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Light-Induced Circadian Expression of Starch Granule-Bound Starch Synthase Gene in Cell Suspension Cultures of Sweet Potato

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ABSTRACT: Attempts were made to evaluate whether suspension cell cultures of sweet potato could be used as a tool for studying gene expression controlled by circadian clocks. Starch granule-bound starch synthase (*GBSS*) gene, which exhibited a circadian rhythm in the leaf tissue but expressed constantly in dark-adapted cell cultures, was used as a marker gene for the study. Because light is considered as a major signal to set circadian clocks, dark-adapted cells were entrained by 16 h light/8 h dark photoperiod for 7 days before determination for the expression of *GBSS* gene. Results of RNA blot analysis showed that fluctuations of *GBSS* mRNA during a diurnal cycle could be induced in cell cultures and the pattern of rhythm was similar to that of the leaf tissue. In addition to light effect, other environmental factors such as osmotic stress were also evaluated. Expression of *GBSS* gene was enhanced by 600 mM sorbitol in the dark-adapted cells; however, the pattern of circadian rhythm established under 16 L/8 D photoperiod was repressed by the high osmotic effect. These results not only indicate that the circadian clock could be set by light but also provide evidence that suspension cells might be used for studying the regulation of circadian rhythm.

KEY WORDS: Circadian rhythm, Granule-bound starch synthase (GBSS), Osmotic effects, Suspension cells, Sweet potato (*Ipomoea batatas*).

INTRODUCTION

Starch granule-bound starch synthase (GBSS) preferentially transfers the glucose moiety from ADP-glucose or UDP-glucose to non-reducing ends of amylose molecules (Tsai, 1973). Studies with several *waxy* mutants of maize have established the role of this enzyme in the synthesis of amylose (Nelson and Tsai, 1964; Tsai, 1974). Subsequent studies in rice (Sano, 1984), potato (Hovenkamp-Hermelink *et al.*, 1987), sorghum (Hsieh, 1988) and antisense-*GBSS* transgenic plants (Salehuzzaman *et al.*, 1993; Visser *et al.*, 1991b) also confirm the importance of GBSS in amylose formation. In some cereal and pea plants, the presence of different GBSS isoforms has been observed (Denyer *et al.*, 1997; Nakamura *et al.*, 1998; Tomlinson *et al.*, 1998), and expressions of these GBSS isoforms may be tissue-specific. For example, the GBSS of wheat endosperms and pollens played little or no function in the

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pericarp. Instead, another GBSS isoform was detected specifically in starch granules of pericarp (Nakamura *et al.*, 1998). However, in sweet potato, there is only one GBSS present in tuberous roots, leaves, and stems (Wang *et al.*, 1999b). Jacobsen *et al.* (1989) also indicated that amylose synthesis in different tissues of potato was catalyzed by the same GBSS. Although the function of GBSS is well known, regulation of *GBSS* expression in different tissues has not been established.

We have recently isolated a *GBSS* cDNA from tuberous roots of sweet potato (Wang *et al.*, 1999b). Mechanisms involved in regulating *GBSS* gene appeared to be different between tuberous roots and leaves. In tuberous roots, the expression of *GBSS* gene correlated well with the developmental stages. However, expression of this gene was controlled by endogenous biological clock(s) in leaves (Wang *et al.*, 1999b). Circadian rhythms are ubiquitous in eukaryotes (Feldman, 1982) and some prokaryotes (Hung and Chou, 1991). Light and temperature are major input signals to set the biological clock(s), and circadian rhythms have been observed to affect various biological functions, including physiological changes (e.g., stomata opening and leaf movement) (Hennessey and Field, 1992), biochemical processes (e.g., ethylene production and starch accumulation) (Emery *et al.*, 1994; Li *et al.*, 1992), and gene expressions (Kathiresan *et al.*, 1996; Sander *et al.*, 1995). However, molecular mechanism of how environmental factors turn on biological clock(s) is still unknown. Some studies indicate that phytochromes are mediators for light entraining oscillations (Millar *et al.*, 1995; Somers *et al.*, 1998). Since cell suspension cultures could be established for analysis of various parameters and provide an excellent tool for studying the light-dependent signal transduction (Harter *et al.*, 1993), attempts were made to determine whether suspension cells of sweet potato might be suitable for studying the circadian regulation of *GBSS* gene.

