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ORIGINAL PAPER

Molecular regulation of starch accumulation in rice seedling leaves in response to salt stress

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Abstract In this report we show that the starch content decreased in NaCl-stressed rice (*Oryza sativa* L.) seedling leaves during the daytime. Because photosynthetic efficiency and starch degradation enzyme activity were not significantly affected by the high salt, it is likely that this effect results from repression of starch biosynthesis. To determine the regulatory mechanism, the activities of enzymes such as ADP-glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthase (SSS) and starch branching enzyme (SBE) involved in starch synthetic pathway were examined. Data suggest that NaCl-induced repression of GBSS activity was the most significant factor reducing starch accumulation. Based on real-time RT-PCR analysis, the effect of salinity on GBSS expression was primarily controlled on the transcriptional level. Furthermore, the salt-induced decrease of both *GBSSI* and *GBSSII* gene expressions could be mostly contributed by ion-specific effect and not by osmotic stress. Although the mRNA accumulation of *GBSSI* and *GBSSII* can be down-regulated by exogenous ABA, the negative influence of salt stress on *GBSSI* and *II* gene expression could be chiefly mediated via an ABA-independent pathway.

Keywords Abscisic acid · Granule-bound starch synthase · *Oryza sativa* · Salt stress · Starch

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Abbreviations

ABA	Abscisic acid
AGPase	ADP-glucose pyrophosphorylase
GBSS	Granule-bound starch synthase
PEG	Polyethylene glycol
SBE	Starch branching enzyme
SSS	Soluble starch synthase

Introduction

Soil salinity is one of the key environmental factors that limit crop growth and agricultural productivity. Several physiological pathways, i.e., photosynthesis, respiration, nitrogen fixation and carbohydrate metabolism have been observed to be affected by high salinity (Soussi et al. 1998). Starch serves as a carbon and energy source for seed germination, seedling establishment and plant growth. Moreover, it is a dominant factor in crop yield. Changes in the starch content of plant tissues in the presence of high NaCl have been reported (Rathert 1985). However, little is known about the molecular mechanism by which NaCl regulates starch accumulation.

Three groups of enzymes are involved in starch biosynthesis: ADP-glucose pyrophosphorylase (AGPase), starch synthase and starch branching enzyme (SBE). AGPase is a heterotetrameric enzyme composed of two each large and small subunit. It is the enzyme that catalyzes the production of ADP-glucose, which functions as a glucosyl donor to elongate the amylose and amylopectin chains. The enzyme activity is allosterically regulated by 3-phosphoglyceric acid and inorganic orthophosphate balance (Hwang et al. 2005). Starch synthases are responsible for incorporating the glucose unit provided by ADP-glucose into starch

molecules. Based on their protein localization in amyloplast/chloroplast, starch synthases are classified into two types: granule-bound starch synthase (GBSS) and soluble starch synthase (SSS). GBSS is tightly bound on starch granules. Two GBSS isoforms, GBSSI and GBSSII, have been identified in several plants (Edwards et al. 1996; Hylton et al. 1996). GBSSI is also named WAXY protein, and it controls amylose synthesis in plant storage organs (Tsai 1974; Visser et al. 1991). GBSSII is the major bound form of starch synthase in non-storage tissues (Vrinten and Nakamura 2000). The nucleotide sequence of rice *GBSSII* shares around 70% homology with that of *GBSSI* (Hirose and Terao 2004). The SSS is localized in plastidic stroma and contributes to elongation of the branching chains in amylopectin. There are at least four kinds of SSS isoforms in rice (SSSI, SSSII, SSSIII and SSSIV) and each isoform is encoded by a small gene family (Hirose and Terao 2004). SBE functions to form α-1,6 glucan branch chains of amylopectin. Currently, three distinct classes of SBE (SBEI, SBEIII and SBEIV) have been identified in rice (Nakamura et al. 1992; Mizuno et al. 1993). According to analyses of mutants and transgenic plants, it is well known that expressions of these starch-synthesis enzymes determine both starch content and structure (Visser et al. 1991; Craig et al. 1998).

