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Circadian Expressions of Starch Branching Enzyme Gene in Sweet Potato Leaves

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Diurnal changes of transcript levels of starch branching enzyme (SBE) involved in building up of amylopectin structure were detected in sweet potato leaves during 16 h light/ 8 h dark (16 L/8 D) photoperiod cycles, and the accumulation peak of SBE mRNA presented at the end of light period. Furthermore, the fluctuation of the transcript levels in leaves was still remained when the 16 L/8 D adapted plants were shifted to the 24-h constant light condition, just the periodic length of gene expression in constant light was slightly shorter than that in 16 L/8 D. These results indicated that the diurnal oscillation of SBE transcripts could be controlled by an endogenous biological clock. However, the expression of SBE gene presented the different diurnal rhythm with that of granule-bound starch synthase I gene that was in charge of amylose synthesis. To understand the circadian regulations of SBE gene, an effort was made to isolate and characterize the SBE promoter. Several putative circadian and light regulatory elements were found in the SBE promoter sequence.

Key words: circadian clock, circadian regulatory elements, starch branching enzyme, sweet potato, transitory starch

Introduction

Transitory starch synthesized in leaves is the end product of photosynthesis, and it would be conversed to sucrose in dark for exporting to storage organs or providing energy for plant growth [1]. Starch is a complex consisting of amylose, linear α-1,4 linkage glucan chains, and highly branched amylopectin. Three groups of enzymes, ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS) and starch branching enzyme (SBE), have been characterized to involve in starch synthesis. AGPase is responsible for generation of ADP-glucose provided as the substrate for starch polymerization. SS plays a role to elongate the glucan chains, and it is classified to two major forms, granule-bound starch synthase (GBSS) and soluble starch synthase (SSS), according their distributions in amyloplasts. *SBE* is the key enzyme for building up the structure of amylopectin. It catalyzes the cleaving of partial α-1,4 linkage glucan chains and re-linking the chain by α-1,6 bonds to form the branch chains of

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amylopectin [2]. In spite of the functions of starch synthetic enzymes have been well established, little was known about the coordination of genes encoded these proteins for starch synthesis. Especially, the regulatory mechanisms of transitory starch synthesis in leaves during diurnal cycles still need to be elucidated.

It has been found the synthesis of transitory starch in plant leaves could be regulated by a circadian clock [3,4]. Circadian oscillators have been demonstrated to control several metabolic reactions and gene expressions in plants [5]. Furthermore, some genes encoded the components of biological clocks or transcription factors involved in the circadian regulations have been identified, e.g. circadian clock-associated 1 (CCA1) has been found as a transcription factor to control the circadian rhythm of *light-harvesting complex (LHC)* gene expressions [6]. Circadian rhythm of starch accumulation during diurnal cycles in sweet potato leaves has been presented in our previous report, and the circadian expression of a gene encoded GBSSI responsible for amylose synthesis has also been performed [4,7]. However, the circadian regulation of starch synthesis in leaves has not been fully revealed. Although diurnal changes of some starch synthetic gene expressions have been observed [8], only *GBSSI* and *GBSSII* have been identified that the fluctuations of expressions were circadian oscillators-controlled [7,9,10]. It is still unclear about the regulatory mechanisms of other starch synthetic genes in leaves during diurnal cycles. In this study, it was attempted to analyze the expression of *SBE* gene in sweet potato leaves and identify whether the expressions of *SBE* was controlled by a circadian clock.

Materials and Methods

Plant material

Sweet potato (*Ipomoea batatas* Lam. cv. Tainong 57) was grown at 28 ˚C in a growth chamber under 16 L /8 D. For treatments with different photoperiods, the plants were moved to a separated growth chamber with 24-h light (LL) after entraining under 16 L/8 D at least for two weeks. Leaf samples were harvested at time intervals indicated.

RNA gel blot and semiquantitative reverse transcriptase-PCR (RT-PCR) analysis

Total RNA was isolated from sweet potato leaves using Trizol reagent (Invitrogen, CA, USA). RNA gel blot analysis were used the digoxigenin (DIG)-labeled partial *SBE, GBSSI* and *LHC* cDNA as probes and processed according to the DIG application guide for filter hybridization (Roche, Penzberg, Germany). Semiquantitative RT-PCR analysis was performed by OneStep RT-PCR Kit included Omniscript/ Sensiscript Reverse Transcriptases mix and HotStar Taq polymerase following the instruction (Qiagen, CA, USA).

