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Regulations of granule-bound starch synthase I gene expression in rice leaves by temperature and drought stress

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Abstract

Effects of temperature (15/10, 25/20, 30/25, and 35/30 °C) and drought stresses on the expression of *granule-bound starch synthase I* (*GBSSI)* gene were examined in rice (*Oryza sativa* L.) seedlings. The *GBSSI* expression was higher at the low temperature (15/10 °C), and the transcript level decreased at temperatures higher than 30 °C. Protein phosphorylation was involved in the low temperature-stimulated signal transduction of *GBSSI* regulation. The expression of *GBSSI* in rice seedling was reduced under a drought stress. Even though exogenous ABA played a role to reduce the *GBSSI* transcript accumulation under non-stress condition, the reducing of *GBSSI* expression by drought stress appeared to be mediated by an ABA-independent pathway.

Additional key words: abscisic acid, *Oryza sativa*.

Introduction

Accumulation of starch has been recognized as a major factor for determining the yield of agronomic crops. Starch is a polymer complex consisting of amylose and amylopectin. In general, the ratio of amylose to amylopectin is about 1:3, but the ratio vary in different plant species (Manners 1985). Starch synthase is a group of important enzymes involved in the synthesis of amylose and amylopectin, and it may be further classified into two major types according to their distributions in the amyloplast, namely granule-bound starch synthase (GBSS) and soluble starch synthase (SSS). For GBSS, there are two isoforms, GBSSI and GBSSII. GBSSI, also known as the WAXY protein, is tightly bound to starch granules and has a molecular mass of 58 - 60 kDa. This enzyme provides the largest proportion of total GBSS activity (Dry *et al.* 1992), and is the major enzyme responsible for amylose synthesis (Tsai 1974). The role of SSS is not clear; however, this enzyme also contains several isoforms, SSI, SSII, SSIII and SSIV (Marshall *et al.* 1996, Dian *et al.* 2005).

Environmental factors are also important in affecting starch quality and quantity. Water deficits up to -0.72 MPa stimulated sucrose synthesis and decreased starch synthesis in potato tubers (Geigenberger *et al.* 1999a,b). Temperature stress also reduced starch content in wheat grains (Chinnusamy and Khanna-Chopra 2003). A decrease in the activity of SSS under high temperatures was observed in wheat (Keeling *et al.* 1993), but not in maize and rice (Cao *et al.* 2000, Jiang *et al.* 2003). However, decreases in activities of ADP-glucose pyrophosphorylase has been observed both in maize kernels and wheat endosperms under heat stresses, thus resulting in a reduction of starch synthesis (Keeling *et al.* 1993, Wilhelm *et al.* 1999). It has been shown that the amylose content in rice grains was reduced under a heat stress, but increased at cool conditions (Asaoka *et al.* 1985, Hirano and Sano 1998). Besides, the branch chain pattern of amylopectin could also be changed by temperatures, and it was suggested that a reduced activity of branching enzyme at high temperatures is a significant factor limiting the branching frequency (Jiang *et al.* 2003). Although environmental factors have significant effects on starch synthesis, their regulatory mechanisms remain unclear.

In order to better understand the effect of abiotic stresses on starch synthesis, *GBSSI* was used as a marker gene to study its expression and regulation in rice seedlings under temperature and drought stresses.

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Abbreviations: ABA - abscisic acid; CHX - cycloheximide; GBSSI - granule-bound starch synthase I; SSS - soluble starch synthase.
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Materials and methods

Plants and stress treatments: Rice (*Oryza sativa* L. cv. Tainung 67) seeds were sterilized in 2.65 % sodium hypochlorite with Tween 20 for 15 min and subsequently washed with distilled H_2O for three times. Seeds were then germinated at 28 °C in dark for 2 d, and then they were moved to the phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) for growing in half-strength Kimura B nutrition solution (Chu and Lee 1989) at 30/25 °C under the natural light and 90 % of relative humidity. The seedlings with 3 leaves were moved to other phytotrons with various temperatures, *i.e.*, 15/10, 25/20 or 35/30 °C for 24, 48 and 72 h, respectively. Seedlings left at 30/25 °C were used as the control. For drought stress treatments, rice seedlings were removed from medium and desiccated in Petri dishes with filter paper for various periods at 30/25 °C (Seki *et al.* 2002). For detection the effect of abscisic acid (ABA) on *GBSSI* expression, rice seedlings were cultured on 50, 100 and 200 μM ABA - contained Kimura B solution for 24 h before the leaf samples were collected for *GBSSI* expression analysis. To analyze the role of ABA on *GBSSI* expression under a drought stress condition, rice seedlings were pre-cultured with 200 μM ABA or 200 μM fluridone (an ABA-synthesis inhibitor) - contained nutrition solutions for 24 h before the air-drying treatment for 12 h. To evaluate effects of various inhibitors on protein synthesis, protein kinases and phosphatases on the low temperature-stimulated *GBSSI* expression, rice seedlings were pretreated with various inhibitors contained nutrition solutions for 1 h before the low temperature treatment. The final concentrations of inhibitors of protein synthesis, kinase and phosphatase were 100 μM, 1 μM and 0.5 μM, respectively.