MATERIALS AND METHODS

Plant materials

Suspension cells derived from tuberous roots of sweet potato (*Ipomoea batatas* Lam. cv. Tainong 57) were maintained in MS medium (Murashige and Skoog, 1962) containing 3% sucrose, and kept in the dark at 25℃ with constant shaking at 120 rpm (Wang *et al.*, 1999a). Before assaying for *GBSS* gene expression during a diurnal cycle, cell cultures were placed in an incubator with 16 h light/8 h dark (16 L/8 D) photoperiod, and harvested after 7 days of adaptation. For determining the osmotic effect on *GBSS* gene expressions, suspension cells were subcultured in medium containing 600 mM sorbitol (Wang *et al.*, 1999a).

Isolation of RNA from leaves and suspension cells

RNA was isolated according to the procedure described by Yeh *et al.* (1991). A sample of suspension cells (0.5 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, 0.5% (w/v) lauroyl sarcosine and 0.1 M β-mercaptoethanol). The mixture was incubated at room temperature for 10 min before centrifugation at 12000*g* for 10 min. The supernatant was treated in succession with equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v)

and chloroform/isoamylalcohol (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℃ for 30 min. After centrifugation, the pellet was dissolved in 0.5 ml H_2O, and treated again with phenol/chloroform/ isoamylalcohol (25:24:1, v/v) and chloroform/isoamylalcohol (24:1, v/v) before precipitation. Finally, the RNA pellet was dissolved in 100 μl H_2O.

Northern blot hybridization

Total RNAs extracted from suspension cells were separated on 1% formaldehyde-agarose gels and transferred onto nitrocellulose membranes (Sambrook *et al.*, 1989). Expressions of *GBSS* gene were determined by probing with *GBSS* cDNA (Wang *et al.*, 1999b) which was labeled with α-^{32}P-dCTP (111 TBq/mmol, NEN™, USA) using a random primer labeling kit (Amersham, UK). After hybridization, the membranes were washed twice with 2× SSC (1L 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℃ for 30 min (Sambrook *et al.* 1989). All experiments were repeated at least twice and rRNAs were used as internal standards. Accumulation of *GBSS* 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 that was harvested at 06:00 a.m. (100%).

RESULTS AND DISCUSSION

Light-induced circadian expressions of *GBSS* gene in suspension cells

When suspension cells were cultured in the dark condition, referring to as dark-adapted cells, the *GBSS* gene was constantly expressed (Fig. 1). In order to determine whether circadian regulation of this gene could be induced by light, dark-adapted cell cultures were moved to a 16 L/8 D condition. After entraining under this condition for 7 days, RNAs were isolated from the cultured cells every 4 hrs, and *GBSS* transcripts were determined by probing with ^{32}P-labeled *GBSS* cDNA. The result showed that *GBSS* mRNA accumulation fluctuated during a diurnal cycle (Fig. 2). The peak of *GBSS* transcript accumulation was at 8 hr after illumination and then decreased until the end of the photoperiod, a situation similar to that of the leaf tissue. When sweet potato plants were grown in a growth chamber under 16 L/8 D at 28℃, the peak of *GBSS* mRNA accumulation in the leaf tissue was at 4 hr after light was turned on and then decreased gradually until disappeared completely at 16 hr; however, accumulation of the transcript reappeared at the end of the period (Fig. 3). A similar pattern of *GBSS* expression was also observed when the 16 L/8 D-entrained plants were moved to continuous light or continuous dark conditions (Wang *et al.*, 1999b). Although the peak of oscillation rhythm was shifted to 8 hr for the suspension cells (Fig. 3), these observations indicated that light could reset the endogenous clock of the dark-adapted cells to induce a circadian rhythm of *GBSS* expression similar to that found in leaves.

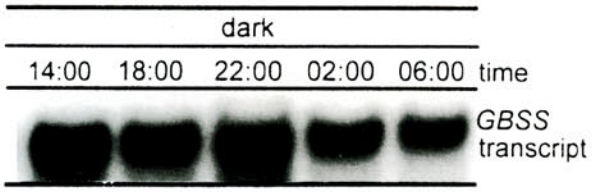

Fig. 1. Expression of *GBSS* gene in dark-adapted cells. Cells were cultured in dark and harvested at indicated time points before RNA was isolated. Total RNAs (20 μg for each sample) were separated on a 1% formaldehyde agarose-gel and transferred onto a nitrocellulose membrane. Accumulations of *GBSS* transcript were determined by probing with ^{32}P-labeled *GBSS* cDNA.