Genomic DNA walking for finding the SBE promoter

Genomic DNA was extracted from tuberous roots of sweet potato using Plant DNAzol (Invitrogen, CA, USA). The 5' upstream sequence starting from the open reading frame of sweet potato *SBE* was isolated by using the GenomeWalker™ kit (BD Biosciences Clontech, CA, USA). The *SBE* specific primers for primary and secondary PCR are 5'-TGATAATGCCTCAGCAACCTCTAACTG ATC-3' and 5'-GATGATCCCTCGCCCTCGCCACCAGG AACA-3', respectively, designed according to the sweet potato *SBE* cDNA sequence (accession number: DQ066739). The putative *cis*-elements in promoter were characterized with the Database of Plant *Cis*-acting Regulatory DNA Elements (PLACE)

[11].

Results and Discussion

There are several isoforms of *SBE* were isolated from various plant species, respectively, and they are in charge of making the different length of branch chain of amylopectin [12]. However, only one *SBE* gene was isolated from sweet potato, and there is no information about its expression and regulation. To study the effect of photoperiod on *SBE* gene expression in sweet potato leaves, the expression pattern of the *SBE* transcript was determined from plants grown under 16 L/8 D. RNA gel blot analysis showed the *SBE* transcripts presented regular fluctuations during 16 L/8 D cycles, and the mRNA accumulation peaked at the end of light period (Fig. 1). *GBSSI* is one of GBSS isoform, also known as WAXY, and it is the key enzyme responsible for amylose synthesis [13,14]. Even the *GBSSI* expression during diurnal cycles was observed previously [7]; it was analyzed again and presented here for comparing the expression pattern with that of *SBE* transcripts. *GBSSI* highly expressed after light was turned on for 4 h, and then the expressions were decreased gradually until the end of the cycles (Fig. 1). In addition, the transcripts of *LHC*, a gene encoding the major component of photosynthetic systems, were increased rapidly at the early light period and decreased at dark period (Fig. 1). The expression pattern of *LHC* was similar to that of *GBSSI*. Although the expression pattern of *SBE* was different with that of *GBSSI* and *LHC*, generally all of them were highly expressed during the daytime and then

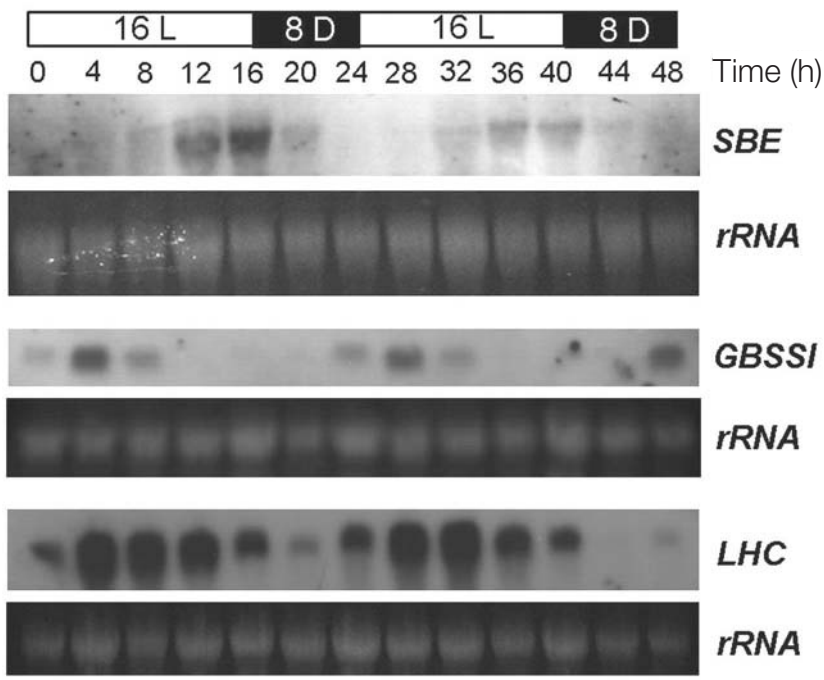

Fig. 1. Expressions of sweet potato SBE, *GBSSI* and *LHC* genes during diurnal cycles. Plants were maintained in 16 L/8 D with light turned on at 6:00 a.m. (e.g. sampling time 0 and 24). Total RNA (10 μg for each sample) of sweet potato leaves harvested at the 4 h intervals were separated on a 1% formaldehyde-agarose gel and probed with DIG-labeled partial SBE, *GBSSI* and *LHC* cDNA, respectively. EtBr-stained 28S rRNA patterns were used as RNA loading control.

reduced the transcript levels in dark. The diurnal changes of these starch synthetic and photosynthetic gene expressions in sweet potato were similar to that were observed in *Arabidopsis* leaves [8,15]. Understanding the regulatory mechanism of the genes involved in carbohydrate metabolism during diurnal cycles may help to realize the regulatory mechanism of the coordination between the carbon fixation and assimilation in leaves. *GBSSI* is the protein tightly associated the starch granules. Since the starch granules were degraded at dark period, the *GBSSI* proteins were digested. Therefore, it was suggested the *GBSSI* expression at early morning for protein resynthesizing [8]; the diurnal oscillation of *GBSSI* protein as the same as the transcripts in sweet potato leaves supported this hypothesis [4]. The expression of *SBE* at late light period following that of *GBSSI* indicated the branch chains of amylopectin could be formed later than amylose synthesized.