RNA extraction from rice leaves: RNA was isolated from rice leaves using *Trizol* reagent (*Invitrogen*, CA, USA). The second and third leaves (counting leaves were began from the leaf next to coleoptile as the first leaf) were harvested, and 100 mg of leaves was homogenized in 1 cm^3 *Trizol* reagent before centrifuging at 10 000 *g*. The supernatant was treated with 0.2 cm^3 chloroform, and shake for 15 s before incubating at room temperature for 3 min. The upper layer solution was transferred to a new tube after centrifugation at 12 000 *g* for 15 min at 4 °C. RNA was precipitated with 0.5 cm^3 isopropanol and incubated for 10 min at room temperature. After centrifugation, the pellet was dissolved in H_2O completely.

Northern blot analysis: Total RNAs (10 μg) were separated on 1 % formaldehyde-agarose gels and transferred to the nylon membrane. The partial *GBSSI* cDNA probe was radioactively labeled with α-^{32}P-dCTP using a random primer labeling kit (*Amersham Biosciences*, Buckinghamshire, UK). After hybridization, the membrane was washed twice with 2× SSC (1 dm^3 of 20× SSC stock solution contained 175.3 g of NaCl and 88.2 g of sodium citrate, pH 7.0) containing 0.1 % (m/v) SDS at room temperature for 30 min and twice with 0.1× SSC containing 0.1 % (m/v) SDS at 55 °C for 30 min. EtBr-stained rRNA patterns were used as internal standards in all Northern blot analysis data. Accumulation of *GBSSI* mRNA was quantified from the Northern blot using image analysis system (Wang *et al.* 2001).

Results and discussion

Effects of temperature on the GBSSI expression: Temperature is an important factor to determine crop growth and development. For example, high temperature reduced the seed germination percentage and rate in pearl millet and maize; moreover, heat stress also significantly affected the vegetative growth of maize (Ashraf and Hafeez 2004). Temperature stress affected gene expressions were also observed in several species (Sun *et al.* 2002, Shinozaki *et al.* 2003). In this study report, Northern blot hybridization showed that the expression of *GBSSI* gene was higher at the low temperature (15/10 °C) than at the control (30/25 °C) or high temperature (35/30 °C) treatment for 24 h (Fig. 1). The differences became more significant as the treatment was extended to 72 h (Fig. 1). Basically, this result was consistent with the observation where amylose content in rice endosperm was found to increase at cool conditions (Asaoka *et al.* 1985). Increase in the GBSSI activity at low-temperature presumably would cause the accumulation of more amylose during grain development, thus reducing the grain quality (Suzuki *et al.* 2002). Although a rice mutant insensitive to cool temperatures on amylose synthesis has been isolated (Suzuki *et al.* 2002), which could facilitate the study of temperature effect, the molecular mechanism of low-temperature regulating *GBSSI* expression is still unclear. In order to study the regulatory mechanism of low-temperature effect on *GBSSI* gene expression,

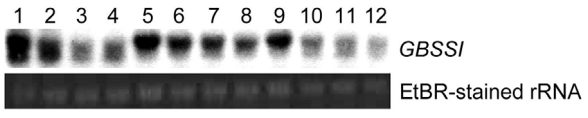

Fig. 1. Effects of temperature on the *GBSSI* expression in rice leaves. Rice seedlings with three leaves were moved from 30/25 °C to 15/10, 25/20, and 35/30 °C, respectively. After different temperature-treatment for 24 (*lane 1 - 4*), 48 (*lane 5 - 8*) and 72 h (*lane 9 - 12*), the RNAs were extracted from leaves. *GBSSI* expressions were determined by Northern blot hybridization with α-^{32}P-dCTP-labeled partial *GBSSI* cDNA as a probe. Seedlings were exposed at 15/10 °C (*lane 1, 5, 9*), at 25/20 °C (*lane 2, 6,* and *10*), at 30/25 °C (*lane 3, 7* and *11*), and at 35/30 °C (*lane 4, 8* and *12*) before samples were collected.

