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Detection of Sugar-Regulated Gene Expression and Signaling in Suspension-Cultured Rice Cells

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Abstract

To better understand the mechanism of sugar signaling in rice cell, the suspension-cultured rice cells were transferred from sucrose-containing (+S) to sucrose-free (−S) of MS culture medium, we found that ribosomal RNAs (rRNAs) were degraded progressively. This suggests that carbon, nitrogen, and phosphate were recycled in this process and the reduction in cellular rRNAs might lead to decreased translation to save energy in response to sugar starvation. Differential screening revealed that two groups of genes, sugar-starvation-repressed (SSR) and sugar-starvation-activated (SSA) genes, were regulated by sugar in an opposing manner. Northern-blot analysis showed that two major hybridization signals of 0.8 and 1.9 kb were induced strongly under sugar starvation. The two populations of genes corresponded with homologs of α-amylases (1.9 kb) and the glycine-rich proteins (GRPs) gene family (0.8 kb), and all were SSA genes. Expression of GRP genes was strongly induced in sugar-starved cells, which suggests that GRPs may help to protect cells against nutritional stress. Treatment of +S and −S cells with the protein kinase (PK) inhibitor staurosporine (St) and the serine/theronine phosphoprotein phosphatases 1 (PP1) and 2A (PP2A) inhibitor okadaic acid (OA) revealed that PP1 and PP2A (PPs) might be involved in increasing SSR gene expression in +S cells, and that activation of the majority of the SSA genes in −S cells might be due to PKs activity. These results suggested that PKs and PPs might be involved in the sugar regulation of SSR and SSA gene expression. An in-gel PK activity assay demonstrated that the activity of two classes of PKs (50 and 66 kDa) may be induced rapidly after transfer of +S cells to −S medium. Following transfer of −S cells to +S medium, a novel class of 38 kDa PK was induced rapidly and showed high activity. The 38 kDa PK might play a role in sugar sensing, and the 50 and 66 kDa PKs might play roles in signal sensing under sugar starvation in rice cells. These results provide valuable information on three classes of protein kinases that might play key roles in sugar sensing and signaling in rice.

Keywords

Suspension-Cultured Rice Cells, Glycine-Rich Proteins, Sugar-Starvation Repressed, Sugar-Starvation Activated, Protein Kinases, Phosphoprotein Phosphatases

1. Introduction

Carbohydrates are important nutrients in metabolism and important structural constituents in plants. Sugars, which are the basic units of carbohydrates, can serve as an energy source or a synthetic building block. Sugar availability may determine the direction of cellular metabolism and developmental responses [1] [2]. Many studies indicate that the expression of a variety of genes is regulated by sugar. In rice suspension-cultured cells, two sets of genes, namely growth-related and stress-related genes, are up- and down-regulated, respectively, by sugars [3]. In general, sugars favor the expression of genes in connection with the biosynthesis, respiration, and storage of reserves, and conversely repress the expression of genes associated with photosynthesis and reserve mobilization [4] [5]. For example, sugars positively regulate the expression of genes that encode storage proteins, such as patatin in potato and sporamin in sweet potato [6] [7]. Accumulation of the mRNAs that encode proteins associated with starch biosynthesis, such as ADP glucose pyrophosphorylase, is also dependent on sugars [8]. In contrast, a variety of genes involved in key metabolic processes are negatively regulated by sugars, such as *α*-amylase in rice [9] [10], and the ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (*rbcS*) and chlorophyll *a*/*b* binding protein (*Cab*) in maize protoplasts and suspension-cultured *Chenopodium* cells [11] [12]. A GeneChip analysis detected transcriptional up-regulation of 343 genes in sugar-starved suspension-cultured *Arabidopsis* cells [13]. These genes are involved in the recycling of cellular components and nutrient scavenging, in a variety of defense and stress-response pathways, with specific protein kinases and transcription factors regulating these processes.

One of the most extensively studied sugar-regulated genes in plants is that for *α*-amylase. Expression of the gene is induced by sugar deprivation in suspension-cultured rice cells, germinating embryos, and aleurone layers, and is suppressed by addition of sucrose [9] [10]. Sugar repression of *α*-amylase gene expression is reported to involve the control of both transcription and mRNA stability [14] [15] [16]. All *α*-amylase genes isolated from a variety of plant species contain a TATCCA element, or variants, in the promoter region, to which MYB nuclear proteins bind in a sequence-specific and sugar-dependent manner [5] [17] [18]. Transient expression assays with barley half-seeds showed that OsMYBS1 and OsMYBS2 activate a promoter containing the TATCCA element when sugar is added. With sugar starvation, OsMYBS3 represses transcription of the same promoter [19]. However, the details of sugar signaling pathways in plants require clarification.

Many studies indicate that hexokinase acts as the primary sugar sensor [1] [20] [21] [22] [23] [24]. In *Arabidopsis*, antisense suppression and overexpression of AtHXK1 resulted in insensitivity and hypersensitivity to exogenous glucose, respectively [20]. Other sugar-sensing pathways in plants have been proposed, such as hexose-dependent but hexokinase-independent, and sucrose-dependent pathways [21] [25] [26] [27] [28] [29]. Moreover, a gene (*SnRK*1) that encodes the yeast SNF1 homolog was cloned from potato and shown to be required for activation of sucrose synthase gene expression [30]. The expression of antisense SnRK1 in wheat embryos represses the activity of an *α*-amylase gene promoter, which suggests that SnRK1 is required for activation of glucose-repressing genes [31]. Rice SnRK1A functions upstream of the interaction between the transcription factor MYBS1 and a sugar response complex in the *αAmy3* promoter to relieve glucose repression [32]. These results suggest that SnRK1 plays a role in sugar signal reception. An additional molecule involved in sugar signaling is trehalose 6-phosphate (T6P), which is generated from glucose 6-phosphate and UDP-glucose by trehalose 6-phosphate synthase (TPS) [33]. Overexpression of *AtTPS1* in *Arabidopsis* results in insensitivity to glucose during seed germination [34], and increased quantities of T6P represses SnRK1 activity [35], which suggests that T6P and SnRK1 play contrasting roles in sugar responses.