Since it was demonstrated that the diurnal expressions of *GBSSI* and *GBSSII* genes were controlled by a biological clock and related to the diurnal changes of starch content in leaves [7,9,10], it was suggested that the endogenous oscillator could play an important role to coordinate the expressions of genes encoded the proteins involved in the starch synthetic pathway. Therefore, it was interesting to know whether the diurnal change of *SBE* expression was controlled by environmental stimuli or circadian rhythm. To achieve this, the 16 L/8 D photoperiod adapted plants were shifted to 24-h constant light (LL) before the *SBE* transcripts levels were determined. The data showed the *SBE* expression pattern in LL was similar to that in day-night cycles (Fig. 2), and this result suggested that the diurnal changes of *SBE* expression were regulated by a circadian clock. The periodic length of *SBE* expressions under LL was slightly shorter than that presented under 16 L/8 D (Fig. 2), and this phenomenon was consistent with our previous observation on *GBSSI* [4]. The periodic length of circadian rhythm under constant conditions was often different with that were investigated in light-dark cycles [16].

To understand the regulatory mechanism of *SBE* gene, the upstream sequence of 988 bps starting from the coding region of *SBE* was isolated (accession number: DQ066740). After sequence characterizing, numerous regulatory elements were found in *SBE* promoter (Fig. 3). Since the *SBE* expression could be regulated by a circadian clock, as expect, there were several clock control-related elements were found in *SBE* promoter. Such as three CCA1 binding sites (CBS; AATCT) were located at -211, -316 and -560, respectively. "CAANNNNATC" was an important motif identified in tomato *LHC* promoters to involve in circadian regulation [17], and this element was also

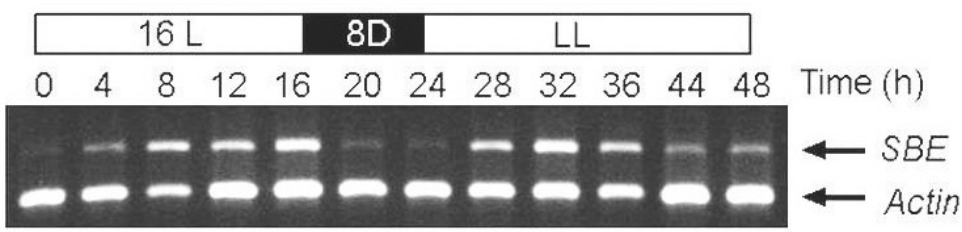

Fig. 2. Circadian expressions of *SBE* gene under LL condition. Sweet potato plants were moved to LL conditions after entraining under 16 L/8 D. Leaf samples were harvested at the 4 h intervals and *SBE* expressions were determined by semiquantitative RT-PCR. *Actin* was used as an internal control to normalize for variations in RNA template amounts and for RT-PCR reaction efficiency.

