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Sugar-modulated gene expression of sucrose synthase in suspension-cultured cells of rice

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In suspension-cultured cells of rice (*Oryza sativa* L. cv. Tainung 67), two sucrose synthase genes, *RSus1* and *RSus2*, were found to be differentially regulated by sugars. Exogenously provided sugars triggered marked accumulation of the *RSus1* transcripts and this required de novo synthesized proteins. In addition to being regulated at the transcriptional level, other regulatory mechanisms might be involved in the sugar-modulated expression of *RSus1* since the RSuS1 proteins were extremely stable in sucrose-depleted cells. Glucose analogues, 3-*O*-methyl glucose, 6-deoxyglucose and mannose, did not trigger the induction of *RSus1* expression, suggesting that hexose uptake per se and hexokinase are not involved in the sugar-sensing pathway controlling *RSus1* expression. The accumulation of *RSus2* mRNA was observed regardless of the presence or absence of sugars. However, newly synthesized proteins induced by sucrose and/or starvation were involved in the regulation of *RSus2* expression at transcriptional and/or post-transcriptional levels. The protein level of RSuS2 was elevated under prolonged starvation condition but a reciprocal change was observed in the sucrose-fed cell. The constant presence of RSuS1 and RSuS2 proteins in both sucrose-starved and sucrose-provided cells may allow the cells to respond to sugar availability immediately.

Introduction

In higher plants, sucrose is a principle product of photosynthesis, a major form of translocated carbon and an important substrate for growth. Utilization of sucrose as a source of carbon and energy depends on its cleavage into hexoses. Both sucrose and hexoses can also act as direct or indirect regulators of gene expression (Koch 1996). The cleavage of sucrose in plants is catalysed either by invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) or by sucrose synthase (UDP- glucose: D-fructose 2-α-D-glucosyl transferase, EC 2.4.1.13, SuS). The former hydrolyses sucrose into fructose and glucose and the latter converts sucrose and UDP into fructose and UDP-glucose. Both enzymes are crucial for plant development, growth and carbon partitioning (Sturm and Tang 1999).

Although the reaction catalysed by SuS is reversible, it is thought that the enzyme mainly acts in the cleavage direction and its activity has been linked to starch synthesis (Chourey and Nelson 1976, Déjardin et al. 1997), cellulose synthesis (Amor et al. 1995, Carlson and Chourey 1996, Chourey et al. 1998, Nakai et al. 1999, Salnikov et al. 2001), phloem loading/unloading (Yang and Russell 1990, Martin et al. 1993, Nolte and Koch 1993, Fu and Park 1995, Guerin and Carbonero 1997, Wang et al. 1999) and sink strength (Sun et al. 1992, Wang et al. 1993, Zrenner et al. 1995). Multiple isoforms of SuS that are encoded by two or three genes have been found in a number of plant species, such as maize (Echt and Chourey 1985, Shaw et al. 1994, Carlson et al. 2002), sugarcane (Buczynski et al. 1993), carrot (Šebková et al. 1995), barley (Guerin and Carbonero 1997), rice (Yu et al. 1992, Huang et al. 1996, Huang and Wang 1998) and pea (Barratt et al. 2001). Expression of different *Sus* genes is spatially and temporally regulated and is differentially modulated in response to anoxia, low temperatures and osmotic stress (Maraña et al. 1990,

Abbreviations – ActD, actinomycin D; CHX, cycloheximide; 2-dG, 2-deoxyglucose; 6-dG, 6-deoxyglucose; 3-OMG, 3-*O*-methyl glucose; *Sh1*, *shrunken 1*; SPS, sucrose phosphate synthase; *Sus*, gene for sucrose synthase; SuS, protein encoded by *Sus*.

Chourey et al. 1991, Crespi et al. 1991, Zeng et al. 1998, Déjardin et al. 1999). Moreover, the expression of *Sus* genes is also differentially affected by sugars (Salanoubat and Belliard 1989, Koch et al. 1992, Fu and Park 1995, Fu et al. 1995, Godt et al. 1995, Huang et al. 1996, Koch 1996, Komatsu et al. 2002). For example, in maize, the *shrunken 1* (*Sh1*) gene is maximally expressed under conditions of limited carbohydrate supply, whereas *Sus1* is upregulated when sugars are abundant (Koch et al. 1992). In detached potato leaves, only transcription of *Sus4* gene is inducible by sucrose, while expression of *Sus3* remains unaffected under the same conditions (Fu and Park 1995).