In this study, we quantified the effect of sugar on cellular transcripts, and observed a high proportion of GRP homologs induced in suspension-cultured rice cells under sugar starvation. We showed that the protein kinase (PK) inhibitor staurosporine (St) and protein phosphatase (PP) inhibitor okadaic acid (OA) differentially affected the expression of genes subject to sugar-mediated regulation. Notably, addition or withdrawal of sugar from the culture medium resulted in rapid and transient activation of the specific activity of 38, 50, and 66 kDa PKs, which indicates that PKs may perform important roles in sugar sensing in rice.

2. Materials and Methods

2.1. Plant Material

The rice cultivar *Oryza sativa* L. cv Tainung 67 was used. Immature seeds were dehulled, sterilized with 2.4% NaOCl for 1 h, washed thoroughly with sterile water, and placed on N6D agar medium for callus induction. After 1 month, the callus derived from scutella was transferred to liquid Murashige and Skoog (MS) medium [36] supplemented with 3% sucrose and 10 μM 2,4-Dichlorophenoxyacetic acid (2, 4-D, a synthetic plant hormone auxin, which is necessary for rice cell division) to establish a suspension cell culture. Cells were cultured on a reciprocal shaker at 120 rpm and incubated at 26˚C in the dark.

2.2. Differential Screening of cDNA Library

Suspension-cultured rice cells were subcultured in fresh MS medium supple-

mented with sucrose (+S) and cultured for 72 h, then the cells were transferred to sucrose-free (−S) medium for 4 h to isolate the candidate genes in response to sugar signaling in earlier phase. The cells were harvested and total RNA was purified. Poly $(A)^+$ RNA was purified from the total RNA using an oligo (dT)-cellulose spin column. The Poly $(A)^+$ RNA was used to construct a cDNA library with the λ gt22A vector and the SuperScript™ II reverse transcriptase cDNA library construction system (Invitrogen, Carlsbad, CA, USA). The pool of ^{32}P-labeled single-stranded cDNA probes were prepared from Poly $(A)^+$ RNA derived from 24 h of +S or −S cells, respectively, using an oligo (dT) primer and SuperScript™ II reverse transcriptase. Duplicated filters prepared from high-density platings of the cDNA library were differentially screened with the differential cDNA probes. The phage plaques that showed hybridization signals strongly associated with the cDNA probes from 24 h −S cells and weakly associated with the cDNA probes from 24 h +S cells were isolated. The cDNAs inserted in the λ gt22A vector were cleaved with *Not*I and *Sal*I and subcloned into the *Not*I–*Sal*I sites of pBluescript vectors before being subjected to sequencing analysis.

2.3. Plasmids

The inserted DNA fragments of *Act*, *ADH*2, *G3PD*, *HSP*86, and *SSP*2 were digested from the pBluescript vector with *Sal*I and *Not*I. The DNA fragments of *salT*, *OsGRP*1, *OsGRP*2, *OsGRP*3, *OsGRP*4, and *OsGRP*5 were digested with *Eco*RI. The *αAmy*3 and *αAmy*8 gene-specific DNA fragments were prepared as described by Sheu *et al.* [15]. The inserted DNAs were individually isolated, labeled with ^{32}P, and used as probes. A DNA fragment containing the 28S, 18S, and 5.8S rDNAs was excised from the pRY18 plasmid using *Bam*HI, labeled with α-^{32}P, and used as a probe to evaluate the quantity of the rRNA.

2.4. Northern-Blot Analysis

Total RNA was isolated from suspension-cultured cells using the TRIzol® Reagent (Invitrogen). The RNA gel-blot was analyzed as described by Ho *et al.* [3]. Ten micrograms of total RNA was electrophoresed in 1% agarose gel containing 10 mM sodium phosphate buffer (pH 6.5), transferred to a nylon membrane, and hybridized with random-primer-labeled [α-^{32}P]dCTP cDNA probes at 42˚C. The membranes were exposed to X-ray film at −80˚C.

2.5. In-Gel Protein Kinase Activity Assay

Rice suspension-cultured cells were harvested and ground into powder. Proteins were purified with precooled extraction buffer (50 mM Hepes·KOH [pH 7.6], 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM Na_3MoO_4, 20 mM β-glycerophosphate, 20% glycerol, 2 mM DTT, and 2 mM PMSF), supplemented with 2 μg/mL of three different protease inhibitors, namely leupeptin, E64, and pepstatin. After thorough mixing and centrifugation at 18,000 × *g* for 10 min,

the supernatants were collected and the protein concentration was quantified using the method of Bradford [37]. The in-gel PK activity assay was conducted in accordance with the methods of Mizoguch *et al.* [38] and Usami *et al.* [39] with minor revision. In brief, 50 μg proteins were separated by 10% SDS-PAGE with or without 0.5 mg/mL histone III-S (Sigma). After electrophoresis, the gel was rinsed in the wash solution (20% β-propanol and 50 mM Tris-HCl [pH 8.0] for 1 h to remove SDS, followed by soaking with buffer containing 50 mM Tris-HCl (pH 8.0) and 5 mM β-mercaptoethanol for removal of β-propanol residues. The gel was denatured at 25˚C for 2 h with denaturation solution (6 M guanidine hydrochloride, 50 mM Tris-HCl [pH 8.0] and 5 mM β-mercaptoethanol), then renatured at 4˚C for at least 16 h by neutralization solution (50 mM Tris-HCl [pH 8.0], 5 mM β-mercaptoethanol, and 0.04% Tween 40). For kinase activity detection, the gel was soaked in kinase reaction buffer (40 mM Hepes·KOH [pH 7.6], 2 mM DTT, 15 mM $MgCl_2$, 30 mM ATP, 100 μ*Ci* [γ-^{32}P] ATP, and 0.1 mM EGTA) and incubated at 25˚C for 1 h, then rinsed in stop solution (5% TCA and 1% sodium pyrophosphate) for 30 min to terminate the reaction. The gel was washed several times with sterilized water to remove the free isotope, then the gel was vacuumed dried and exposed to X-ray film.