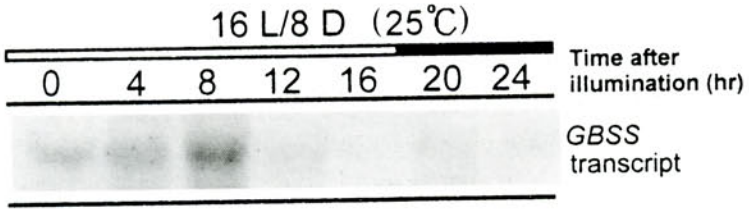

Fig. 2. Diurnal fluctuations of *GBSS* mRNA in 16 L/8 D photoperiods-entrained cells. The dark-adapted cells were entrained by 16 L/8 D photoperiods for 7 days. Then cells were harvested at 4-h intervals from 06:00 a.m. Total RNAs (10 μg for each sample) were separated on a 1% formaldehyde agarose-gel and transferred onto a nitrocellulose membrane. Accumulations of *GBSS* transcript were determined by probing with ^{32}P-labeled *GBSS* cDNA.

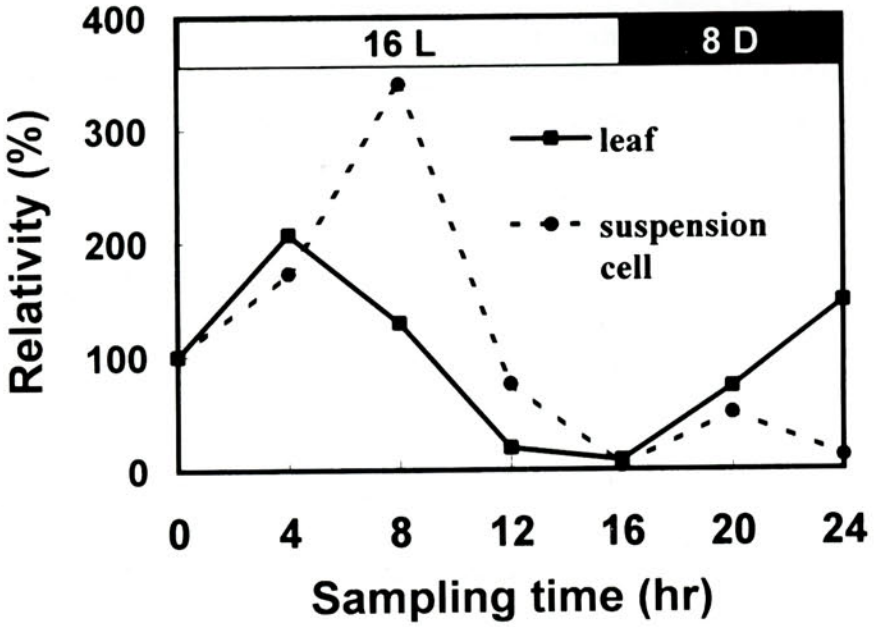

Fig. 3. Comparisons of the fluctuations of *GBSS* mRNA in leaves and suspension cells during 16 L/8 D photoperiods. Samples were harvested after entraining by 16 L/8 D photoperiods for 7 days. The leaf data was published elsewhere (Wang *et al.*, 1999b) but was transferred and included here for the purpose of comparison. Suspension cells data in Fig. 2 were quantified by a densitometer. Relative levels were based on the measurement determined for the samples that were harvested at 06:00 a.m. (100%).

A study on sugar metabolism in sugar beet indicated that the pattern of starch accumulation in leaves might be controlled by endogenous clock(s) (Li *et al.*, 1992), and the pattern might be controlled by enzyme(s) involved in the starch synthetic pathway. This study on circadian regulation of *GBSS* gene might help us to understand the regulatory mechanism of starch metabolism in leaves during diurnal cycles.

