行政院國家科學委員會專題研究計畫　成果報告

生物時鐘調控甘藷葉部澱粉合成酵素基因表現之分子機制

計畫類別：個別型計畫
計畫編號：NSC92-2313-B-002-100-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學農藝學系暨研究所

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 9 月 27 日

（二）、中、英文摘要

中文摘要：

在甘藷葉部組織中，澱粉結合性澱粉合成酵素基因(*granule-bound starch synthase I; GBSSI*)受到生物時鐘的調控，而使其表現呈現概日韻律現象。且藉由 nuclear run-on transcriptional assay 證明此調控主要是藉由控制 *GBSSI* 基因之啟動子轉錄作用之速率。為進一步的探討 *GBSSI* 基因的受生物時鐘調控之分子機制機制，利用 GenomeWalker PCR 之方法篩選出 *GBSSI* 之啟動子，並對 *GBSSI* 基因的啟動子進行序列特性分析，經由序列比對，發現 *GBSSI* 啟動子序列含有四個 circadian clock-associated 1 protein-binding sites (AATCT)、兩個 putative circadian regulation elements (CAANNNNATC) 及六段與光線調控相關的 I-box 。利用轉殖阿拉伯芥進行不同長度之甘藷 *GBSSI* 啟動子功能及活性測試，結果顯示即使將啟動子縮短至 690 bp，其仍受生物時鐘調控。另一方面，以阿拉伯芥為材料，發現大部分參與澱粉生合成之基因（如 *soluble starch synthase, ADP-glucose pyrophosphorylase*）的表現均呈現概日韻律，因而推論植物內在生物時鐘在調控暫存性澱粉生合成之過程中，擔任協調基因表現之功能。

關鍵字：

生物時鐘，澱粉結合性澱粉合成酵素，甘藷，澱粉。

英文摘要：

A starch granule-bound starch synthase I (*GBSSI*) gene is regulated by a circadian clock in sweet potato leaves. In order to examine whether the promoter region is responsible for controlling a circadian expression of the *GBSSI* gene, the sweet potato *GBSSI* promoter was isolated and deleted to different lengths for functional analysis with a *GUS* reporter gene in transgenic *Arabidopsis* plants. Nuclear run-on transcriptional assays showed that the circadian control was regulated at the transcriptional rate level, and *de novo* synthesized proteins were necessary for controlling the rhythm. Promoter assays showed that the *GBSSI* promoter fragments containing six I-boxes, two putative circadian regulation elements (CAANNNNATC) and four circadian clock-associated 1 protein-binding sites (AATCT) maintained the activity to induce the circadian expression of the *GUS* gene. Similar to the *GBSSI* in sweet potato, *GBSSI*, *soluble starch synthase* and *ADP-glucose pyrophosphorylase* genes in *Arabidopsis* leaves also exhibited a circadian rhythm. These results suggested that common signals may exist in dicotyledonous plants to coordinate the circadian expression of genes involved in the transitory starch synthetic pathway.

Key words:

Circadian clock, Granule-bound starch synthase, *Ipomoea batatas*, Starch.

（三）、報告內容

此計畫之報告之內容已發表於

Plant Growth Regulation 42:161-168 (2004)

報告內容如下所附：

Plant Growth Regulation **42:** 161–168, 2004.

Circadian control of sweet potato *granule-bound starch synthase I* gene in *Arabidopsis* plants

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Received 2 May 2003; accepted 2 September 2003

Key words: Circadian clock, Granule-bound starch synthase, *Ipomoea batatas*, Run-on transcription, Starch

Abstract

A starch *granule-bound starch synthase I* (*GBSSI*) gene is regulated by a circadian clock in sweet potato leaves. In order to examine whether the promoter region is responsible for controlling a circadian expression of the *GBSSI* gene, the sweet potato *GBSSI* promoter was isolated and deleted to different lengths for functional analysis with a *GUS* reporter gene in transgenic *Arabidopsis* plants. Nuclear run-on transcriptional assays showed that the circadian control was regulated at the transcriptional rate level, and *de novo* synthesized proteins were necessary for controlling the rhythm. Promoter assays showed that the *GBSSI* promoter fragments containing six I-boxes, two putative circadian regulation elements (CAANNNNATC) and four circadian clock-associated 1 protein-binding sites (AATCT) maintained the activity to induce the circadian expression of the *GUS* gene. Similar to the *GBSSI* in sweet potato, *GBSSI*, *soluble starch synthase* and *ADP-glucose pyrophosphorylase* genes in *Arabidopsis* leaves also exhibited a circadian rhythm. These results suggested that common signals may exist in dicotyledonous plants to coordinate the circadian expression of genes involved in the transitory starch synthetic pathway.