3. Results

3.1. Sugar Modulates Ribosomal RNA Turnover

Carbon metabolite-regulated gene expression is known in a variety of living organisms. Gene expression, through the processes of mRNA transcription followed by protein translation by the ribosome in accordance with the coding sequence of mRNA, results in biologically active polypeptides. Ribosomal RNA (rRNA) is the main component of the cellular ribosome and constitutes more than 80% of the total cellular RNA. Therefore, the rRNAs may be used as a measure of total mRNA stability. In addition, suspension-cultured cells represent a convenient experimental system with which to study mechanisms involved in gene regulation owing to their sensitivity of responses to exogenous stimuli. We therefore used suspension-cultured rice cells and rRNA to dissect the global changes in total RNA stability in the presence or absence of exogenous sucrose in the culture medium. The suspension cells were cultured in sucrose-containing (+S) medium for 72 h, then transferred to sucrose-free (−S) medium for 72 h to let sugar was depleted completely in cells, followed by provision with sucrose for 24 h. Total RNA was purified from these cells at specific time points (**Figure 1**) and subjected to northern-blot analysis using rDNA (a rice genomic rDNA cluster, including the 18S, 5.8S, and 28S rRNA genes) as a probe. As shown in **Figure 1(a)**, after RNA electrophoresis, when cells were transferred to −S medium, the amount of small RNAs gradually increased from time 0 to 12 h (lanes 5 - 10, the position indicated by arrowhead), dramatically increased at 24 h (lane 11), and peaked at 36 - 72 h (lanes 12 - 14). Accumulation of the small RNAs progressively decreased 24 h after the transfer of cells from −S

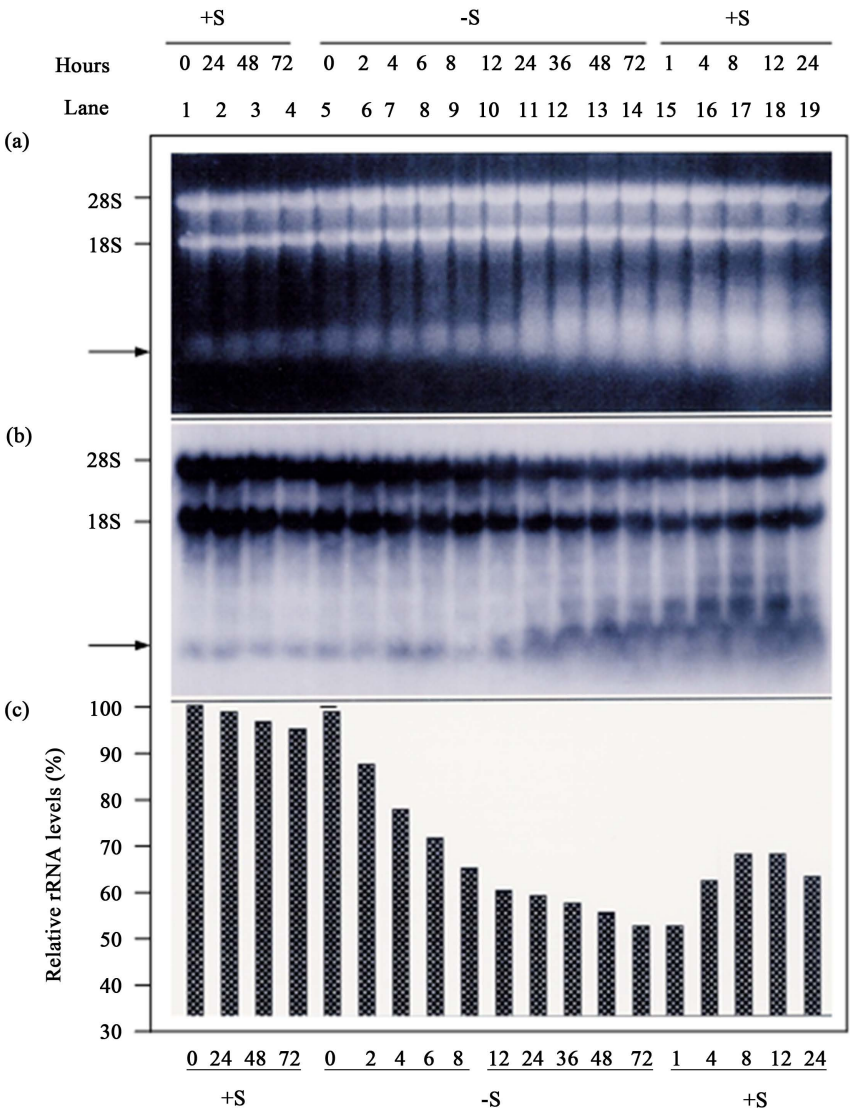

Figure 1. Increased rRNA degradation in rice suspension-cultured cells under sucrose starvation. After incubation for 72 h in sucrose-containing (+S) medium, cells were transferred to sucrose-free (−S) medium for 72 h, and then transferred to +S medium for an additional 24 h. Total RNA was purified from the cells and agarose gel electrophoresis was conducted. (a) After electrophoresis, the gel was stained with ethidium bromide and photographed; (b) The gel blot was hybridized with a ^{32}P-labeled rDNA probe; (c) Levels of 28S and 18S rRNAs were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). The positions of 28S and 18S rRNAs are as indicated. Arrows indicate the position of RNA degradation products.

to +S medium (lane 19). These small RNAs hybridized with the rDNA probe, which suggests that their accumulation was derived from the degradation products of rRNAs (Figure 1(b)). These results are also supported by quantitative estimation of the concentration of remaining intact rRNAs using phosphor-imaging software (Figure 1(c)). After cells were transferred from +S to −S medium, in contrast to small RNAs, the accumulation of rRNAs progressively decreased (−S, 0 - 48 h), attaining the lowest level 72 h after sugar starvation, and

progressively increased after cells were transferred from −S to +S medium. These results suggest that reduction in the amount of rRNAs might be due to increased degradation rather than decreased synthesis during sucrose starvation. Given that rRNAs are functionally correlated with protein synthesis, reduction in cellular rRNAs content probably leads to reduction in translation to conserve energy in response to sugar starvation.