In rice, sucrose synthase (RSuS) is encoded by three genes, *RSus1*, *RSus2* and *RSus3* (Wang et al. 1992, Huang et al. 1996). The gene products of *RSus1* and *RSus2* are ubiquitously present in suspension-cultured cells, roots and leaves of etiolated seedlings while those of *RSus3* are predominantly found in rice seeds (Wang et al. 1999). According to their tissue-specific and temporal distributions in the developing rice seeds, different roles have been suggested for these three RSuS isoforms. RSuS1 has been postulated as playing a role related to sucrose transport into endosperm cells. The reaction catalysed by RSuS3 has been suggested as providing the precursor for starch synthesis. Finally, RSuS2 may play a housekeeping role since it is ubiquitously and constitutively expressed (Wang et al. 1999). In the present study, the suspension-cultured cells of rice were investigated as to whether *RSus1* and *RSus2* expression is affected by sucrose transported into cells. Suspension-cultured cells of rice are heterotrophic and respond to exogenously applied sugars readily, and complicated results due to the spatial and developmental regulation can be avoided. The results indicate that *RSus1* is induced by sucrose, glucose and fructose, and its expression is regulated at the transcriptional and the post-transcriptional levels. An unexpected finding of the study is that the expression of *RSus2* requires newly synthesized proteins induced by sucrose and/or starvation. *RSus2* may have an important biological function in rice cells other than as a housekeeping gene.

Materials and methods

Plant materials

The suspension-cultured cells of rice (*Oryza sativa* L. cv. Tainung 67) were maintained in modified liquid N6 media containing Chu basal salts (Chu et al. 1975) and supplemented with 3% (w/v) sucrose, 0.1% (w/v) casamino acid, 1 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine-HCl and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid. Cultures were incubated at 25°C in the dark with a rotation of 120 r.p.m.

Before sugar treatment, the 3-day-old, logarithmically growing cells were rinsed and incubated with a fresh medium devoid of sucrose but containing 1.64% (w/v) mannitol for 48 h to lower the internal level of sugars. Cells were then transferred to fresh media with or without sugars. Sugars, sugar analogues and other chemicals were added to the medium at the reported concentrations. Cells were collected at desired time points by filtration through filter papers, quickly washed with sucrose-free media, blot-dried on paper towels and immediately frozen in liquid nitrogen. The frozen cells were stored at − 80°C until use.

Isolation of total RNA and Northern analysis

The frozen suspension-cultured cells were ground in liquid nitrogen to a fine powder using a pestle and mortar. Total RNA was isolated using TRIZOL reagent (Life Technologies/Gibco-BRL, Cleveland, USA) according to the protocol suggested by the manufacturer. Fifteen micrograms of total RNA from each sample were separated on 1.2% (w/v) agarose gels containing formaldehyde (Lehrach et al. 1977) and then blotted to positively charged nylon membranes (Immobilon-Ny +, Millipore, Bedford, USA) using 10 × SSPE (1.5 *M* NaCl, 100 m*M* sodium phosphate, 10 m*M* EDTA, pH 7.4). The transferred nucleic acids were UV cross-linked to membranes. Prehybridization and hybridization were done as described by Sambrook et al. (1989). After hybridization, the blots were washed twice for 10 min at room temperature with 2 × SSPE, 0.1% SDS, washed once for 15 min at 42°C with 1 × SSPE, 0.1% SDS, and then washed once with 0.1 × SSPE, 0.1% SDS for 15 min at 61°C for the *RSus2* gene-specific probe or at 68°C for the *RSus1* and the 18S rRNA gene-specific probes. The blots were exposed to X-ray films or phosphor-imaging plates and the captured images were analysed with a Bio Imaging Analyser (Fujix BAS1000, Fuji Photo Film, Tokyo, Japan).