Abbreviations: 16 L/8 D – 16 h light/8 h dark; CAB – chlorophyll a/b-binding protein; CCA1 – circadian clock-associated 1; CBS – CCA1-binding site; CHX – cycloheximide; GBSSI – granule-bound starch synthase I; SSS – soluble starch synthase

Introduction

A starch *granule-bound starch synthase I* (*GBSSI*) gene encodes a key enzyme responsible for amylose synthesis (Tsai 1974; Hovenkamp-Hermelink et al. 1987). In sweet potato, the expression of *GBSSI* in tuberous roots was found to be developmentally regulated, i.e., accumulation of mRNA continued to increase as the fresh weight of tuberous root increased (Wang et al. 1999). On the other hand, light played a major factor in leaves to regulate the expression of this gene through two independent pathways. First, light induced a circadian expression of the *GBSSI* gene controlled by the biological clock setting, and the peak of *GBSSI* mRNA accumulation occurred in 2–4 h after illumination (Wang et al. 1999). Second, light functioned as an indirect regulator to control the level of *GBSSI* transcripts through the product of photosynthesis, sucrose, as a direct signal (Wang et al. 2001).

Biological clocks have been demonstrated to regulate gene expression and to coordinate metabolic and physiological reactions in several eukaryotes as well as in some prokaryotes (Harmer et al. 2000;

Schaffer et al. 2001). Circadian expression of a gene encoding chlorophyll a/b-binding protein (*CAB*) was widely observed in dicotyledonous and monocotyledonous plants (Meyer et al. 1989). Light was found to be a major factor that modulates the rhythm of *CAB* expression where phytochrome- and blue light-responsive photoreceptor pathways were suggested to be involved in the regulatory mechanism (Millar et al. 1995b). Several genes related to starch-mobilization, phenylpropanoid biosynthesis and cell elongation were also observed to be regulated by biological clocks (Harmer et al. 2000). In addition, expressions of nitrate reductase and nitrite reductase were observed to exhibit a circadian rhythm (Deng et al. 1990; Sander et al. 1995).

Although the molecular mechanism of circadian control is still unclear, three basic components have been postulated to be involved in the regulatory pathway (Takahashi 1993). First, input signals must be supplied from the environment to set or reset biological clocks, and the signal may be either light or temperature. Second, the clock(s) triggers a circadian rhythm. Third, output signals regulate gene expression and metabolism. Several gene products have been suggested as components of biological clocks or as a regulator of clocks based on analyses of mutants. These include CCA1 (circadian clock-associated 1), LHY (late elongated hypocotyl), TOC1 (timing of *CAB* expression), ZTL (ZEITLUPE) and FKF (flavin-binding, kelch repeat, F box). In these mutants, periodic length of gene circadian expressions were modified when compared with their normal plants (Millar et al. 1995a; Wang et al. 1997; Schaffer et al. 1998; Nelson et al. 2000; Somers et al. 2000). Some of these clock components might function as transcriptional factors to regulate circadian transcription of other genes (Wang et al. 1997). In addition to transcriptional regulation, post-transcriptional mechanisms and protein phosphorylation may also play important roles in regulating the circadian rhythm (Millar and Kay 1991; Sugano et al. 1999).

In sweet potato leaves, light was identified as an input signal to reset the biological clock for a circadian regulation of *GBSSI* gene expressions (Wang et al. 2001). In order to elucidate the signal transduction of circadian regulation of *GBSSI* gene and its relationship with carbon redistribution in leaves, regulatory elements of the *GBSSI* promoter were examined in this study.

Materials and methods

Plant materials and chemicals treatments

Sweet potato (*Ipomoea batatas* Lam. cv. Tainong 57) was grown at 28 °C in a growth chamber under 16 h light (6:00 a.m.–22:00 p.m.)/ 8 h dark (22:00 p.m.–6:00 a.m.) (16 L/8 D). To determine whether *de novo* protein synthesis was required for the circadian regulation of the *GBSSI* gene, leaf-petioles (10 cm) were cut from plants (about 20 cm in height) and incubated in 100 μM of cycloheximide (CHX) at the beginning of the dark period for a total of 12 h. Total RNA was then isolated from leaves harvested at 10:00 a.m. (4 h after the light was turned on).