3.2. Global Gene Expression in Sugar-Starved Cells

To evaluate global gene expression in rice cells under sugar starvation, total RNA was purified from suspension-cultured rice cells treated with or without sugar and subjected to northern-blot hybridization. The total poly (A^+) RNA (mRNA) was prepared from cells cultured in +S or −S medium for 24 h. The purified mRNA was used as the template to synthesize the pool of [α-^{32}P]-labeled cDNA probes. Two major hybridization signals were distinctly induced in cells under sugar-starvation treatment (Figure 2, indicated by arrows). One induction signal was similar in size to that of 18S rRNA (1.9 kb), which was induced abundantly when cells were starved of sucrose for 48 - 72 h and declined rapidly 1 - 4 h after cells were transferred from −S to +S medium. The other signal was about 0.8 kb in size. The mRNA populations gradually accumulated and attained the highest amounts at 12 - 72 h after sugar starvation (lanes 10 - 14) and rapidly decreased 1 - 24 h after transfer to culture medium supplemented with sucrose (lanes 15 - 19). These results suggested that the two distinct populations of 1.9 kb and 0.8 kb mRNAs might play important roles in the response to sugar starvation in rice cells.

To verify the two populations of corresponding genes induced under sugar starvation, differential screening of a cDNA library prepared from rice cells sugar-starved for 4 h was conducted. After screening, more than 100 cDNA

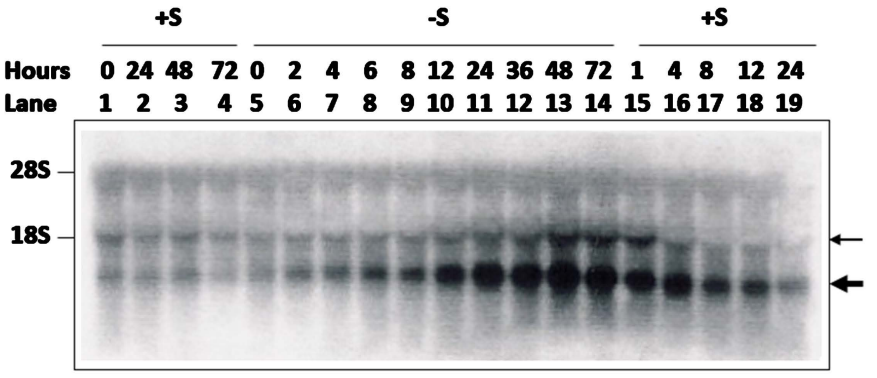

Figure 2. Northern-blot analysis of global gene expression in rice suspension cells cultured in sucrose-containing (+S) or sucrose-free (−S) medium. Total RNA isolation and gel electrophoresis was performed as described in the legend of Figure 1. After blotting, the membrane was hybridized with the pool of ^{32}P-labeled single-stranded cDNA probes, which were prepared from Poly $(A)^+$ RNA derived from cells cultured under sugar starvation for 24 h, using an oligo (dT) primer and reverse transcriptase. The thin and thick arrows indicate the positions of 1.9 and 0.8 kb RNAs, respectively.

clones that showed stronger signals (spots) after hybridization with either the pool of −S or +S cDNA probes were selected (Supplementary **Figure S1**) and cloned into plasmid vectors. Among these cDNA clones, five clones showed strongest hybridization signals with the +S cDNA probes and a weak signal with the −S cDNA probes (defined as the sugar-starvation-repressed [SSR] genes), and 12 clones showed stronger hybridization signals with the −S cDNA probes (defined as the sugar-starvation-activated [SSA] genes). These 17 cDNA clones were partially sequenced and analyzed. As described in our previous study [3], the five SSR cDNA clones were identified as actin (Act), alcohol dehydrogenase 2 (ADH2), glyceraldehyde-3-phosphate dehydrogenase (G3PD), heat shock protein 86 (HSP86) and sucrose synthase P2 (SSP2), and the corresponding full-length cDNA clones were obtained from the Japan Rice Genome Research Program [40]. Among the 12 SSA cDNA clones, three clones were *α*-amylase homologous genes (two clones were *αAmy*3 [XM_015794560, the predicted mRNA length in GenBank was 1834 bp], one clone was *αAmy*8 [XM_015795212, the predicted mRNA length was 1687 bp]), and one clone was identical to *sal T* (S45168, 724 bp). Unexpectedly, the other eight clones were highly homologous to the glycine-rich proteins (GRPs) gene family of unknown function, of which three identical clones were designated OsGRP1 (X54449, predicted mRNA length in GenBank was 834 bp), two clones were OsGRP2 (D21281, 852 bp), and one clone each as OsGRP3 (AF010579, 713 bp), OsGRP4 (U40708, 551 bp) and OsGRP5 (AJ002893, 780 bp). The predicted mRNA lengths of all the isolated SSA clones were consistent with the two major hybridization signals detected by −S cDNA probes at the positions of higher molecular weight (corresponding to the length of *αAmy*3 and *αAmy*8) (**Figure 2**, thin arrow) or lower molecular weight (this position similar to the length of *GRP*s and *salT*) (**Figure 2**, thick arrow). These results suggest that the expression of a high proportion of *α*-amylase and GRP homologs was induced abundantly during sugar starvation.