Expressions of clock-controlled genes were observed in some plant species, and most of these genes were involved in photosynthesis and nitrogen assimilation (Deny *et al.*, 1990; Nagy *et al.*, 1988; Pilgrim *et al.*, 1993; Sander *et al.*, 1995). However, little information is available regarding signal transduction from environmental signals to clocks setting and gene regulations. Light is an exogenous timing cue which may set the biological clock (Nagy *et al.*, 1993) as well as regulate the period and amplitude of circadian rhythms (Millar *et al.*, 1995). Plants sense the light which in turn mediates multiple photoreceptors to affect many light-dependent processes (Kuhlemeier *et al.*, 1987). Phytochromes and blue-light photoreceptors have been shown to involve in maintaining periods of circadian rhythm (Millar *et. al.*, 1995). Clock proteins isolated from *Neurospora* and *Drosophila* both have a common motif – PAS (for PER, ARNT, SIM) domain (Kay, 1997). Lagarias *et al.* (1995) indicated that plant photoreceptors also contained the PAS domain. These studies suggested that light receptors and clock regulations might be closely related. The observation that expression of the *GBSS* gene in dark-adapted cells maintained a constant rate but fluctuated in the 16 L/8 D entrained cells indicates that light may set the clock. Appearance of phytochrome has been observed in soybean suspension cells after light induction (Tanada, 1977), and active phytochromes may regulate light-dependent gene expressions (Lam *et al.*, 1989; Harter *et al.*, 1993).

Osmotic effect on *GBSS* gene expression

Osmotic pressure is an important factor affecting starch and storage protein synthesis (Oparka and Wright, 1988; Tsai, 1983; Tsai *et al.*, 1978). Increase in starch accumulation by osmotic stress was also found in suspension cells of sweet potato (Wang *et al.*, 1999a). Expression of *GBSS* gene was enhanced in the suspension cells under an osmotic stress condition. When the dark-adapted cells were transferred to a medium containing 600 mM sorbitol and continuously cultured in the dark, the level of *GBSS* transcript was higher than that in the normal medium (Fig. 4). However, light-inducible circadian expressions of *GBSS* disappeared in the osmotic stressed cells (Fig. 5). This result suggests that either the osmotic effect could repress the circadian regulation of *GBSS* gene under the 16 L/8 D photoperiod or the osmotic effect might enhance the *GBSS* gene expression to a saturated level at any time points, under which the circadian fluctuation of *GBSS* mRNA could not be observed.

Expressions of several starch-related genes could be induced by osmotic stress and/or sucrose effect (Sokolov *et al.*, 1998; Visser *et al.*, 1991a; Nakata and Okita, 1995). An increase in expression of the large subunit of ADP-glucose pyrophosphorylase gene (*ApL1*) in *Arabidopsis* was found to be caused by osmotic effect (Sokolov *et al.*, 1998), while other genes were enhanced by metabolic sugars (e.g., sucrose, glucose) but not by osmotic stress (Visser *et al.*, 1991a; Nakata and Okita, 1995). Wang *et al.* (1999a) indicated that the level of sucrose in the dark-adapted cells increased three-fold when treated with 600 mM sorbitol; however, no significant change was observed for glucose. This observation, therefore,

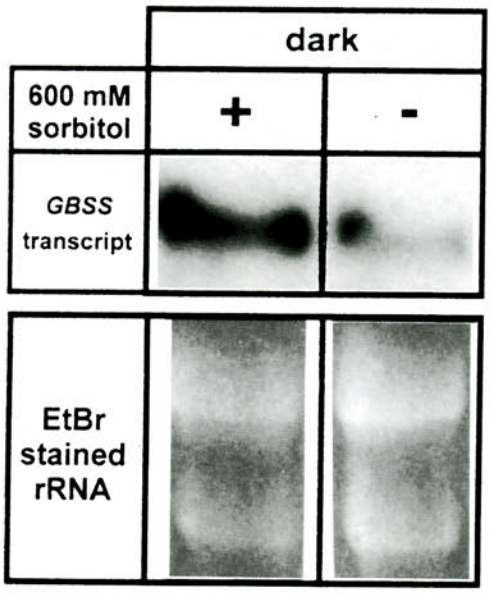

Fig. 4. Osmotic effect on *GBSS* gene expression. Before cells were harvested, some cell cultures were transferred to a medium containing 600 mM sorbitol and maintained in the dark for 7 days. Total RNAs (10 μg for each sample) were separated on a 1% formaldehyde agarose-gel and *GBSS* transcripts were determined by probing with ^{32}P-labeled *GBSS* cDNA. EtBr-stained rRNA patterns were used as internal standards.