Rice plant growth is particularly sensitive to salinity at the seedling stage (Maas and Hoffman 1977). The aim of this study was to clarify the regulatory mechanisms governing starch accumulation in the leaves of young rice seedlings under high-NaCl condition. To achieve this, the effect of salinity on the activities of enzymes involved in starch biosynthesis was investigated. Expression of the genes encoding starch synthesis enzymes was then analyzed to gain insight into the molecular mechanisms underlying starch synthesis. An additional goal was to determine whether the effect of high-NaCl on gene expression was caused by an ion-specific event or by osmotic stress. Finally, in order to understand the salt stress-signal transduction for controlling starch-synthesis genes, the effect of ABA on NaCl-regulated gene expressions was discussed.

Materials and methods

Plant materials and treatments

Rice (*Oryza sativa* L. cv. Tainung 67) seeds were sterilized in a 2.65% sodium hypochlorite solution with Tween 20 for 15 min and subsequently washed three times with distilled H_2O. They were germinated at 37°C in the dark for two days and then moved to the phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan), where they continued to grow in a half-strength Kimura B nutrient solution (Chu and Lee 1989) at 30/25°C in natural daylight. For salt or osmotic stress treatments, 3-leaf-old seedlings were transferred to a medium containing either 200 mM NaCl or 20% (w/v) polyethylene glycol (PEG, average molecular weight 6,000) for 24 h. The starch content, chlorophyll fluorescence, enzyme activity, and gene expressions were then analyzed in the third leaf above the coleoptile. The 20% (w/v) PEG was iso-osmotic with the medium containing 200 mM NaCl (Ueda et al. 2004).

To determine the effect of ABA on starch-synthesis gene expressions, rice seedlings were cultured for 24 h in a kimura B solution containing 100 μM ABA. Leaf samples were then collected for gene expression analysis. To analyze the effect of ABA on the salinity-related signal transduction for regulation of gene expressions, ABA biosynthesis was inhibited with fluridone (Yoshioka et al. 1998). Rice seedlings were pre-cultured in nutrient solution containing 200 μM fluridone for 2 h. They then received a 24-h treatment with a solution containing 200 mM NaCl plus fluridone. For all experiments, three replicates were processed and the final data are presented as the mean ± SE.

Measurement of chlorophyll fluorescence

The efficiency of photosynthesis was determined by chlorophyll fluorescence, measured with a Chlorophyll Fluorometer (WALZ) according to the method of Oquist and Wass (1988). Three-leaf-old seedlings were kept in darkness for 30 min before recording the fluorescence of the third leaves.

Analysis of starch content

For starch extraction, leaf samples (1 g) were ground with liquid nitrogen, and the sample powder was extracted twice with 5 ml of 80% (v/v) ethanol at 80°C for 5 min. After centrifugation at 3,000×*g* for 5 min, samples were washed twice with H_2O at room temperature. Each sample was re-suspended with 3 ml H_2O and boiled for 2 h for starch content analysis. Amyloglucosidase and pullulanase were used to digest starch samples at 55°C for 3 h. Samples were then boiled for 5 min to stop enzyme activity. Starch content was determined using the procedure described by Keppler and Decker (1974).

Preparation of enzyme extract

Enzymes were prepared using a modification of the method described by Nakamura et al. (1989). Third leaf blades

(1 g) from 3-leaf-old seedlings were powdered in liquid nitrogen with a mortar and pestle. The powder was mixed with 5 ml of extraction buffer (100 mM Tricine–NaOH, pH 8.0, 2 mM EDTA, 8 mM $MgCl_2$, 12.5% (v/v) glycerol, 5% polyvinylpolypyrrolidone-40, 50 mM 2-mercaptoethanol) before filtering through two layers of Miracloth (Calbiochem, San Diego, CA, USA). After centrifuging at 10,000×*g* for 5 min at 4°C, soluble proteins in the supernatant were precipitated by 80% (v/v) cold-acetone. The pellet, including starch granules, was washed two times with ice-cold extraction buffer before being air-dried.