We verified whether the isolated clones that showed gene expression patterns affected by sugar were also involved in protein phosphorylation and protein dephosphorylation events. The rice cells were cultured in +S medium for 72 h, then transferred to +S or −S medium without exogenous treatment and further incubated for 2 h and 12 h (**Figure 3** and **Figure 4**, lanes 1 - 4), respectively, or exogenous supplementation with either the PK inhibitor staurosporine (St) (**Figure 3** and **Figure 4**, lanes 5 - 8) or the serine/theronine phosphoprotein phosphatases 1 (PP1) and 2A (PP2A) inhibitor okadaic acid (OA) (**Figure 3** and **Figure 4**, lanes 9 - 12), and cultured for an additional 2 h and 12 h, respectively. Cells were collected and total RNA was purified and subjected to northern-blot hybridization by using the 3'-untranslated region (3'-UTR) of the examined gene as a gene-specific probe. The accumulation of all five SSR genes was increased in cells cultured in +S medium, but was repressed in cells cultured in −S medium (**Figure 3**, cf. lanes 2 and 4). Furthermore, expression of each of the predicted SSA genes was more strongly activated in cells cultured for 12 h in −S medium than in cells cultured in +S medium for 12 h (**Figure 4**, cf. lanes 2 and 4). These

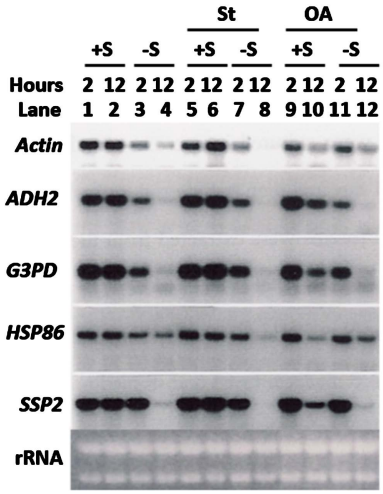

Figure 3. Protein kinase and protein phosphatase inhibitors alter expression of sucrose-starvation-repressed genes. Rice suspension cells were cultured in sucrose-containing (+S) medium for 72 h and transferred to +S or sucrose-free (−S) medium lacking an inhibitor, or treated with the protein kinase inhibitor staurosporine (2 μM; St), or the phosphoprotein phosphatase 1 (PP1) and 2A (PP2A) inhibitor okadaic acid (100 nM; OA), and incubated for an additional 2 or 12 h. All media contained 0.2% dimethyl sulforide (DMSO), which was used to dissolve St and OA. Total RNA was purified and subjected to RNA gel blot analysis. Sucrose-starvation-repressed genes were used as probes. Act, actin; ADH2, alcohol hydrogenase 2; G3PD, glyceraldehydes-3-phosphate dehydrogenase; HSP86, heat shock protein 86; SSP2, sucrose synthase P-2; *α*Amy, *α*-amylase.

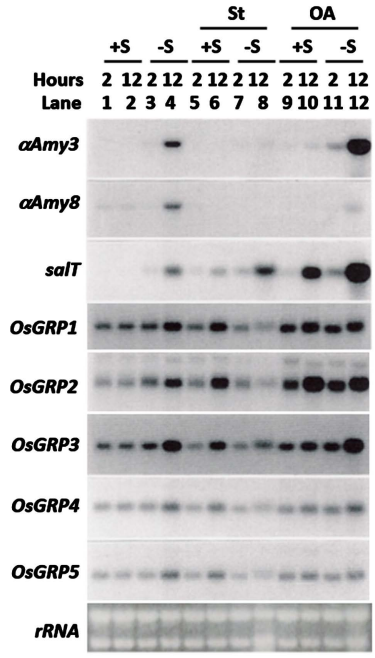

Figure 4. Protein kinase and protein phosphatase inhibitors alter expression of sucrose-starvation-activated genes. The treatment of rice suspension-cultured cells and the gel blotting procedure was identical to that described in the legend of **Figure 3**. The blotted membranes were hybridized with the probes prepared from the 3'-untranslated gene-specific regions of the sucrose-starvation-activated genes. *α*Amy, *α*-amylase; GRP, glycine-rich protein.

findings indicate that the expression of all SSA clones was sensitive to sugar content in the culture medium and was activated in −S cells, whereas activation was diminished in +S cells, and the expression patterns are all converse in those SSR genes. Therefore, the SSR and SSA genes were considered to be an ideal sample to study the regulatory mechanisms of sugar regulation pathways.

3.3. Protein Kinase and Protein Phosphatase Effects on the Sugar Regulation of Gene Expression

In the present study, we observed that two groups of genes (SSR and SSA genes) were regulated by sugar in opposing manners. To further verify whether the effects of sugar were mediated by protein phosphorylation or dephosphorylation, suspension-cultured rice cells treated with PK or PP inhibitors were subjected to northern-blot analysis. In +S cells, accumulation of mRNAs of all five SSR genes was not affected by St (**Figure 3**, cf. lanes 2 and 6), but was repressed by OA (**Figure 3**, cf. lanes 2 and 10). In −S cells, the mRNA level for all of the SSR genes was repressed by St (**Figure 3**, cf. lanes 4 and 8), but was not altered by OA (**Figure 3**, cf. lanes 4 and 12). On the basis of these results, the activity of PPs may positively regulate SSR gene expression in +S cells, whereas the activity of PKs somewhat activated expression of SSR genes in −S cells.