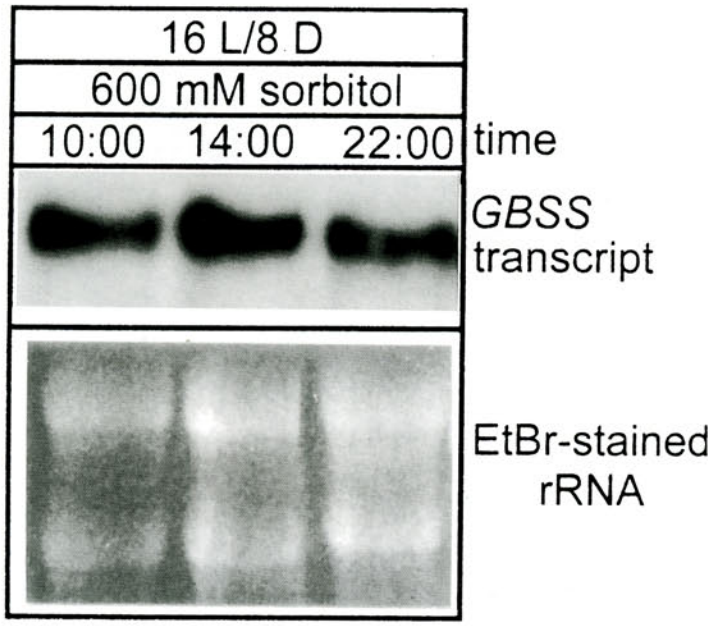

Fig. 5. Abolition of circadian *GBSS* expression by osmotic stress. Before cells were harvested, some cell cultures were transferred to a medium containing 600 mM sorbitol and entrained by 16 L/8 D photoperiods for 7 days. Total RNAs (10 μg for each sample) were separated on a 1% formaldehyde agarose-gel and *GBSS* transcripts were determined by probing with ^{32}P-labeled *GBSS* cDNA. EtBr-stained rRNA patterns were used as internal standards.

provides evidence to suggest that a high concentration of sorbitol might serve as an indirect signal to enhance the *GBSS* expression mediated by sucrose. This hypothesis was supported by an observation that *GBSS* mRNA was enhanced in leaves of sweet potato when grown in the presence of 175 mM sucrose, and the effect could not be repeated by the same

concentration of glucose (unpublished data). Further studies of the dark-adapted cells supplemented with various concentrations of exogenous sucrose, glucose or sorbitol coupling with analysis of endogenous sugar content should be helpful to clarify whether the osmotic effect is a direct or indirect signal to enhance *GBSS* mRNA accumulation.

This study indicates that circadian rhythm of *GBSS* expression could be induced by light in suspension cells to a situation similar to that was found in the whole plant. Therefore, suspension cells might serve as a suitable system for studying the signal transduction of circadian regulation.

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光線誘導甘藷懸浮細胞中澱粉粒結合性澱粉合成酵素基因概日韻律表現之探討

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摘　　要

本研究之主要目的在探討甘藷懸浮細胞是否適合做為探討概日韻律調控機制之有效工具。我們以澱粉粒結合性澱粉合成酵素 (granule-bound starch synthase; *GBSS*) 基因做為此研究之標誌基因。*GBSS* 基因在葉部的表現呈現概日韻律，但在黑暗環境連續培養的懸浮細胞中則表現恆定。因光線為啟動生物時鐘的因子之一，故將黑暗培養之懸浮細胞轉移至十六小時光照、八小時黑暗的生長箱中培養七天再進行北方雜合分析，其結果顯示經光線誘導後 *GBSS* 基因的表現與在葉部所觀察到的概日韻律相似。除了光照因子外，我們亦探討其他環境因子（例如：滲透逆境）對 *GBSS* 基因表現的影響。將細胞培養在高滲透壓的培養基中會使 *GBSS* 基因的表現增強。但在此高滲透壓的環境下，*GBSS* 基因受光線誘導的韻律性表現則會受到抑制。在此研究中不僅指出光線可做為一環境訊息啟動生物時鐘，亦證明懸浮細胞可做為研究概日韻律的一便利系統。

關鍵字: 概日韻律、澱粉粒結合性澱粉合成酵素、滲透逆境、懸浮細胞、甘藷。

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