Analyses of enzyme activities

AGPase activity was assayed using the method of Nakamura et al. (1989). The reaction buffer contained 100 mM HEPES–NaOH (pH 7.4), 5 mM $MgCl_2$, 1 mM ADP glucose, 3 mM sodium pyrophosphate, 4 mM DTT. Protein crude extract (200 µl) was mixed with 850 µl of reaction buffer and incubated at 30°C for 30 min before being boiled to inactivate enzymatic activity. After centrifugation, the supernatant was mixed with $NADP^+$. Phosphoglucomutase and glucose-6-phosphate dehydrogenase were added, and the increase of OD_{340} was measured. Data are presented as the rate of glucose-1-phosphate (G1P) production.

The activity of SSS was assayed using the method of Nishi et al. (2001). Protein crude extract was first incubated with 50 mM HEPES–NaOH (pH 7.4), 1.6 mM ADP glucose, 0.23% (W/V) glycogen, and 16.7 mM DTT at 30°C for 30 min. Samples were then boiled to inactivate enzymatic activity. Following, the mix were supplied with 50 mM HEPES–NaOH (pH 7.4), 10 mM phosphocreatine, 200 mM KCl, and two unit creatinephosphokinase. After incubation at 30°C for 30 min, the sample was boiled. The sample were then mixed with 50 mM HEPES–NaOH (pH 7.4), 20 mM $MgCl_2$, 10 mM glucose, 2 mM $NADP^+$, and glucose-6-phosphate dehydrogenase. After 30 min incubation at 30°C, samples were boiled in a water bath to stop enzymatic activity. Hexokinase was added, and the increase of OD_{340} was measured. The assay for GBSS activity was similar to that for SSS, except that GBSS containing starch granules was added to the enzyme reaction buffer as a reaction primer instead of the glycogen primer.

The SBE activity was measured using the method of Guan and Preiss (1993). Protein crude extract was incubated with 0.1 mg starch and 100 mM HEPES–KOH (pH 7.2) at 30°C for 30 min before being boiled to halt enzymatic activity. After centrifugation, the supernatant (900 µl) was mixed with 200 µl of an I_2–KI solution (0.1% I_2 and 1% KI). The decrease in OD_{600} was then recorded.

RNA extraction from rice leaves

Leaf samples (100 mg) were homogenized in 1 ml Trizol reagent (Invitrogen, San Diego, CA, USA) for total RNA extraction according to the protocol described in a previous report (Wang et al. 2006).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA (200 ng) was used as the template for quantitative real-time RT-PCR analyses using Brilliant SYBR Green QRT-PCR Master Mix (Stratagene, La Jolla, CA). The PCR reactions were performed in using a Multiplex 3000P Real-Time PCR System (Stratagene). RT-PCR was performed with the gene-specific primer pairs for *GBSSI* (Fwd 5′-GCGAAGAACTGGGAGAATG-3′, Rev 5′-CACTACAACAAACAAACCACTG-3′) and *GBSSII* (Fwd 5′-AACTGCATGGCTCAAGACCT-3′, Rev 5′-ACAGG CAAATGCATGCCATC-3′). RT-PCR was carried out as follows: 50°C for 30 min; 95°C for 10 min; 40 cycles of 95°C for 1 min; annealing temperature for 1 min; and 72°C for 1 min. To accurately quantify the relative expression levels of the genes, the C_T value for each identified gene was normalized to the C_T value of *Actin*. For all real-time RT-PCR analyses, three independent experiments were carried out, and the data are presented as mean ± SE.

Promoter characterization

To analyze the *cis*-acting elements on promoter regions of the *GBSSI* and *II* genes, the regions 1.5 kb upstream from the putative ATG translation start codons for *GBSSI* (accession number: AP008212) and *GBSSII* (AP008213) were characterized with the Database of Plant *cis*-acting Regulatory DNA Elements (PLACE) (Higo et al. 1999).

Analysis of ABA content

Leaf samples were extracted with 80% (v/v) methanol combined with 2% glacial acetic acid. The ABA content of the leaf extracts was then measured with a Phytodetek ABA test kit (Agdia Incorporated, Elkhart, IN, USA), which uses a competitive antibody binding method.