several protein-synthesis and protein-modification inhibitors were applied to culture media. A pretreatment with 100 μM cycloheximide (CHX) for 1 h had no significant effect on the expression of *GBSSI* gene stimulated by the low temperature (Fig. 2*A*). This result

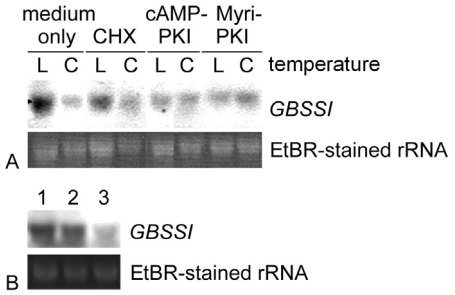

Fig. 2. Northern blot analysis of the low temperature-stimulated *GBSSI* expressions following treatments with cycloheximide (CHX), protein kinase or phosphatase inhibitors. *A* - Effects of CHX and protein kinase inhibitors on the low temperature-stimulated *GBSSI* gene expressions. Rice seedlings were pretreated for 1 h with 100 μM of CHX, 1 μM cAMP-dependent protein kinase inhibitor (cAMP-PKI) or 1 μM myristoylated protein kinase C inhibitor (Myri-PKI), before transferring to the 15/10 °C. After the low temperature treatment for 24 h, the RNA was extracted from leaves. L - indicated low temperature (15/10 °C) treatment. C - indicated control temperature (30/25 °C) treatment. *B* - Effect of phosphatase inhibitors on the low temperature-stimulated *GBSSI* gene expressions. Rice seedlings were pretreated with 0.5 μM okadaic acid for 1 h before they were moved to the 15/10 °C (*lane 1*) and 25/20 °C (*lane 2*), respectively. Rice seedlings were maintained in normal medium at 30/25 °C (*lane 3*) as the control. *GBSSI* mRNA was determined by the Northern blot hybridization.

suggested that synthesis of *de novo* protein was not necessary for regulating the transcription of *GBSSI* gene at low-temperature environments. Therefore, modifications of pre-existed proteins might be involved in the signal transduction of temperature-regulated *GBSSI* transcription. For analyzing whether protein phosphorylation was involved in the low temperature-related signal transduction for *GBSSI* gene expressions, effects of cAMP-dependent protein kinase inhibitor and myristoylated protein kinase C inhibitor were examined. The results showed that the low temperature effect on *GBSSI* gene expression was repressed by these two kinds of protein kinase inhibitors (Fig. 2*A*). On the other hand, okadaic acid, a selective inhibitor of protein phosphatase types 1 and 2A (Cohen 1989), did not affect the *GBSSI* expression response to low temperature (Fig. 2*B*). These results suggested an involvement of protein phosphorylation in the transduction pathway. However, we could not rule out the possibility that changes in *GBSSI* expression due to inhibitor treatments might be the result of an indirect effect, especially since GBSSI is the final enzyme in the biosynthetic pathway of starch.

Effects of drought stress on the GBSSI expression in rice leaves: While low temperatures appeared to stimulate *GBSSI* expressions, *GBSSI* transcript accumulation was found to be reduced under a drought stress (Fig. 3). The *GBSSI* expression in the control condition showed a fluctuation pattern during a diurnal cycle. The peak of *GBSSI* accumulation was observed in the morning (08:00), and then the expression of *GBSSI* decreased gradually (Fig. 3). This pattern was similar

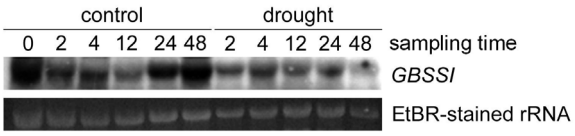

Fig. 3. Effects of drought stress on the *GBSSI* expression in rice leaves. Rice seedlings were treated with drought stress by air-drying for 2, 4, 12, 24 and 48 h, respectively. The control samples were cultured in the culture medium, and the leaf samples were harvested from 08:00 (sampling time was indicated as 0). Expressions of *GBSSI* gene were determined by Northern blot hybridization.