Nuclear run-on transcription

Nuclei were isolated from sweet potato leaves according to the method of Luthe and Quatrano (1980). Nuclei isolated from 3 g of leaves were suspended in 350 μl of nuclei storage buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 20% glycerol (Sheu et al. 1994). The *in vitro* transcription mixture (200 μl) contained 100 μl of nuclei suspension and 100 μl of 2× reaction buffer (50 mM Tris–HCl, pH 7.8, 5 mM DTT, 20 mM $MgCl_2$, 120 mM $(NH_4)_2SO_4$, 1 mM each of ATP, CTP, GTP, 20% (v/v) glycerol, 200 U of RNasin (Promega, USA), 50 μg of yeast tRNA) and 3.7 MBq [α-^{32}P]UTP (110 TBq mmol^{-1}, Amersham, Buckinghamshire, UK). Run-on reactions were carried out at 25 °C for 30 min. After incubation, 1 μl of UTP (10 mM) was added and the mixture was allowed to stand for 30 min at 25 °C. Subsequently, 5 U of RNase-free DNase I was added and the mixture was incubated at 25 °C for 30 min before treating with 20 μg of proteinase K by incubating at 25 °C for an additional 30 min. Finally, the labeled RNA was extracted by phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) and precipitated by ethanol. For DNA filter hybridization, 10 μg of *GBSSI* cDNA and 5 μg of plasmid pRY18 containing a rice genomic rRNA cluster (Sano and Sano 1990) were denatured with 0.4 N NaOH at room temperature for 30 min. The plasmids were neutralized with 4.5× SSC. Subsequently, the DNA was blotted onto Hybond-N membranes (Amersham,

Buckinghamshire, UK) using a MillBlot^{TM}-S slot-blot apparatus (Millipore, Bedford, MA, USA). Each membrane was hybridized with an equal count (5×10^7 cpm) of labeled RNAs that was *in vitro* synthesized from isolated nuclei. The run-on transcriptional data of *GBSSI* and *rRNA* were quantified from the autoradiograph using Nobel ABC-Tiger Gel Documentation and Analysis System version 2.0 (Taigen, Taiwan). The run-on transcriptional assays were repeated in three independent experiments.

RNA extraction and analysis by northern blotting

RNA was extracted according to the method described by Yeh et al. (1991). For the northern blot analysis, total RNAs (10 μg) were separated on 1% formaldehyde-agarose gels (Sambrook et al. 1989). cDNA probes were 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 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 (Sambrook et al. 1989). EtBr-stained 28S rRNA patterns were used as loading control in northern blot analysis data.

Genomic DNA walking for finding the GBSSI *promoter*

The 5′- upstream region containing the *GBSSI* promoter was isolated by using the GenomeWalker^{TM} kit (Clontech, USA) according to the method described by Siebert et al. (1995). Genomic DNA extracted from tuberous roots of sweet potato was digested with *Dra*I, *Eco*RV, *Puv*II, *Sca*I and *Stu*I, respectively. The digested DNA fragments were ligated with adaptors. The primary PCR was carried out using a *GBSSI* cDNA specific primer (GSP1; 5′-TGGCTCCTCAGGGCTAATTGACC-3′) and the outer adaptor primer (AP1) provided in the kit. For the secondary (nested) PCR, a nested *GBSSI* cDNA specific primer (GSP2; 5′-TGAGAAACAAAGTGTGAGGC-3′) and the nested adaptor primer (AP2) provided in the kit were used. The sequences of the GSP1 and GSP2 primers were both complementary to the sequences near the 5′ end of *GBSSI* coding region. Primary and secondary PCR were performed using *Taq* Polymerase Mix (Clontech, USA). Finally, the PCR product was cloned into the *Sma*I site of pUC19 vector for sequencing.

