugar coupling with a re-synthesis of sucrose or the prolonged treatment with glucose might cause an osmotic stress to induce an accumulation of sucrose. In *Arabidopsis* seedlings, synthesis of sucrose was activated by glucose added in the incubation medium [28]. Also, osmotic stress is known to induce sucrose accumulation in plant tissues. For example, sorbitol-induced osmotic stress and caused an accumulation of sucrose in sweet potato suspension cells [29]. A prolonged treatment of sweet potato leaf-petiole cuttings with sorbitol for 24 h did enhance accumulation of *GBSSI* mRNA (data not shown). It should also be noted that enhancements of *GBSS* and branching enzyme gene expressions in cassava were observed to be stimulated by culturing in glucose for 24 h in a dark condition [18]. In potato, expressions of *GBSSI* gene could also be enhanced when leaf explants were cultured in fructose or glucose [17].

The sugar sensing for *GBSSI* expression was not mediated by hexokinase pathway because accumulations of *GBSSI* transcripts could not be stimulated by glucose or fructose alone (Fig. 4B, D) and glucosamine did not block the sucrose effect on *GBSSI* gene expressions (Fig. 4C, D). This possibility was further supported by the observation that increases in *GBSSI* transcripts could be resulted from a treatment with glucose and fructose combination (Fig. 4B, D), presumably, the combination promoted the synthesis of sucrose to activate an intracellular sensor for stimulating the expression of *GBSSI* gene.

In addition to *GBSSI*, several other genes in sweet potato, e.g. sporamin, ADP-glucose pyrophosphorylase and β-amylase were also found to be responding to sucrose for expressions, and phosphorylation and dephosphorylation of several proteins were involved [16,30]. The observation that CHX was ineffective to affect the sucrose-regulated *GBSSI* gene expressions (Fig. 5A, C) suggests that *de novo* protein synthesis was not necessary for regulating the transcription of *GBSSI* gene. Therefore, modifications of pre-existed proteins might be involved in the signal transduction of sucrose-regulated *GBSSI* transcription. Okadaic acid, a selective inhibitor of protein phosphatase types 1 and 2A [31], inhibited the sucrose-stimulated *GBSSI* gene expression strongly (Fig. 5B, C). This inhibition suggests either an involvement of dephosphorylation in the transduction pathway or a block in sucrose uptake, since okadaic acid was also shown to block the function of sucrose transporters to inhibit sucrose uptake in sugar beet [32]. However, the observations that the level of *GBSSI* mRNA was enhanced by treating with glucose and fructose combinations, but the enhancement was inhibited by okadaic acid (data not shown), suggest that the decrease of sucrose-stimulated *GBSSI* expressions by okadaic acid might not be attributable to an inhibition in sucrose uptake. Instead, it is more likely that the decrease in expression was due to the result of a block in the internal signal cascade of sucrose-regulated pathway, presumably involving phosphatases. Cantharidin at low concentrations will inhibit protein phosphatase 2A, but not protein phosphatase 1 [33]. The observation that cantharidin at the concentration tested was ineffective to inhibit the sucrose-stimulated

GBSSI transcription (Fig. 5B, C) suggests that protein phosphatase type 1 was involved in the signal transduction of sucrose-regulated *GBSSI* expressions.

In addition to dephosphorylation, phosphorylation might also be involved in the signal transduction pathway, because myristoylated protein kinase C peptide inhibitor, a specific inhibitor for calcium- and phospholipid-dependent protein kinase C, was found to reduce slightly the level of sucrose-induced *GBSSI* transcripts (Fig. 5B, C). On the contrary, cAMP-dependent protein kinase inhibitor enhanced sucrose-regulated *GBSSI* transcriptions (Fig. 5B, C). The different effects of protein kinase inhibitors on the sucrose-stimulated *GBSSI* expressions suggest that calcium-, phospholipid-, or cAMP-dependent protein kinase C might be involved in the pathway of sucrose regulation on *GBSSI* expressions. Since expressions of genes related to carbohydrate metabolism could be affected by inhibitors of protein kinase and phosphatase, which might result in changes in levels of metabolic intermediates; in turn, these changes might produce an indirect effect on *GBSSI* gene expressions, especially since GBSSI is the final enzyme in the biosynthetic pathway of starch. Therefore, we could not rule out the possibility that changes in *GBSSI* expression due to inhibitor treatments were measurements of indirect effects.