In +S cells, St slightly activated the expression of SSA genes, but not that of *αAmy3* and *αAmy8* (**Figure 4**, cf. lanes 2 and 6). In contrast, OA dramatically increased the expression of *salT*, *OsGRP1*, *OsGRP2*, and *OsGRP3*, and slightly activated *OsGRP4* and *OsGRP5*. No effect of OA on *αAmy3* and *αAmy8* was observed (**Figure 4**, cf. lanes 2 and 10). In –S cells, St enhanced expression of *salT* but suppressed that of the other seven SSA genes (**Figure 4**, cf. lanes 4 and 8). Okadaic acid enhanced the expression of *αAmy3*, *salT*, *OsGRP2*, and *OsGRP3*, but suppressed *αAmy8* expression. Okadaic acid had no effect on expression of *OsGRP1*, *OsGRP4*, and *OsGRP5* (**Figure 4**, cf. lanes 4 and 12). On the basis of these results, in +S cells the activities of both PKs and PPs may suppress the expression of SSA genes, but not that of *αAmy3* and *αAmy8*. In –S cells, the activity of some PKs may negatively regulate the expression of *salT* and positively regulate expression of other SSA genes. Some PPs suppressed the expression of *αAmy3*, *salT*, *OsGRP2*, and *OsGRP3*, but activated the expression of *αAmy8*, and no effect on the expression of *OsGRP1*, *OsGRP4*, and *OsGRP5* was observed. These results suggest that PKs, PP1, and PP2A might be involved in the regulation of SSR and SSA gene expression by sugars.

3.4. Detection of Protein Kinase Activity Involved in Sugar Sensing

To determine the types of PKs that may be involved in sugar signaling in rice cells, an in-gel PK activity assay was conducted. Rice suspension cells were cultured in +S medium for 72 h, then the culture was divided into two equal portions: one portion was transferred to fresh +S medium, and the other portion was transferred to −S medium; each portion was cultured for 24 h. The cells

cultured in +S medium were then transferred to either +S or −S medium, and cultured for an additional 5, 10, 20, and 60 min (Figure 5(a)). Similarly, the cells cultured in −S medium were then transferred to either +S or −S medium, and cultured for an additional 1, 2, 5, and 10 min (Figure 5(b)). The cells were harvested at the specified time points and total proteins were purified for the in-gel PK activity assay by supplementation with (Figure 5(a) and Figure 5(b), upper panel) or without (Figure 5(a) and Figure 5(b), lower panel) Histone III-S in the gel as the PK substrate. We therefore could distinguish the signals of PKs activity derived from autophosphorylation of PKs (without Histone III-S in gel) (Figure 5, lower panel) or phosphorylation of the substrate by the specific PK activity (with Histone III-S in gel) (Figure 5, upper panel). After the +S cells were transferred to +S medium, no obvious difference in the specific signal of PKs activity was detected, nor in the presence or absence of Histone III-S in the gel (Figure 5(a)). However, after cells were transferred from +S to −S medium, two specific signals of PKs activity, one stronger signal located in the position of about 66 kDa and a weaker signal at about 50 kDa, were induced rapidly within 5 - 10 min, and thereafter declined quickly and were only weakly detected at 20 min (Figure 5(a), lanes 6 - 9). Moreover, four constitutive PKs activity detected

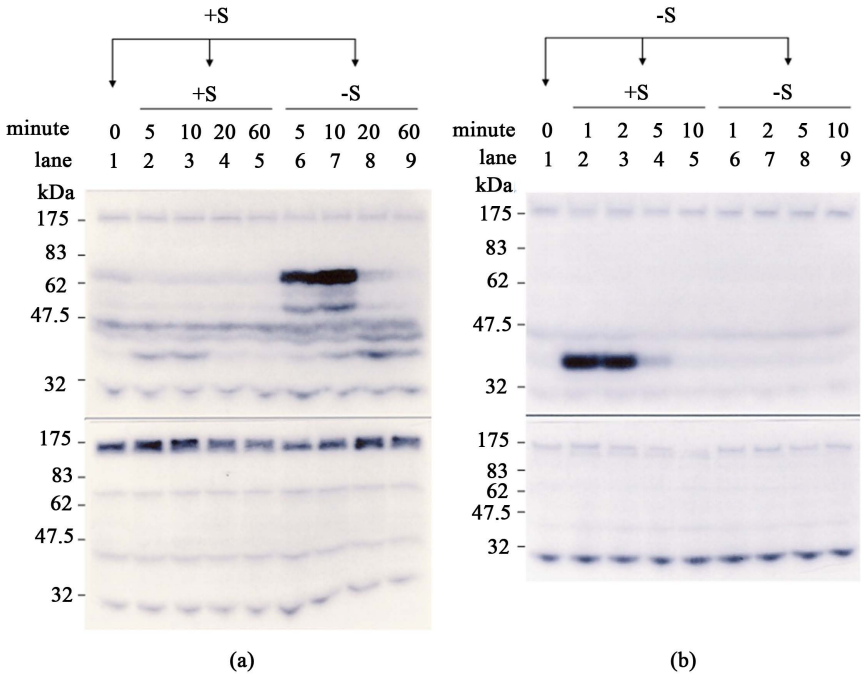

Figure 5. Activation of protein kinases in response to alteration in sucrose level. Rice suspension cells were cultured in sucrose-containing (+S) medium for 72 h, then transferred to +S or sucrose-free (−S) medium for 24 h, and followed by transfer to fresh +S or −S medium for the indicated time periods. Proteins were extracted from the harvested cells and subjected to an in-gel protein kinase activity assay by addition of histone III-S in the gel as a substrate. (a) Cells transferred from +S medium to +S or −S medium; (b) Cells transferred from −S medium to +S or −S medium. Upper panel: with addition of exogenous histone III-S in the gel as a substrate. Lower panel: without addition of histone III-S in the gel as the control.