Construction of promoter/GUS fusions and generation of transgenic Arabidopsis *plants*

Different promoter fragments were generated by PCR. Promoter fragments of varying length were constructed into a pBI101 vector carrying the *GUS* gene. The pGBSS(−1944/−7)/GUS construct contained the *GBSSI* promoter fragment from −1944 to −7 relative to the translation start site fused to the *GUS* gene. The pGBSS(−695/−7)/GUS construct contained the −695 to −7 *GBSSI* promoter, and the pGBSS(−149/−7)/GUS construct contained the −149 to −7 promoter region. *Arabidopsis* plants were transformed by *Agrobacterium tumefaciens* C58 using the floral dip method (Clough and Bent 1998). The seeds harvested from the dipped plants were selected on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 30 μg kanamycin/ml. Plants were then grown under 16 L/8 D conditions. The expression of the *GUS* gene in transgenic *Arabidopsis* leaves was analyzed.

Results

Changes of transcriptional rates for GBSSI *gene in sweet potato leaves during a diurnal cycle*

In order to demonstrate whether or not the periodic fluctuation of *GBSSI* mRNA observed in sweet potato leaves was controlled by transcriptional rate, nuclei were isolated from leaves harvested at 10:00 a.m. (4 h after illumination) and 22:00 p.m. (16 h after illumination), respectively. Transcriptional rates of *GBSSI* gene from these isolated nuclei were then determined by *in vitro* nuclear run-on transcriptional assays. The result showed that the transcriptional activity of the *GBSSI* gene at 10:00 a.m. was about 27 times higher than the activity determined at 22:00 p.m. (Figure 1), and the pattern of transcriptional activity in the second cycle was similar to that of the first cycle (data not shown). A comparison of patterns

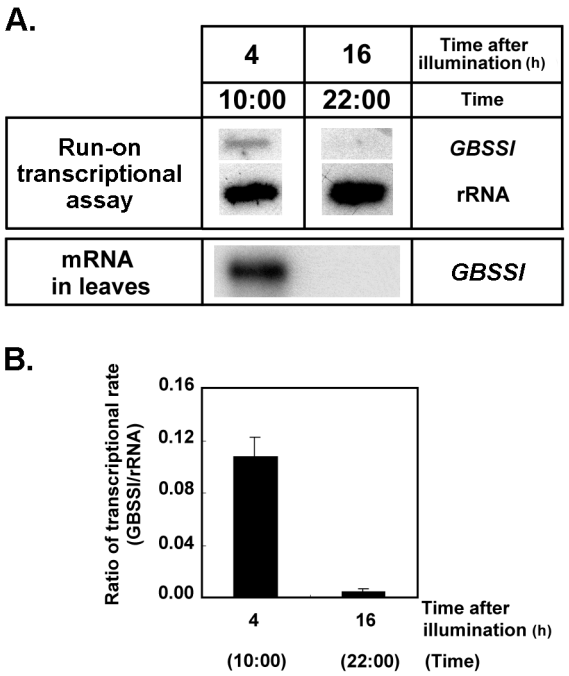

Figure 1. Transcriptional rates of sweet potato *GBSSI* gene under 16 L/8 D. (A) Light was turned on at 6:00 a.m. and sweet potato leaves were harvested at 10:00 a.m. (4 h after illumination) and 22:00 p.m. (16 h after light was turned on). Newly synthesized mRNAs (α-^{32}P labeled) from nuclei isolated from leaves at the indicated time points were hybridized to the *GBSSI* cDNA. rRNA was used as a loading control. Northern blot analysis showing accumulation of *GBSSI* mRNA in leaves at the same sampling time. (B) A comparison between the transcriptional rates of *GBSSI* gene at 10:00 a.m. and 22:00 p.m.

between the *GBSSI* gene transcriptional rate and mRNA accumulation in leaves showed that the change in transcriptional rates exhibited a pattern according to the rhythm of *GBSSI* mRNA accumulation (Figure 1A).

Effects of CHX on the circadian regulation of GBSSI *gene*

In order to determine whether *de novo* protein synthesis was required to regulate the circadian expression of *GBSSI* gene in sweet potato leaves, leaf-petiole cuttings were treated with 100 μM CHX for 12 h. RNA was then extracted from leaves harvested at 4 h after illumination when *GBSSI* mRNA level should reach its maximum in a rhythmic cycle in the control cuttings. Results showed that CHX significantly suppressed the