Results

Effect of salinity on photosynthesis and starch content in rice seedling leaves

The growth of the rice seedlings was highly sensitive to saline conditions at the early seedling stage. After 24 h treatment with 200 mM NaCl, the 3-leaf-old seedlings were slightly weaker than the seedlings grown in normal culture solution. After 120 h of NaCl treatment, the leaves were rolled and completely wilted (data not shown). Moreover, even though the photosynthetic efficiency of the salt-stressed seedlings was not significantly different than that of the control seedlings after 24 h of treatment (Fig. 1a), the starch content of the NaCl-stressed seedling leaves was only 36% of that of the unstressed plants (Fig. 1b).

Effect of salt stress on activities of starch-synthesis enzymes

The catalytic activities of AGPase, GBSS, SSS, and SBE, were measured in leaf tissues from seedlings treated with 200 mM NaCl. GBSS activity was significantly repressed under high salinity condition (Fig. 2b). Although AGPase activity was also decreased by NaCl, the reduction was not statistically significant (Fig. 2a). In contrast, the activities of SSS and SBE were almost unaffected by salt stress (Fig. 2c, d). These results indicate that GBSS may be more NaCl-sensitive than the other starch-synthesis enzymes.

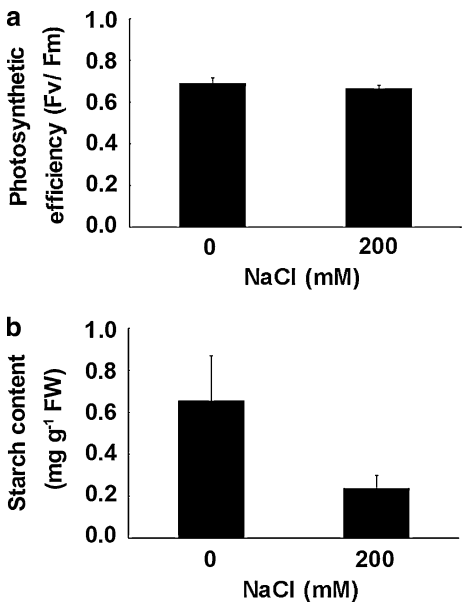

Fig. 1 NaCl effect on chlorophyll fluorescence and starch content in leaves of rice seedlings. Chlorophyll fluorescence (**a**) and starch accumulation (**b**) were measured after samples were treated by 200 mM NaCl for 24 h. Standard errors are indicated with *vertical bars*

Effects of NaCl on *GBSSI and II* gene expressions

The GBSS gene family contains two isogenes, *GBSSI* and *GBSSII*. The effect of salt on the expression of these two isogenes was evaluated in order to understand the molecular regulation of starch accumulation under salt stress. Real-time RT-PCR data showed that mRNA levels both *GBSSI* and *GBSSII* in NaCl-treated seedling leaves were lower than in the control seedlings. The expression of *GBSSI* was repressed 4.1-fold (Fig. 3a), and the expression of *GBSSII* was reduced 5-fold (Fig. 3b). The next goal was to determine whether the effects of salt were due to ionic or osmotic stress. To accomplish this, gene expression in seedlings treated with 20% PEG was measured. The results indicated that PEG did not reduce the expression of either isogene (Fig. 3a, b).

Effect of NaCl on endogenous ABA content in seedling leaves

Changes in ABA content were analyzed in salt-stressed and control seedling leaves. As shown in Fig. 4, the ABA content in leaf tissues was enhanced 2.2-fold after 24 h NaCl treatment. The salt-induced ABA increase was, however, completely inhibited by 200 μM fluridone (Fig. 4).

Analysis of *cis*-acting element on *GBSSI* and *II* gene promoters

The molecular regulation of *GBSSI* and *II* gene was further explored by characterizing the *cis*-acting elements on their gene promoters. Several ABA- or abiotic stress-responsive elements were found: ABA-responsive element (ABRE; ACGT), dehydration-responsive element (DRE; G/ACCGAC), MYB binding-related elements (A/TAACCA, C/TAACG/TG, CNGTTG/A, A/CACCA/TAA/CC, CCA/TACC, GGATA), MYC binding-related elements (CANNTG), and the ABA-regulated DPBF element (ACACNNG) (Table 1). The MYB and MYC binding-related elements were present on both *GBSSI* and *II* gene promoters.