at the positions of about 30, 45, 66, and 170 kDa, irrespective of +S and –S cells and with or without Histone III-S in the gel (**Figure 5**), revealed that at least four classes of autophosphorylation of PKs were exhibited in rice cells. Similar to the experimental results shown in **Figure 5(a)**, after –S cells were transferred to +S and –S medium followed by incubation for an additional 5, 10, 20, and 60 min, only a weaker signal was detected specifically at the position of about 38 kDa at 5 min after the transfer of –S cells to +S medium. This result indicates that activity of the 38 kDa PK may be induced rapidly within 5 min. We therefore shortened the incubation time points to 1, 2, 5, and 10 min after –S cells were transferred to +S and –S medium. As shown in **Figure 5(b)**, after –S cells were transferred to +S medium, we observed that a strong signal of PKs activity was induced rapidly at the position of 38 kDa, and that no inducible signal of PKs activity was observed after –S cells were transferred to –S medium. Altogether, the present results demonstrate that activity of two classes of PKs, of 50 and 66 kDa, were induced after +S rice cells were transferred to –S medium, and activity of a 38 kDa class of PK was induced after –S cells were transferred to +S medium. These results indicate that the 38 kDa class of PK might play a role in sugar sensing, and that the 50 and 66 kDa classes of PKs might play roles in signal transduction of sugar starvation in rice cells.

4. Discussion

Sugars can modulate gene expression at the transcriptional and post-transcriptional levels through a complex signal transduction network and through certain common mechanisms [3]. Many reviews summarize evidence for sugar control of gene expression through the activities of a variety of protein kinases, protein phosphatases, transcription factors, and *cis*-acting regulatory elements [5] [21] [22] [24] [27] [29]. Sugars also show interactions with light, stress, and phytohormone signaling pathways. In *Arabidopsis* suspension-cultured cells, the mRNA levels of 343 genes increase in response to sugar starvation [13]. Three of these genes were identified as putative GRPs. Interestingly, the three genes respectively showed a 33-, 23-, and 96-fold increase in transcript level after 24 h of sugar starvation [13]. The present study reveals that a group of low-molecular-weight mRNAs (about 0.8 kb) showed a dramatic increase in expression after 12 - 72 h of sugar starvation, and the expression decreased after the cells were provided with sugar (**Figure 2**). Eight of 12 cDNA clones showed expression patterns similar to that of the low-molecular-weight mRNAs, and all were highly homologous to GRPs. These findings are consistent with those of Conteno *et al.* [13] and may explain the high proportion of GRP genes cloned in sugar-starved cells. The GRPs are widely distributed in plants, of which some are developmentally regulated and some are involved in response to stresses, such as wounding, virus infection, drought, and flooding [41] [42] [43] [44] [45]. Sugar starvation is a severe nutritional stress in a plant cell, and during this condition many symptoms may be exhibited to conserve and supply energy, such as retarded cell division

and growth, autophagy, reduced protein synthesis, and strong activation of *α*-amylase and proteolytic enzymes [29]. Similar to the aforementioned studies, the present results show that extension of the duration of sugar starvation of rice cells was accompanied by global degradation of rRNAs and mRNAs (**Figure 1**), which might be intended to supplement the nitrogen and carbon sources in the cell. Moreover, two populations of mRNAs, namely those of *α*-amylases and GRPs, were induced and expressed abundantly during 12 - 72 h of sugar starvation, which suggests that *α*-amylases and GRPs might play important roles in the response to sugar starvation (**Figure 2**). The *α*-amylases hydrolyze starch into glucose to provide a carbon source [5]. Although GRPs form a group of structural protein components in the cell walls of many higher plants [46] [47], the biological functions of GRPs in response to biotic and abiotic stresses in plant cells is still largely unknown. We observed that under the energy-limited condition of sugar starvation, a population of GRPs is still generated, which suggests that GRPs might play novel roles in adaptation or response to nutritional stress in plant cells. In addition, the synchronized induction and repression of *GRP* homologs in −S and +S cells, respectively (**Figure 3** and **Figure 4**), might imply that a common signaling pathway is operational in sugar sensing. Given that *GRP* transcript levels are synergistically regulated and sensitive to sugar supply, GRPs be a useful indicator in investigations of the sugar signaling pathway.

One of the most advanced studies of sugar signaling in plants is on the hexokinase-dependent pathway [22]. In *Arabidopsis*, phosphorylation of mannose by hexokinase leads to the repression of genes needed for germination, but capability for germination can be restored by a specific hexokinase inhibitor, mannoheptulose [48]. Transgenic *Arabidopsis* plants that carry the antisense hexokinase are hyposensitive to sugar and show normal development comparable to that of the wild type, whereas plants that overexpress hexokinase are hypersensitive to sugar, leading to developmental arrest in the seedling [20]. In maize protoplasts and suspension-cultured cells of *Chenopodium rubrum*, incorporation of OA (a PP1 and PP2A inhibitor) in −S medium may mimic the repression of photosynthesis gene expression by sugars [49] [50]. These results reveal that protein phosphorylation and dephosphorylation events are involved in the sugar signal transduction pathway. In +S cells, we observed that the sugar-induced expression of SSR genes was repressed by OA (**Figure 3**). In addition, St and OA may diminish the effects of sugar-induced suppression of SSA gene expression (**Figure 4**). In −S cells, neither St nor OA altered the expression of SSR genes (**Figure 3**). In contrast, we observed that St repressed expression of SSA genes but enhanced expression of *salT*. Okadaic acid enhanced the effect of sugar starvation on *αAmy3*, *salT*, OsGRP2, and OsGRP3 mRNA levels, but reduced the −S induction of *αAmy8* expression (**Figure 4**). These findings suggest that in +S cells, PKs and PPs coordinately down-regulate expression of SSA genes, but not that of *αAmy3* and *αAmy8*. In −S cells, the expression of all *GRP* homologs was coordinately up- and down-regulated by PKs and PPs, respec-

tively. Interestingly, although *αAmy*3 and *αAmy*8 are homologous genes, the expression of *αAmy*3 and *αAmy*8 may be coordinately up-regulated by PKs, whereas PPs differentially down- and up-regulate the expression of *αAmy*3 and *αAmy*8, respectively. These results demonstrate that sugar signaling pathways function via a complex crosstalk between phosphorylation and dephosphorylation to control expression of a variety of genes.