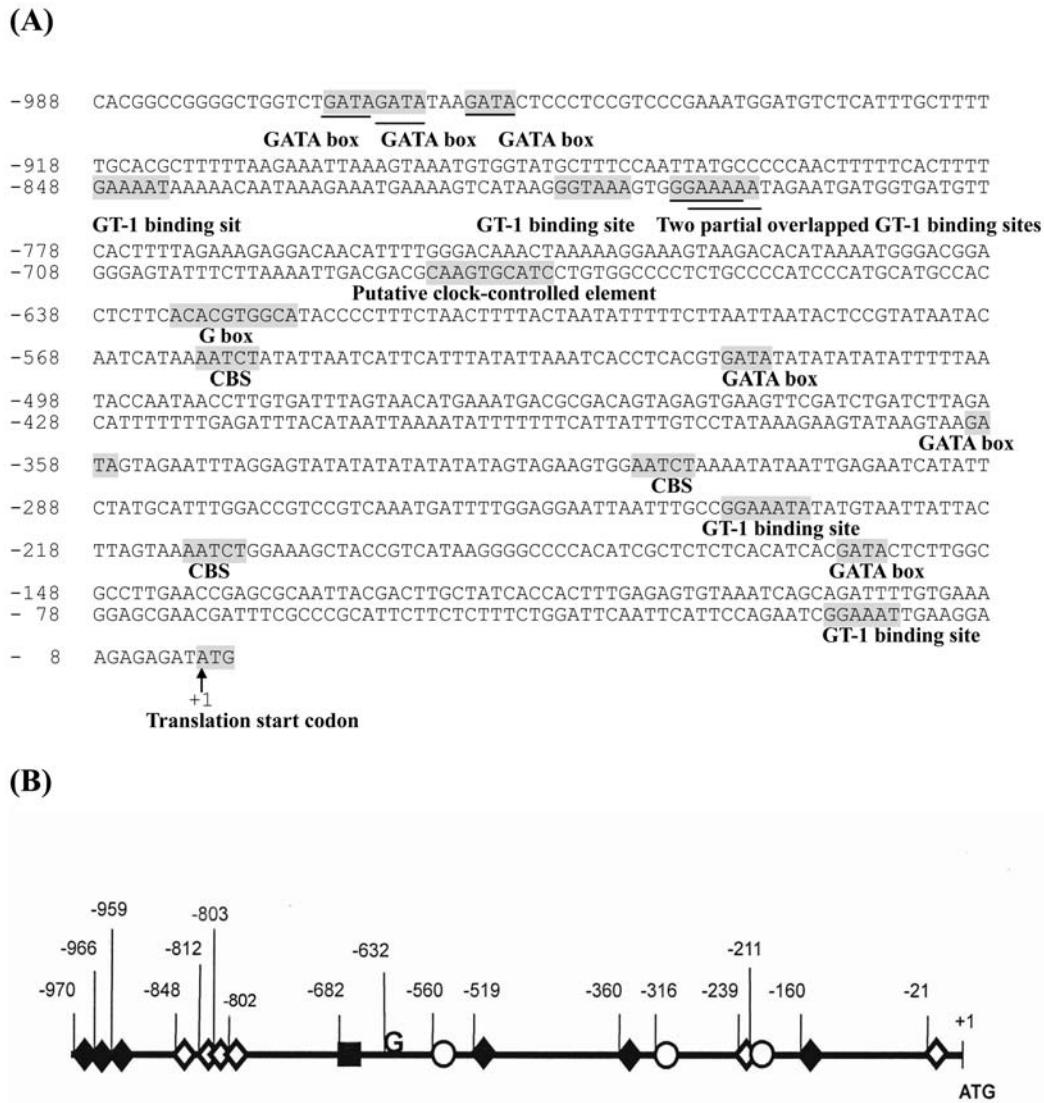

Fig. 3. *Cis*-acting elements related light/circadian clock regulations in *SBE* promoter. (A) The sequence of the *SBE* promoter. (B) The light/circadian regulatory elements in *SBE* promoter. The number of nucleotides was based on the A base of the initiated codon of translation, and designated as +1. Circules indicated the CCA1-binding site (CBS). Black sequare containing CAANNNNATC sequence is the putative clock-controlled element. The *SBE* promoter also contains six GATA boxes showed as black diamands , six GT-1 binding sites showed as white diamands and one G box indicated as G.

found at -682 of the *SBE* promoter. In addition, the GATA-box (or I-box) is a conserved element presented in several light or circadian oscillator-regulated promoters, e.g. *Rubisco* and *GBSSI* promoter [18,19], and it could modulate the amplitude of circadian rhythm [20]. There are six GATA-boxes located in *SBE* promoter. Some other light/phytochrome-responsive elements included GT1-binding site and G-box (CACGTG) [21,22] also presented in the promoter. All of these elements were also

investigated in the *GBSSI* promoter of sweet potato [19]. However, the circadian rhythm of *GBSSI* and *SBE* expression patterns were different, hence, it could not be ruled out there are other clock-controlled elements related to the circadian regulation of *GBSSI* and *SBE*, respectively, have not been identified. Since the *GBSSI* and *SBE* were the key enzymes for amylose and amlyopectin synthesis, the coordination of *GBSSI* and *SBE* expression could be important to establish the starch structure during diurnal cycles. Biological clock may play one of the factors to coordinate these gene expressions.

In conclusion, the diurnal changes of the expression of *SBE*, *GBSSI* and *LHC* genes in sweet potato leaves were investigated in this study. The fluctuation of *SBE* expression during diurnal cycles was regulated by a circadian clock, and the *SBE* gene was highly expressed at the end of light time. On the other hand, the accumulation of *GBSSI* and *LHC* transcripts were peaked at early light period. Moreover, the *SBE* promoter was isolated, and several light/circadian clock-regulated elements were found in the sequence. In the future, functional analysis of *SBE* and *GBSSI* promoters would be helpful to understand how endogenous oscillator(s) coordinate the circadian expressions of the genes involved in starch synthetic pathway.

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