Effect of ABA on *GBSSI* and *II* gene expressions

The role of the stress hormone ABA in regulating *GBSSI* and *GBSSII* gene expression was evaluated by treating rice

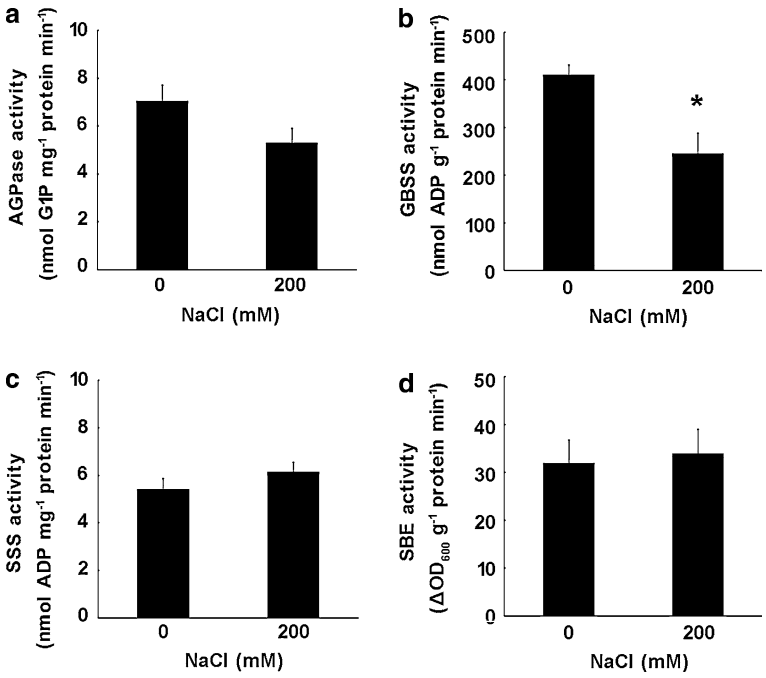

Fig. 2 Enzyme activity of AGPase, GBSS, SSS and SBE in salt stressed-rice seedling leaves. Activities of AGPase (**a**), GBSS (**b**), SSS (**c**) and SBE (**d**) were examined in leaves of 3-leaf-old rice seedlings under control and 200 mM NaCl conditions. *indicates that there is statistically significant difference at $P = 0.05$ according to the Student's *t*-test

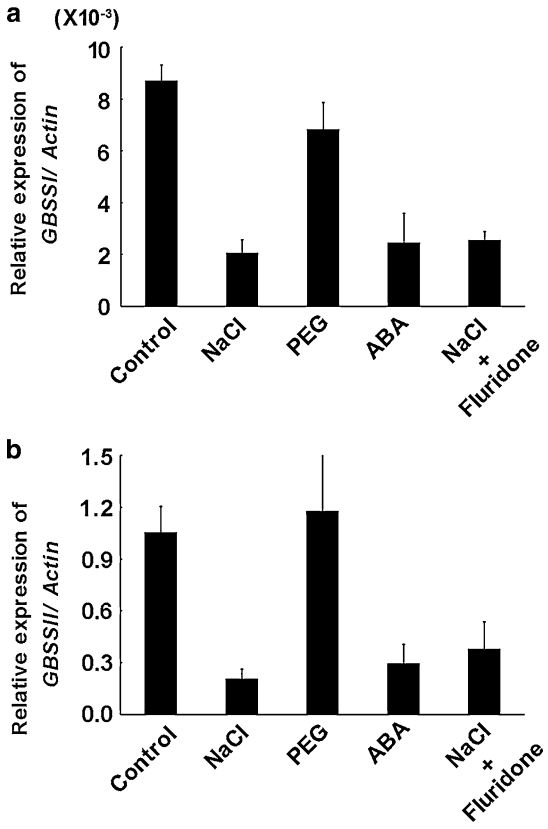

Fig. 3 Expression patterns of *GBSSI* and *GBSSII* genes. Transcript levels of *GBSSI* (**a**) and *GBSSII* (**b**) were detected in leaves of 3-leaf-old rice seedlings treated with 200 mM NaCl, 20% PEG, 100 μM ABA, and 200 mM NaCl plus 200 μM fluridone, respectively

seedlings with 100 μM ABA for 24 h prior to determination of *GBSSI* and *II* transcript levels. The expressions of both isogenes declined ~3.5-fold in ABA-treated seedling leaves (Fig. 3a, b).