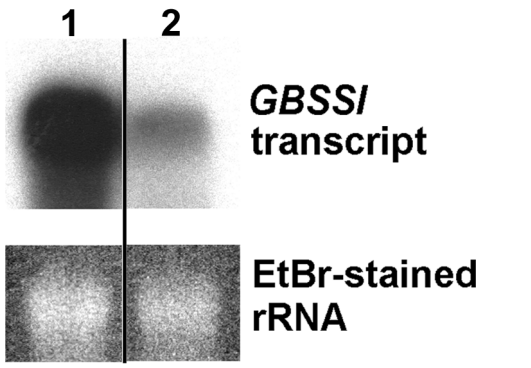

Figure 2. Effect of CHX on light-regulated *GBSSI* gene expression in sweet potato. Lane 1, RNAs were extracted from control leaves without treatment with CHX, and leaves were harvested at 10:00 a.m. (after light was turned on for 4 h). Lane 2, leaf-petiole cuttings were pre-treated with 100 μM CHX for 12 h before RNAs were extracted from leaves, which were also harvested at 10:00 a.m. *GBSSI* mRNA were detected by α-^{32}P labeled *GBSSI* cDNA.

accumulation of *GBSSI* transcripts. The amount of *GBSSI* mRNA accumulated in leaves of the control cuttings was significantly higher than that of the CHX-treated leaves (Figure 2).

Molecular analysis of GBSSI *promoter*

Sequence analysis of the *GBSSI* promoter revealed several regulatory elements (Figure 3). These elements included: (1) six I-box-like elements (GATA or GATT motif, a conserved motif in the 5′-upstream region of light-regulated genes) located at −71, −123, −137, −183, −433 and −482, respectively; (2) one G-box (or named ABA response element, ABRE, with ACGT as the core region of G-box) at −661 position; (3) one sucrose-response element (TTGACGG) located at −857; (4) two sequences characteristic of circadian regulation element, CAANNNNATC, positioned at −204 and −355, respectively; and (5) four CCA1-binding sites (CBSs) (AATCT), located at −210, −348, −394 and −844, respectively.

Circadian regulation of GBSSI *promoter in transgenic* Arabidopsis *plants*

Circadian activity of the *GBSSI* promoters was examined in transgenic *Arabidopsis* plants containing *GUS* with different lengths of *GBSSI* promoter.

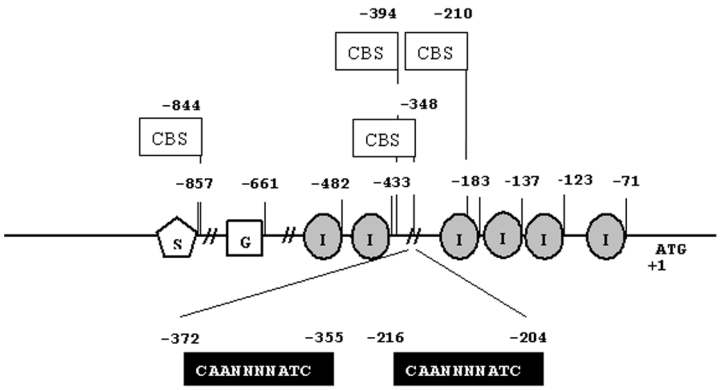

Figure 3. cis-acting elements of *GBSSI* promoter. The number of nucleotides was based on the A base of the initiated codon of translation, and designated as +1. The *GBSSI* promoter contains six I-box-like elements (GATA or GATT) and one G-box (G; CCACGTGGCAC). "S" indicates the sucrose-response element. Black boxes containing CAANNNNATC sequence are the putative clock-controlled elements found in the tomato *Lhc* gene. CBS indicated the CCA1-binding sequence.

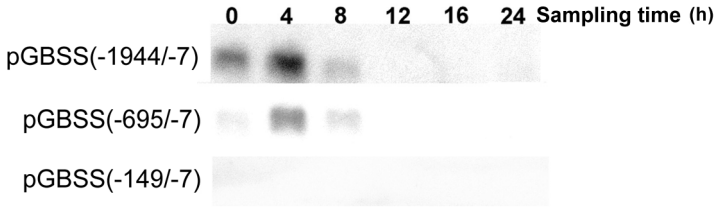

Figure 4. Expression in *Arabidopsis* of a *GUS* reporter gene ligated to a sweet potato *GBSSI* promoter of varying length. Total RNAs (10 μg for each sample) isolated from transgenic *Arabidopsis* leaves that were harvested at 4 h intervals were separated on a 1% formaldehyde-agarose gel and probed with α-^{32}P-labeled *GUS* cDNA.