Although sucrose may function as a stimulator for the transcription, the circadian fluctuation of *GBSSI* mRNA under any photoperiods might not be affected by sucrose. This hypothesis was supported by the observation that the circadian pattern of accumulating *GBSSI* transcripts could be maintained for at least three cycles under DD conditions, although the amplitude of rhythms was significantly lower than that of the 16 L/8 D (data not shown). When the exogenous sucrose was added and subsequently removed to change the sucrose concentration of the cultured condition, the rhythm of *GBSSI* gene expression was also maintained (Fig. 6C). As expected, expressions of *GBSSI* gene was enhanced by exogenous sucrose under DD conditions, but the circadian expressions of this gene was not changed (Fig. 7A). In addition, a treatment with photosynthetic inhibitor DCMU to prevent the synthesis of sucrose reduced the level of *GBSSI* transcripts (Fig. 7B), but it did not change the circadian rhythm (Fig. 7C). These results suggest that the pathways of circadian regulation and sucrose effects on *GBSSI* transcription are independent.

It is not clear from this study whether changes in starch accumulation were directly related to the circadian pattern of GBSSI and its regulation by sucrose since this enzyme is responsible primarily for amylose synthesis; however, other starch synthetic genes such as ADP-glucose pyrophosphorylase in *Arabidopsis* and potato leaves were also shown to be regulated by sucrose and light [15,34]. These observations might suggest that supply and availability of photosynthetic assimilates could serve as an important factor for controlling transitory starch metabolism in the source tissue during a diurnal cycle. Conceivably during the day, leaves should accumulate higher levels of sucrose than in the dark due to an active photosynthesis. Accordingly, greater amounts of mRNAs and proteins for *GBSSI* and other related genes for starch synthesis could be produced to promote conversion of sucrose to starch. Concomitantly, the situation should prevent a feedback inhibition on photosynthesis and osmotic stress. Thus, this sucrose regulation coupling with circadian rhythm should greatly facilitate carbons distribution from the source to sink tissues and prevent the leaf tissue from continuously accumulating starch.

In conclusion, we have demonstrated that light appears to regulate *GBSSI* gene expression through two independent pathways: (1) light plays an input signal to set an endogenous oscillator which controls circadian expressions of *GBSSI* gene; (2) light is an indirect stimulator that quantitatively affects *GBSSI* mRNA levels mediating by the photosynthetic product, sucrose. The sugar sensing of the sucrose-regulated *GBSSI* expression was different from the hexokinase-mediated pathway. In addition, phosphorylated/dephosphorylated modifications of proteins might be involved in the transduction pathway for regulating the expression of *GBSSI*.

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References

[1] O.E. Nelson, C.Y. Tsai, Glucose transfer from adenosine diphosphate-glucose to starch in preparations of Waxy seeds, Science 145 (1964) 1194–1195.
[2] C.Y. Tsai, The function of the *Waxy* locus in starch synthesis in maize endosperm, Biochem. Genet. 11 (1974) 83–96.
[3] J.H.M. Hovenkamp-Hermelink, E. Jacobsen, A.S. Ponstein, R.G.F. Visser, G.H. Vos-Scheperkeuter, E.W. Bijmolt, J.N. de Vries, B. Witholt, W.J. Feenstra, Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.), Theor. Appl. Genet. 75 (1987) 217–221.
[4] S.J. Wang, K.W. Yeh, C.Y. Tsai, Molecular characterization and expression of a starch granule-bound starch synthase gene in the sink and source tissues of sweet potato, Physiol. Plant. 106 (1999) 253–261.