Fluridone was then added to the culture solution to inhibit the ABA increase that occurs in seedling leaves under salt stress. This permitted evaluation of whether endogenous ABA was involved in NaCl-signal transduction to regulate *GBSSI* and *II* transcription. The results demonstrated that even though salt-induced ABA accumulation was inhibited by the fluridone (Fig. 4), the NaCl still diminished *GBSSI* and *GBSSII* expression (Fig. 3a, b).

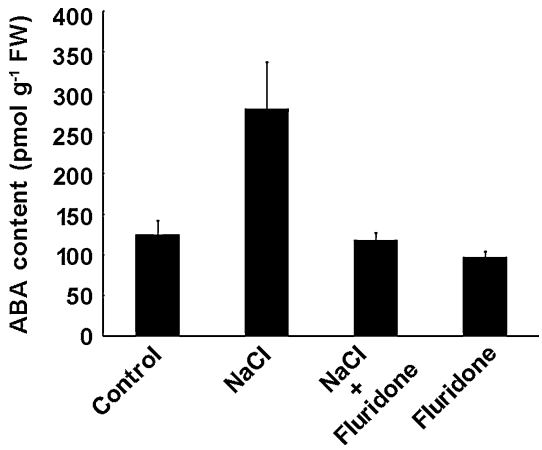

Fig. 4 Changes in ABA content in leaf tissues under salt stress condition. ABA content in leaves were analyzed after the 3-leaf-old rice seedlings treated with 200 mM NaCl, 200 mM NaCl plus 200 μM fluridone, and 200 μM fluridone only, respectively

Table 1 Numbers of ABA- and stresses-related *cis*-acting elements occurring within the putative promoters of *GBSSI* and *GBSSII* genes

Cis-acting element	Sequence[a]	*GBSSI* Numbers of *cis*-acting element	*GBSSII*
ABRE-like element	ACGT	2	0
DRE-like element	G/ACCGAC	0	1
MYB binding-related element	A/TAACCA	0	3
	C/TAACG/TG	3	0
	CNGTTG/A	1	1
	A/CACCA/TAA/CC	0	1
	CCA/TACC	1	1
	GGATA	1	0
MYC binding-related element	CANNTG	4	9
ABA-regulated DPBF element	ACACNNG	1	0

[a] The N in sequence means the base could be A, T, C or G

Discussion

Changes in starch content caused by adverse conditions such as water stress and temperature stress have been observed in various plants. The catalytic activities of some starch synthesis enzymes have also been examined under such stresses (Lafta and Lorenzen 1995; Geigenberger et al. 1998; Ahmadi and Baker 2001; Jiang et al. 2003). However, most studies of the effects of stress on starch accumulation have focused on crop storage organs. Little information exists regarding the effects of stress on transitory starch biosynthesis in photosynthetic tissues. This study demonstrated that the starch content in the leaves of rice seedlings was reduced by salt stress (Fig. 1b).

Reduced starch may be caused by carbon limitation due to diminished photosynthetic activity, or it may be due to alteration of starch biosynthetic and/or degradation efficiency. According to chlorophyll fluorescence measurements, the photochemical efficiency of the leaves was not adversely affected by salt (Fig. 1a). This suggests that the primary result of salt stress is a perturbation of starch metabolism. Libal-Weksler et al. (1994) reported that AGPase activity in citrus callus was not affected by salt stress. The activity of SSS, however, was repressed in salt-sensitive citrus callus and enhanced in salt-tolerant citrus callus. In the study presented here, the catalytic activity of GBSS in rice seedling leaves was significantly down-regulated by NaCl (Fig. 2b). The activities of AGPase, SSS, and SBE, on the other hand, were resistant to salt stress (Fig. 2a, c, d). Thus, the effects of salt stress on starch synthesis enzymes appear to vary by plant species.