In signaling transduction pathways, the signaling molecules usually stimulate a rapid, transient response, and the effects can be rapidly reversed [51]. Consistent with this observation, the present results demonstrated that activity of two classes of PKs, of 50 and 66 kDa, was induced rapidly 5 - 10 min after transfer of +S rice cells to –S medium, and the induced signals were almost non-detectable 20 min after transfer (**Figure 5(a)**). Moreover, when –S cells were transferred to +S medium for 1 - 2 min, a novel class of 38 kDa PK was hypersensitive to sugar and high activity was induced immediately, but then declined quickly and almost vanished at 5 min after transfer (**Figure 5(b)**). These results suggest that the 38 kDa PK class might play a role in sugar sensing, and the 50 and 66 kDa PK classes might play roles in signal sensing in response to sugar starvation in rice cells. To the best of our knowledge, no known class of PK is induced rapidly and is highly expressed in response to sugar and sugar-starvation signaling in plant cells. These results provide valuable information that indicates that 38, 50 and 66 kDa protein kinases might play key roles as sensors in sugar sensing and signaling.

5. Conclusion

Our results provided novel information that a population of glycine-rich proteins might play important roles in response to sugar starvation in rice cells. And a class of 38 kDa protein kinases could act as a sugar sensor, and the class of 50 and 66 kDa protein kinases might function as a sensor in response to sugar starvation in rice cells.

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Supplementary

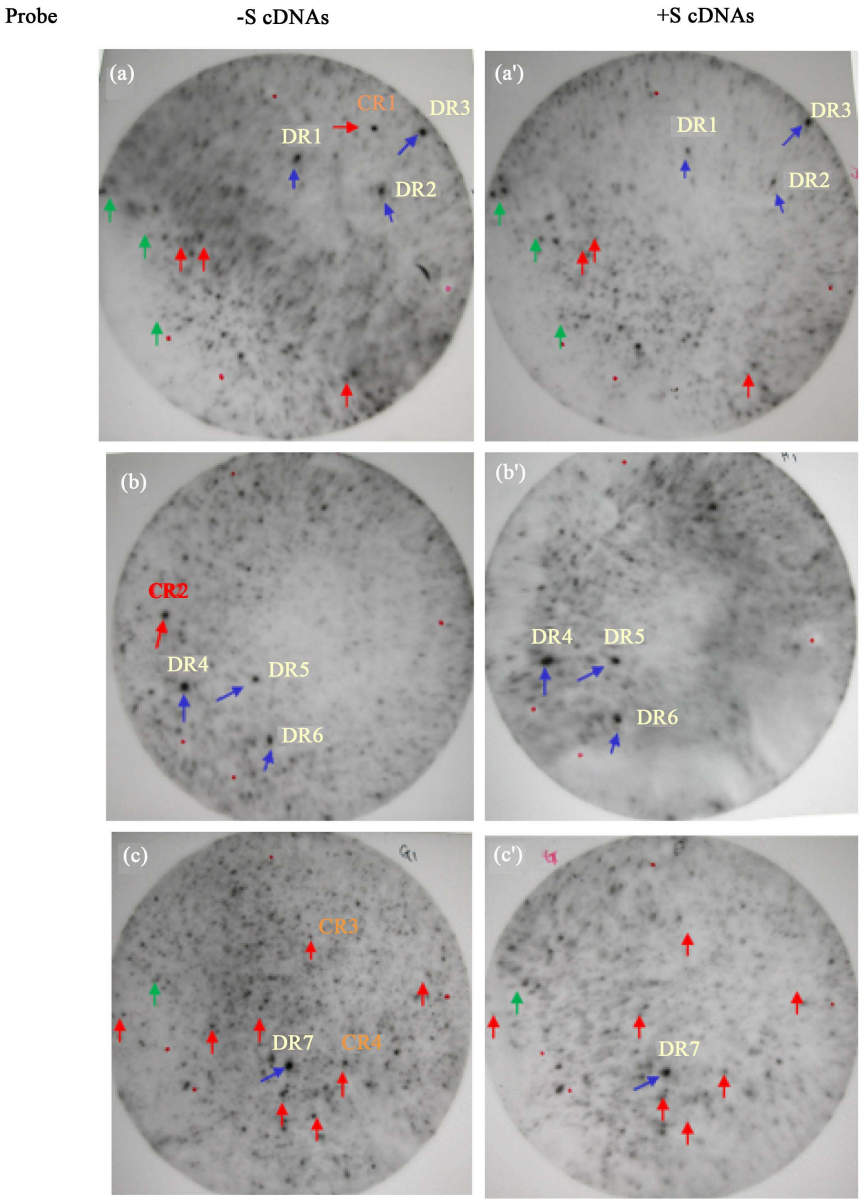

Figure S1. Differential screening to identify sugar-regulated genes. The cDNA library was constructed from rice suspension cells cultured under sugar starvation for 4 h. The phage plaques from the library were plated and transferred to nylon filter membranes. Duplicate filters ((a) and (a'), (b) and (b'), and (c) and (c')) were hybridized with cDNA probes derived from mRNAs isolated from rice cells cultured in MS medium supplemented with (+S cDNAs, right panels; (a'), (b'), and (c')) or without (−S cDNAs, left panels; (a), (b) and (c)) sucrose for 24 h. Red arrows indicate a strong hybridization signal expressed specifically in sugar-starved cells. Green arrows indicate a strong hybridization signal expressed specifically in sugar-containing cells. Blue arrows indicate the strong hybridization signals that were expressed constitutively in both sugar-containing and sugar-starved cells.