Preparation of DNA probes

The DNA probes specific for *RSus1* and *RSus2* were synthesized by PCR using plasmids containing the *RSus1* and *RSus2* cDNAs (Yu et al. 1992, Huang et al. 1996), respectively, as templates. The sequences of the primers that were selected from the 3′-untranslated region of each cDNA were as follows: *RSus1* forward primer, 5′-TAGGTAACCTGGAGAGGCGTGAAAC-3′ (nucleotides 2431–2455). *RSus1* reverse primer, 5′-ATGGAATTCCAGAAACGGCACCAAG-3′ (nucleotides 2855–2831). *RSus2* forward primer, 5′-TGCATCTTCAGCAGGAGAAG-3′ (nucleotides 2531–2550). *RSus2* reverse primer, 5′-CAGGAACCGGCGTTTATTTGA-3′ (nucleotides 2692–2672).

The specificity of each probe was verified by cross Southern hybridization (data not shown). For preparing the probe for 18S rRNA, an 1.6-kb DNA fragment containing a part of the rice 18S rRNA gene was PCR amplified from rice genomic DNA with the forward primer (5′-GAACGAATTCGAACTGTGAAACTGC-3′) and the reverse primer (5′-TACAAACTGCAGGGACGTAGTCAA-3′). The amplified DNA fragment was subjected to sequencing for confirmation.

For use in the Northern hybridization, both the *RSus1*-specific probe and the 18S rRNA-specific probe were labelled with [α-^{32}P] dCTP (3000 Ci mmol^{-1}) (NEN, Boston, USA) using a random prime labelling system (Amersham Pharmacia Biotech, Bucks, UK). The *RSus2*-specific probe was labelled with [α-^{32}P] dATP (3000 Ci mmol^{-1}) by PCR.

Nuclear run-on transcription assays

Nuclei isolation was performed as described by Manzara and Gruissem (1995) but using a different homogenization buffer (10 m*M* Tris-HCl, pH 7.2; 5 m*M* $MgCl_2$, 0.5 *M* sucrose, and 5 m*M* 2-mercaptoethanol) and a different nuclei storage buffer (20 m*M* HEPES-KOH, pH 7.2; 5 m*M* $MgCl_2$, 50% glycerol, 5 m*M* 2-mercaptoethanol).

For RNA synthesis, 75 µl nuclei were mixed with 75 µl of 2 × assay buffer (40 m*M* HEPES-KOH, pH 7.9; 20 m*M* $MgCl_2$, 200 m*M* $(NH_4)_2SO_4$, 20% glycerol, 5 m*M* dithiothreitol, 1 m*M* each of ATP, GTP and CTP) containing 200 units RNasin (Promega, Madison, USA) and 150 µCi [α-^{32}P] UTP (3000 Ci mmol^{-1}). The reaction was incubated at 25°C for 30 min and followed by the addition of 10 µl of 1 m*M* UTP and incubated at 25°C for another 30 min. Five units of RQ1 RNase-free DNase (Promega) were then added and incubated at 25°C for 10 min to remove the template. The synthesized RNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1 [v/v/v], pH 4.5) extraction and then precipitated with 100 µg of yeast tRNA (Life Technologies/Gibco-BRL) using isopropanol. The RNA pellet was dissolved in diethyl pyrocarbonate-treated water.

For dot blot hybridization, unlabelled *RSus1*-specific probe, *RSus2*-specific probe, 18S rRNA-specific probe and the plasmid pBluescript were denatured and dot blotted onto positively charged nylon membranes as described by Ausubel et al. (1987). Blots were hybridized with labelled RNA and washed as described for the Northern blot analysis.

Enzyme extraction, polyacrylamide gel electrophoresis and Western analysis

The frozen suspension-cultured cells were ground to a fine powder in liquid nitrogen. An equal volume (v/w) of PB-7.0 (50 m*M* sodium phosphate, pH 7.0; 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol) was added and mixed. The homogenates were centrifuged at 12 000 *g* and 4°C for 30 min. The resulting supernatants were used for polyacrylamide gel electrophoresis (PAGE) and Western analysis.

The proteins were separated by 10% SDS-PAGE according to Laemmli (1970) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). For the detection of the RSuS1 protein, a monoclonal antibody that recognizes RSuS1 and RSuS3, but not RSuS2, was used. Because the expression of *RSus3* is seed-specific (Wang et al. 1999), only RSuS1 proteins can be detected by this antibody in suspension-cultured cells. In the case of RSuS2, a monospecific antipeptide antibody (Wang et al. 1999) was used for immunodetection.