Starch content in plant tissues is the result of equilibrium between starch biosynthesis and breakdown. Recent data demonstrated that β-amylase was responsible for transitory starch degradation in leaf tissues at night (Lloyd et al. 2005). In the current study, there was no difference in β-amylase activity between the control and salt-stressed rice leaves (data not shown). This is consistent with previous reports showing that α-amylase and β-amylase activities were not significantly changed in salt-stressed plant tissues (Rathert 1983; Libal-Weksler et al. 1994). It is likely; therefore, that salt stress reduces starch content through down-expression of starch synthesis-related enzymes.

The expression of *GBSSI* has been found to be regulated by environmental factors in some plants (Hirano and Sano 1998; Wang et al. 2001, 2006). Expression of *GBSSII* may be controlled by the supply of nitrogen or sugar (Dian et al. 2003). However, the molecular regulation of *GBSSII* under environmental stress is still poorly understood. The level of expression of *GBSSII* in rice leaf tissues was significantly higher than for *GBSSI* (Fig. 3a, b). This finding is consistent with data reported by Hirose and Terao (2004). The research presented here also shows that expression of both *GBSSI* and *GBSSII* was repressed by salt stress (Fig. 3). Clearly the primary control behind this repression is a transcriptional process and/or mRNA stability. Because NaCl repressed both *GBSSI* and *II* mRNA more than GBSS enzyme activity, it is possible that translational regulation was also involved.

Salinity causes both ionic and osmotic stresses, and a number of physiological reactions and gene expressions respond differently to them (Alam et al. 2002; Merchant and Adams 2005). The accumulation of *GBSSI* and *GBSSII* transcripts was affected by salt stress but not by PEG-induced water stress (Fig. 3), providing evidence that ionic effects are the dominant factor repressing *GBSSI* and *GBSSII* expression.

To explore coordinate mechanisms, the *cis*-acting elements on promoters of the *GBSSI* and *II* genes were identified (Table 1). The ABRE element has been found to operate on ABA-dependent regulation and is involved in gene regulation under abiotic stresses such as salinity and dehydration (Narusaka et al. 2003; Shinozaki et al. 2003).

The DRE-like elements act on both ABA-dependent and ABA-independent regulatory systems (Narusaka et al. 2003; Shinozaki et al. 2003). The DREB transcription factor, interacting with the DRE elements, may control stress-induced gene expressions (Dubouzet et al. 2003). Geisler et al. (2006) indicated that the numbers of ABRE and DRE elements in the promoters are correlated to the regulatory signal effect. Since *GBSSI* and *GBSSII* promoters contain only two ABRE and one DRE, respectively, the importance of these two elements in the regulation of gene expression under stress or ABA treatment still need to be identified. Transcriptional factors MYB and MYC are involved in ABA-responsive gene expression and may be induced by dehydration stress (Urao et al. 1993; Abe et al. 2003). The ABA-regulated DPBF element was found by Kim et al. (1997) to be essential for the ABA-inducible expression of the carrot *lea* gene, *Dc*.

Data presented here demonstrate that *GBSSI* and *GBSSII* expression was down-regulated by ABA in rice seedling leaves (Fig. 3). Because endogenous ABA was increased 2.2-fold in the salt-stressed rice seedling leaves (Fig. 4), the question arose as to whether starch metabolism is regulated by ABA signal under salt stress. Treating the seedlings with fluridone did not prevent the adverse effects of NaCl on *GBSSI* and *II* gene expression (Fig. 3). Therefore, the salt stress signal transduction that affects *GBSSI* and *GBSSII* gene expression might operate via an ABA-independent pathway. The effect of salt stress on these two gene expressions might also result from a combination of both ABA-dependent and ABA-independent signals.

In conclusion, this study showed that the coordinate down-expression of *GBSSI* and *GBSSII* genes caused low starch content in the leaves of rice seedlings under salt stress conditions. Ion effects contributed to the impact of high NaCl on the two genes. Although exogenous ABA functions as a signal to regulate *GBSSI* and *GBSSII* gene expression, salt-related changes may be heavily mediated by an ABA-independent pathway. Future research should be aimed at clarifying the salt stress-signal transduction pathway affecting *GBSSI* and *II* gene expression. Such information would be helpful in further elucidating the molecular mechanisms governing starch biosynthesis in rice seedling leaves under high salinity.

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