[5] A. Mérida, J.M. Rodríguez-Galán, C. Vincent, J.M. Romero, Expression of the granule-bound starch synthase I (*Waxy*) gene from snapdragon is developmentally and circadian clock regulated, Plant Physiol. 120 (1999) 401–409.
[6] W.S. Hillman, Biological rhythms and physiological timing, Ann. Rev. Plant Physiol. 27 (1976) 159–179.
[7] J.A. Kreps, S.A. Kay, Coordination of plant metabolism and development by the circadian clock, Plant Cell 9 (1997) 1235–1244.
[8] F. Nagy, S.A. Kay, N.H. Chua, A circadian clock regulates transcription of the wheat *cab-1* gene, Genes Dev. 2 (1988) 376–382.
[9] H. Meyer, U. Thienel, B. Piechulla, Molecular characterization of the diurnal/circadian expression of the chlorophyll a/b-binding proteins in leaves of tomato and other dicotyledonous and monocotyledonous plant species, Planta 180 (1989) 5–15.
[10] M.L. Pilgrim, C.R. McClung, Differential involvement of the circadian clock in the expression of genes required for ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis, assembly and activation in *Arabidopsis thaliana*, Plant Physiol. 103 (1993) 553–564.
[11] M.D. Deng, T. Moureaux, M.T. Leydecker, M. Caboche, Nitrate-reductase expression is under the control of a circadian rhythm and is light inducible in *Nicotiana tabacum* leaves, Planta 180 (1990) 257–261.
[12] A.N. Polidoros, J.G. Scandalios, Circadian expression of the maize catalase *Cat3* gene is highly conserved among diverse maize genotypes with structurally different promoters, Genetics 149 (1998) 405–415.
[13] B. Li, D.R. Geiger, W.J. Shieh, Evidence for circadian regulation of starch and sucrose synthesis in sugar beet leaves, Plant Physiol. 99 (1992) 1393–1399.
[14] J.C. Jang, J. Sheen, Sugar sensing in higher plants, Plant Cell 6 (1994) 1665–1679.
[15] P.A. Nakata, T.W. Okita, Differential regulation of ADP-glucose pyrophosphorylase in the sink and source tissues of potato, Plant Physiol. 108 (1995) 361–368.
[16] S. Takeda, S. Mano, M. Ohto, K. Nakamura, Inhibitors of protein phosphstsaes 1 and 2A block the sugar-inducible gene expression in plants, Plant Physiol. 106 (1994) 567–574.
[17] R.G.F. Visser, A. Stolte, E. Jacobsen, Expression of a chimeric granule-bound starch synthase-GUS gene in transgenic potato plants, Plant Mol. Biol. 17 (1991) 691–699.
[18] S.N.I.M. Salehuzzaman, E. Jacobsen, R.G.F. Visser, Expression patterns of two starch biosynthetic genes in in vitro cultured cassava plants and their induction by sugars, Plant Sci. 98 (1994) 53–62.
[19] J.C. Jang, P. León, L. Zhou, J. Sheen, Hexokinase as a sugar sensor in higher plants, Plant Cell 9 (1997) 5–19.
[20] F. Rook, N. Gerrits, A. Kortstee, M. Kampen, M. Borrias, P. Weisbeek, S. Smeekens, Sucrose-specific signalling represses translation of the *Arabidopsis ATB2 bZIP* transcription factor gene, Plant J. 15 (1998) 253–263.
[21] S. Smeekens, F. Rook, Sugar sensing and sugar-mediated signal transduction in plants, Plant Physiol. 115 (1997) 7–13.
[22] S. Lalonde, E. Boles, H. Hellmann, L. Barker, J.W. Patrick, W.B. Frommer, J.M. Ward, The dual function of sugar carriers: transport and sugar sensing, Plant Cell 11 (1999) 707–726.
[23] K.W. Yeh, R.H. Juang, J.C. Su, A rapid and efficient method for RNA isolation from plants with high carbohydrate content, Focus 13 (1991) 102–103.
[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, New York, 1989.
[25] S.N.I.M. Salehuzzaman, E. Jacobsen, R.G.F. Visser, Isolation and characterization of a cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta* Crantz) and its antisense expression in potato, Plant Mol. Biol. 23 (1993) 947–962.
[26] D.E. Somers, The physiology and molecular bases of the plant circadian clock, Plant Physiol. 121 (1999) 9–19.
[27] S.J. Wang, K.W. Yeh, H.L. Wang, J.C. Su, C.Y. Tsai, Light-induced circadian expression of starch granule-bound starch synthase gene in cell suspension cultures of sweet potato, Taiwania 44 (1999) 202–211.
[28] P.P. Dijkwel, P.A.M. Kock, R. Bezemer, P.J. Weisbeek, S.C.M. Smeekens, Sucrose represses the developmentally controlled transient activation of the plastocyanin gene in *Arabidopsis thaliana* seedlings, Plant Physiol. 110 (1996) 455–463.
[29] H.L. Wang, P.D. Lee, L.F. Liu, J.C. Su, Effect of sorbitol induced osmotic stress on the changes of carbohydrate and free amino acid pools in sweet potato cell suspension cultures, Bot. Bull. Acad. Sin. 40 (1999) 219–225.
[30] M. Ohto, K. Hayashi, M. Isobe, K. Nakamura, Involvement of Ca^{2+} signalling in the sugar-inducible expression of genes coding for sporamin and β-amylase of sweet potato, Plant J. 7 (1995) 297–307.
[31] P. Cohen, The structure and regulation of protein phosphatase, Annu. Rev. Biochem. 58 (1989) 453–508.
[32] G. Roblin, S. Sakr, J. Bonmort, S. Delrot, Regulation of a plant plasma membrane sucrose transporter by phosphorylation, FEBS Lett. 424 (1998) 165–168.
[33] Y.M. Li, J.E. Casida, Cantharidin-binding protein: Identification as protein phosphatase 2A, Proc. Natl. Acad. Sci. USA 89 (1992) 11867–11870.
[34] L.N. Sokolov, A. Dejardin, L.A. Kleczkowski, Sugars and light/dark exposure trigger differenial regulation of ADP-glucose pyrophosphorylase genes in *Arabidopsis thaliana* (thale cress), Biochem. J. 336 (1998) 681–687.