Results

Accumulation of *RSus1* and *RSus2* mRNA in sugar-depleted and sugar-fed cells

To study the effect of exogenously provided sugars on the expression of *RSus* genes, cells cultured in a sucrose-containing medium for 3 days were transferred to a fresh medium devoid of sucrose to deplete the endogenous sugars. Mannitol was added to the medium to maintain the same osmotic pressure and to avoid changes in gene expression caused by osmotic shock. Cells were then collected after different periods of time up to 48 h, and the steady-state levels of *RSus1* and *RSus2* mRNA in cells were examined by Northern analysis using the gene-specific probes. As shown in Fig. 1A, the levels of *RSus1* mRNA gradually decreased and became undetectable within 48 h of culture in the sucrose-free medium, while the changes in *RSus2* mRNA levels were less pronounced. When the 48-h-starved cells were transferred to fresh media containing various sugars at a concentration of 100 m*M*, the *RSus1* mRNA began to accumulate after 2–3 h of sucrose, glucose or fructose feeding (Fig. 1B). After 12–16 h, the *RSus1* transcripts reached a maximal level and then reduced. An accumulation of *RSus1* mRNA was not observed in mannitol-fed cells, indicating that the expression of *RSus1* after cells being transferred to sugar-containing medium was sugar-dependent, and was not a response to changes in osmotic pressure. The accumulation of transcripts caused by sugars was also observed for the *RSus2* gene. However, in contrast to *RSus1*, the steady-state levels of the *RSus2* mRNA in the starved cells increased on transfer to mannitol-containing media. This was not due to the changes in osmotic pressure because the transcript levels of *RSus2* in cells cultured with different concentrations of mannitol were similar (data not shown).

Effect of sucrose on the mRNA stability and transcriptional activity of *RSus* genes

To unravel the mechanism that regulates the expression of the *RSus* genes, both the mRNA stability and the transcriptional activity of *RSus1* and *RSus2* in sucrose-fed and sucrose-depleted cells were examined. The 48-h-starved cells were cultured with sucrose for 24 h, and then treated with 10 µg ml^{-1} actinomycin D (ActD), an inhibitor of transcription, in the presence of sucrose for 12 h. This should result in more than 95% inhibition of transcription (Sheu et al. 1994). The patterns of changes in mRNA level were similar for both *RSus1* and *RSus2* during the subsequent 12-h period of incubation with ActD, regardless of the presence (Fig. 2A) or absence (Fig. 2B) of sucrose in the medium. To estimate the mRNA half-lives of the two genes, the hybridization