A typical pattern of circadian rhythm was observed for *GUS* gene in pGBSS(−1944/−7)/GUS transformants. The peak of *GUS* mRNA accumulations was observed 4 h after illumination and decreased rapidly until the end of the diurnal cycle (Figure 4). A similar pattern of circadian expression was also observed in the pGBSS(−695/−7)/GUS transformants (Figure 4). However, the expression of the *GUS* gene in the pGBSS(−149/−7)/GUS transformant could not be detected either by northern hybridization (Figure 4) or by reverse transcriptase–PCR (data not shown).

Circadian expressions of starch synthesis-related genes in Arabidopsis *plants*

Starch synthesis-related genes in leaves of *Arabidopsis* plants also exhibited a circadian rhythm in a manner similar to the *GBSSI* gene of sweet potato leaves when *Arabidopsis* plants were maintained under 16 L/8 D. As shown in Figure 5, accumulation of the *Arabidopsis GBSSI* transcript

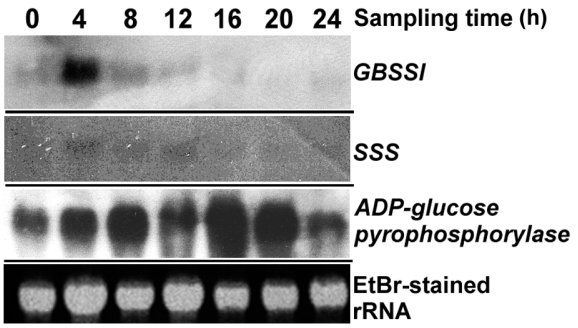

Figure 5. Expression of *Arabidopsis GBSSI*, *SSS* and *ADP-glucose pyrophosphorylase* genes during a diurnal cycle. Total RNA (10 μg for each sample) isolated from *Arabidopsis* leaves that were harvested at the 4 h intervals were separated on a 1% formaldehyde-agarose gel and probed with α-^{32}P-labeled *GBSSI*, *SSS* and *small subunit ADP-glucose pyrophosphorylase* cDNA, respectively.

reached a maximum 4 h after illumination and then it decreased rapidly (Figure 5). A similar circadian rhythm was observed for *soluble starch synthase* (*SSS*) gene expressions in *Arabidopsis* leaves

(Figure 5). Although expression of the small subunit of *ADP-glucose pyrophosphorylase* gene also showed a circadian fluctuation, the pattern was different from that of *GBSSI* and *SSS*. *ADP-glucose pyrophosphorylase* mRNA was present throughout the entire period of the diurnal cycle, but showed a peak at 16 h after the light was turned on (Figure 5).

Discussion

Expression of a *GBSSI* gene and its protein in sweet potato leaves is circadian-regulated by a biological clock that was set by light (Wang et al. 2001). Nuclear run-on assays indicated that the circadian expressions of the *GBSSI* gene in sweet potato leaves were controlled at the transcriptional rate level (Figure 1), and newly synthesized proteins were involved in the regulation (Figure 2). The involvement of *de novo* protein synthesis in regulating the circadian rhythm suggests the presence of specific regulatory element(s) in the promoter of the *GBSSI* gene. The GATA or GATA-like motif termed I-box was identified in the *GBSSI* promoter (Figure 3). The I-box is a conserved motif present in the light/circadian clock-regulated promoter, including *rbcS*, *cab* and *nia* genes (Borello et al. 1993). Teakle and Kay (1995) found an *Arabidopsis* GATA-binding protein, CGF-1. However, Carre and Kay (1995) indicated that although CGF-1 bound to the GATA-motif to modulate the amplitude of circadian oscillation, it was not essential for circadian regulation. In addition to the I-box, the CAANNNNATC homologous element was repeated twice and located at −204 and −355 in the *GBSSI* promoter (Figure 3). This motif was found in the tomato light-harvesting complex (*Lhc*) gene promoter, and was suggested to play an important role for circadian regulation (Piechulla et al. 1998). CCA1 protein was suggested as one of the biological clock components, that was involved in the transcriptional feedback loop of circadian effects (Wang and Tobin 1998; Alabadi et al. 2001). CBS (AATCT) also have been characterized (Wang et al. 1997), and four CBS were located at the sweet potato *GBSSI* gene promoter (Figure 3). In addition to these elements that might be involved in the circadian regulation, a sucrose-response element and a G-box were also located at −857 and −661, respectively, in this promoter. The G-box is conserved in some ABA-regulated gene promoters, including the rice *Rab* and the cotton *Lea* gene (Yamaguchi-Shinozaki et al. 1989; Mundy et al. 1990). The G-box in promoters of plant genes has been shown to respond to environmental stress, and is likely to play a role in the circadian regulation of genes (Xu et al. 2003). The presence of a sucrose-response element was expected, since sucrose was a direct signal to enhance the accumulation of *GBSSI* transcripts (Wang et al. 2001). The sequence TTGACGG is a conserved element in some sucrose-regulated genes of sweet potato (Hattori et al. 1991; Nakamura et al. 1991), and was identified in these two genes using a promoter assay in transgenic tobacco (Maeo et al. 1997).