to the circadian expression of *GBSSI* gene found in sweet potato leaves (Wang *et al.* 1999, 2001, 2004) and snapdragon (Mérida *et al.* 1999). In addition to *GBSSI* gene, the circadian expression of *GBSSII* was also been demonstrated in rice (Dian *et al.* 2003). Besides, the diurnal activity changes of sucrose-phosphate synthase, a key enzyme for sucrose and starch metabolism, have been observed in *Prosopis juliflora* leaves (Pathre *et al*. 2004). But the fluctuation of sucrose-phosphate synthase activity was controlled by environmental factors, was not regulated by circadian clock. Under the drought stress, the expression of *GBSSI* gene could still be detected until air-drying for 48 h; however, the expression levels under drought were lower than that of the control, and also appeared to abolish the circadian expression of *GBSSI* (Fig. 3). Since ADP-glucose pyrophosphorylase was also decreased under water stress in potato (Geigenberger *et al.* 1999a), it was suggested that starch synthesis could be affected by drought stress.

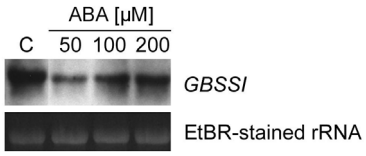

Fig. 4. Effects of ABA on the *GBSSI* expression in rice leaves. Rice seedlings were cultured in 50, 100, and 200 μM ABA-contained medium for 24 h before the leaf samples were collected. *GBSSI* mRNA was determined by Northern blot hybridization. C - indicated the control rice seedlings cultured in normal condition.

Many genes responded to drought stress have been observed, and both ABA-dependent and ABA-independent regulatory pathway were involved in

controlling the expression of drought stress-responsive genes (Shinozaki and Yamaguchi-Shinozaki 2000, Xiong *et al.* 2002). Two major *cis*-acting elements, ABA-responsive element (ABRE) and dehydration-responsive element (DRE)/C-repeat (CRT), have been identified in the promoter of drought-responsive genes with the ABRE element functioning in the ABA-dependent and DRE/CRT in the ABA-independent pathway (Shinozaki *et al.* 2003). In this study, rice seedlings were treated with different concentration of ABA for 24 h, and following the *GBSSI* expression was determined. The result showed that the accumulation of *GBSSI* transcript was reduced by ABA treatment (Fig. 4). In order to determine whether the signal transduction of drought-related *GBSSI* expression was ABA-dependent or independent, ABA and ABA biosynthesis inhibitor, fluridone, were applied to culture medium before seedlings were treated with drought stress. The result indicated that exogenous ABA slightly enhanced the drought effect on *GBSSI* expression (Fig. 5, *lane 3*); however, fluridone was inefficient to improve the effect of drought stress on *GBSSI* expression (Fig. 5, *lane 4*). Since fluridone (200 μM) was shown to reduce the ABA synthesis in rice seedlings (Hsu and Kao 2003), the ineffectiveness of fluridone to affect the drought effect on *GBSSI* expression might not be the result of inability of fluridone to reduced the amount of endogenous ABA. Therefore, the result suggested that the signal transduction of drought stress to affect the *GBSSI* expression might be ABA-independent. However, we could not rule out the possibility that the effect of drought stress on *GBSSI* expression might be coordinated between both ABA-dependent and ABA-independent pathway; in this case, the drought effect could not be recovered when only the ABA-dependent pathway was blocked. In the future, studies on the interaction between rice *GBSSI* promoter and stress-responsive transcription factors should be helpful to furthermore elucidate the regulatory mechanism of *GBSSI* expression controlled by environmental and ABA stresses.

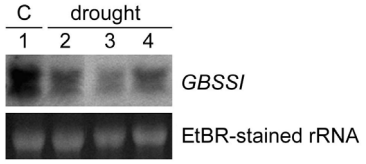

Fig. 5. ABA effects on the drought-regulated *GBSSI* expression in rice seedling. *Lane 1* - rice seedlings were cultured in the normal medium as controls. *Lanes 2*, *3* and *4* - rice seedlings were treated with drought stress. *Lanes 3* and *4* - seedlings pretreated with 200 μM ABA and 200 μM fluridone, respectively, for 24 h before the seedlings were treated with drought. *GBSSI* mRNA was determined by Northern blot hybridization. C - indicated the control sample that was kept in culture medium. Drought indicated that seedlings were air-dried for 12 h before the leaf samples were harvested.

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