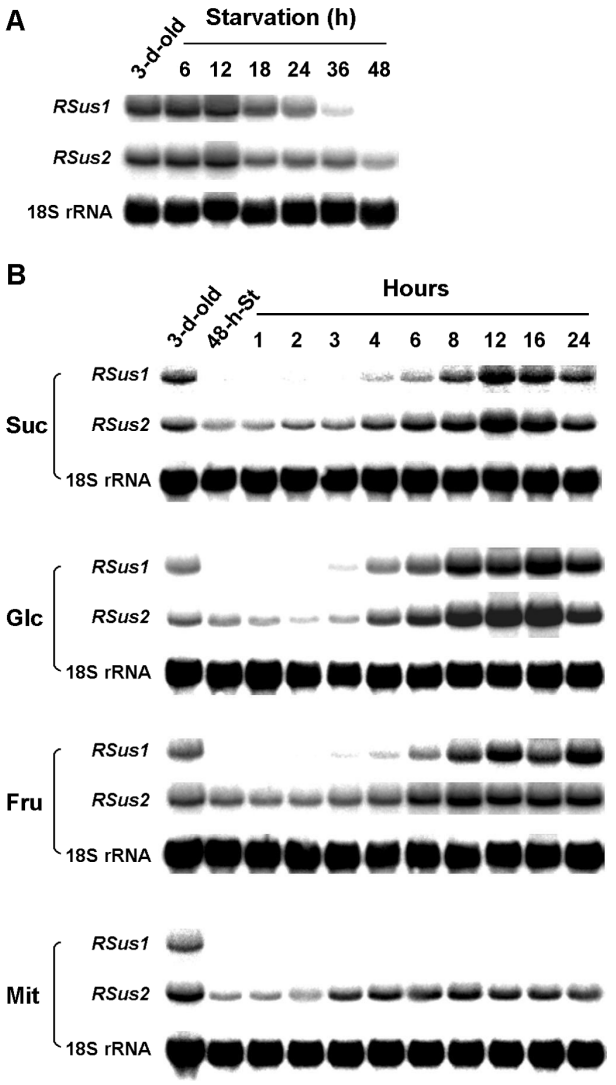

Fig. 1. Time course analysis of changes in the steady-state levels of the *RSus* mRNA in sugar-depleted and sugar-fed suspension-cultured cells. Cells cultured in a sucrose-containing medium for 3 days (3-d-old) were transferred to a sucrose-free medium (containing 1.64% mannitol) for 48 h (A). After 48-h starvation (48-h-St), the cells were incubated in fresh media containing 100 m*M* sucrose (Suc), glucose (Glc), fructose (Fru) or mannitol (Mit) for 24 h (B). Cells were collected at indicated time. Total RNA from the cells was isolated and an equal amount of RNA (15 μg) was loaded into each lane of formaldehyde agarose gels and analysed by Northern blot hybridization. Identical blots were hybridized with *RSus1*-specific, *RSus2*-specific and 18S rRNA-specific probes.

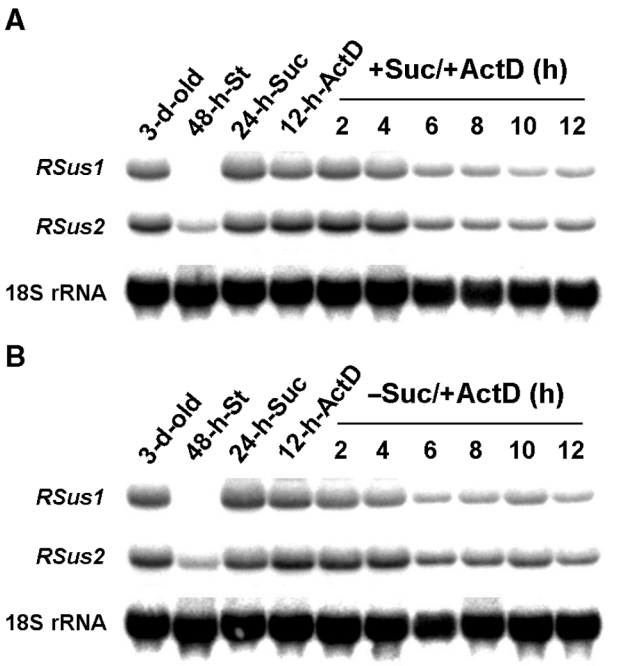

Fig. 2. Effect of sucrose on the stability of *RSus* mRNA. Cells cultured in a sucrose-containing medium for 3 days (3-d-old) were starved for 48 h (48-h-St) and then cultured in a medium containing sucrose for 24 h (24-h-Suc) to achieve a high starting level of mRNA. After transferring to medium containing sucrose (100 m*M*) and ActD (10 μg ml^{-1}) for another 12 h (12-h-ActD), the cells were subsequently incubated in fresh media containing both sucrose and ActD (A) or lacking sucrose but with ActD (B) for 12 h. Samples were collected every 2 h. Total RNA from the cells was isolated and an equal amount of RNA (15 μg) was loaded into each lane of formaldehyde agarose gels and analysed by Northern blot hybridization. Identical blots were hybridized with *RSus1*-specific, *RSus2*-specific and 18S rRNA-specific probes.

signals in Fig. 2 were quantified densitometrically, and the values were normalized relative to the amount of 18S rRNA (data not shown). The estimated half-life of *RSus1* mRNA in sucrose-fed cells and in sucrose-depleted cells was 13.8 h and 14.7 h, respectively. The mRNA half-life of *RSus2* was approximately 1.5-fold longer in sucrose-depleted cells than in sucrose-fed cells, which are 16.6 h and 11.4 h, respectively. The results revealed that changing sucrose levels did not alter the stability of *RSus1* mRNA, but *RSus2* mRNA was more stable under sucrose-deprivation conditions.