The transformant of pGBSS(−1944/−7)/GUS contained a promoter with a sucrose-response element, a G-box, six I-box, two CAANNNNATC elements and four CBS (Figure 3). These showed a circadian rhythm for *GUS* mRNA accumulation and were similar to that of the *GBSSI* expression pattern in sweet potato leaves. A circadian rhythm of the *GUS* gene could still be observed in the transgenic plants harboring the pGBSS(−695/−7)/GUS construct in which the sucrose-response element was deleted. This observation supports a hypothesis indicating that sucrose-stimulated *GBSSI* expression in sweet potato leaves was independent to that of the circadian regulation (Wang et al. 2001). When the promoter fragment was further deleted such that it contained only three I-box as in the construct of pGBSS(−149/−7)/GUS, no *GUS* mRNA could be detected in the leaf tissue of the transgenic *Arabidopsis*, indicating the importance of elements between −695 to −149 for the expression of the *GBSSI* gene.

Since transgenic *Arabidopsis* plants were effective in exhibiting a circadian rhythm for the *GUS* gene ligated to the *GBSSI* promoter of sweet potato, transcription factors and other regulatory proteins necessary for controlling the rhythm should be present in *Arabidopsis*. As expected, when the expression of a *GBSSI* gene native to *Arabidopsis* was studied, it also showed a circadian pattern similar to that of sweet potato leaves. In addition, the accumulation of *Arabidopsis GBSSI* mRNA also peaked 4 h after illumination, and it decreased rapidly thereafter (Figure 5). Besides,

SSS also exhibited a circadian pattern in *Arabidopsis* identical to that of the *GBSSI* gene (Figure 5). The observation that both *GBSSI* and *SSS* genes displayed similar patterns suggested that they might be controlled by one or more of the same regulatory factors in leaves during a diurnal cycle. Since GBSSI was the key enzyme for amylose synthesis (Tsai 1974) and SSS was suggested to play a major function for molecular elongation of amylopectin (Tsai 1973; Taylor 1998), the coordination of *GBSSI* and *SSS* expression could contribute to the maintenance of the ratio of amylose and amylopectin during diurnal cycles in leaves. On the other hand, the *ADP-glucose pyrophosphorylase* gene, which controls the quantity of starch by affecting the amount of ADP-glucose substrate (Tsai and Nelson 1966), showed a diurnal fluctuation pattern different from *GBSSI* and *SSS*. The mRNA accumulation peak of the *ADP-glucose pyrophosphorylase small subunit* gene appeared late in the cycle when compared with that of *GBSSI* and *SSS* genes (Figure 5). These studies suggest that a circadian rhythm of starch accumulation observed in the leaf tissue of several plant species (Li et al. 1992; Mérida et al. 1999; Wang et al. 2001) may be regulated at several steps in the starch synthetic pathway. In addition to the circadian regulation of genes related to starch synthesis, clock-mediated regulations were observed on the expressions of a cluster of starch mobilization genes in *Arabidopsis* plants (Harmer et al. 2000). Therefore, these studies suggest that the circadian clock might play a major role in coordinating the carbon redistribution between starch and sucrose in leaf tissue.

Acknowledgements

Thanks to Dr Jychian Chen for his kind gift of *Arabidopsis SSS* and *ADP-glucose pyrophosphorylase* cDNA as DNA probes. This research was supported by a grant NSC 89-2311-B-002-007 from the National Science Council of the Republic of China.

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