Figure 3 shows the results of nuclear run-on transcription of *RSus* genes with nuclei isolated from sucrose-fed and sucrose-depleted cells. The transcriptional activity of *RSus1* in cells starved for sucrose was undetectable but was high in cells fed with sucrose, showing that transcriptional regulation was involved in the sucrose-modulated expression of *RSus1*. The transcription rates of *RSus2* were similar in cells grown in the sucrose-free and sucrose-containing media, suggesting that there was constitutive expression of *RSus2* mRNA in cells under varying sugar availability.

Requirement of de novo protein synthesis for *RSus* expression

To assess whether newly synthesized proteins are essential for the sugar-modulated expression of *RSus* genes, the 48-h-starved cells were cultured in sucrose-free or sucrose-supplemented media with or without cycloheximide (CHX) added. The addition of CHX completely abolished the induction of *RSus1* expression by sucrose

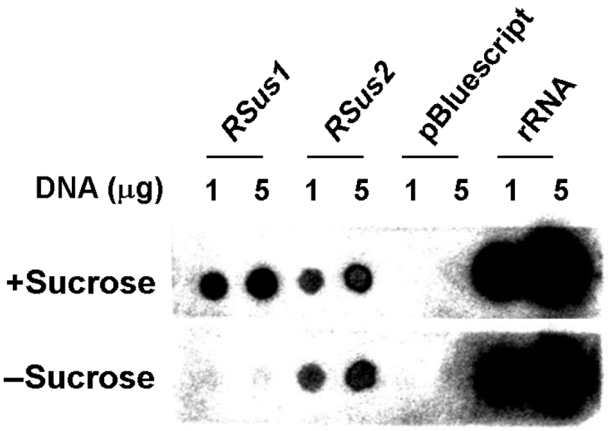

Fig. 3. Transcriptional activity of *RSus* genes in sucrose-fed and sucrose-depleted cells. Nuclei were isolated from cells that had been starved for 48 h and then transferred to fresh media with or without sucrose for 24 h. Run-on transcription reactions were carried out as described in Materials and methods. DNA fragments of unlabelled *RSus1*-specific probe, *RSus2*-specific probe, 18S rRNA-specific probe and the plasmid pBluescript DNA (as a negative control) were denatured and dot blotted onto positively charged nylon membranes in different quantities. ^{32}P-labelled RNA from run-on transcription was hybridized to the DNA immobilized on membranes.

(Fig. 4A). The accumulation of *RSus1* mRNA was restored when the 12.5-h CHX-treated cells were transferred to a CHX-free but sucrose-containing medium (Fig. 4A, the last lane). It indicated that de novo protein synthesis was needed for the induction of *RSus1* by sucrose. Unexpectedly, the accumulation of *RSus2* mRNA observed in sucrose-fed (Fig. 4B) and sucrose-depleted cells (Fig. 4D) was inhibited by CHX (Fig. 4A,C). The transcript levels of *RSus2* were reduced significantly and were undetectable by the 12-h time point in the CHX-treated cells, regardless of the presence or absence of sucrose (Fig. 4A,C). When the 12.5-h CHX-treated cells were transferred to a CHX-free medium, the accumulation of *RSus2* mRNA was observed again (Fig. 4A,C, the last lane). These results showed that addition of CHX not only suppressed any further increase in *RSus2* mRNA levels but also led to a decrease in half-life of *RSus2* mRNA in both sucrose-fed (Fig. 4A,B) and sucrose-depleted cells (Fig. 4C,D). CHX negatively affected the transcription and/or the stability of *RSus2* mRNA.

The protein levels of RSuS in sugar-depleted and sugar-fed cells

To determine whether the protein levels of RSuS were also affected by sugar availability, SDS-Western analyses were performed using a monoclonal antibody specifically recognizing RSuS1 in suspension-cultured cells (Materials and methods) and an RSuS2 monospecific antibody (Wang et al. 1999). Although the accumulation of *RSus1* mRNA was hardly detected after 48-h starvation (Fig. 1A), RSuS1 protein was still present in 48-h-starved

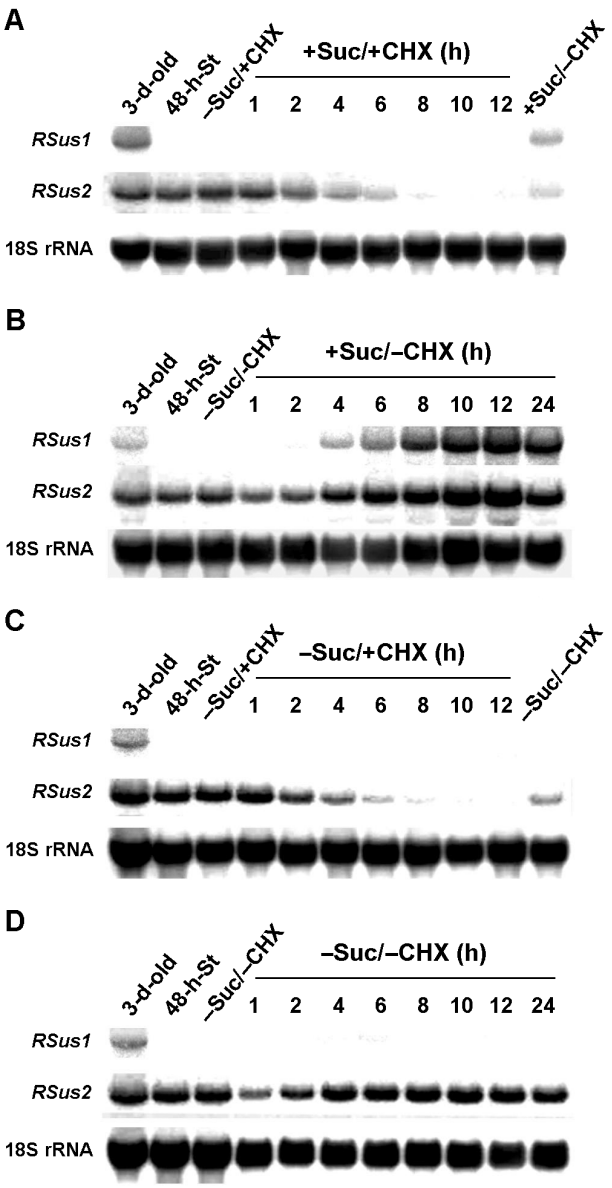

Fig. 4. Effect of cycloheximide on *RSus* expression in a sucrose-free or a sucrose-containing medium. 3-d-old cells were starved for 48 h (48-h-St) and then divided into two halves. Half the cells were preincubated with 200 μ*M* CHX in sucrose-free medium for 30 min (A and C, –Suc/+ CHX). Then cells were subsequently transferred to media with (A) or without (C) sucrose but containing 200 μ*M* CHX for up to 12 h and returned to media without CHX (+ Suc/– CHX in A or –Suc/– CHX in C). The other half was transferred to fresh medium devoid of sucrose and CHX for 30 min (B and D, –Suc/– CHX) and then incubated in media with (B) or without sucrose (D). Cells were collected at indicated time. Total RNA of the cells was isolated and an equal amount of RNA (15 μg) was loaded in each lane and analysed by Northern blot hybridization. Identical blots were probed with *RSus1*, *RSus2* and 18S rRNA probes.

cells (Fig. 5), showing that the turnover rate of RSuS1 proteins was much slower than that of *RSus1* mRNA. We therefore examined the changes in protein abundance over a longer period. As shown in Fig. 5, the RSuS1 protein level was a little higher in sucrose-fed cells than

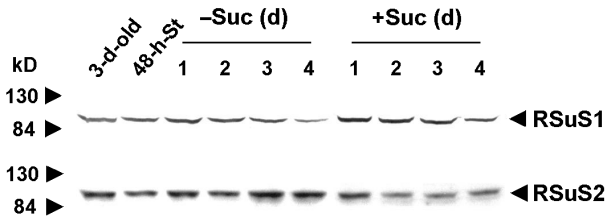

Fig. 5. Effect of sugar availability on the protein levels of RSuS. Cells cultured in a sucrose-containing medium for 3 days (3-d-old) were starved for 48 h (48-h-St) and then transferred to fresh media containing 100 m*M* mannitol (– Suc) or sucrose (+ Suc). Cells were collected at the indicated times. An equal volume of crude protein extract from the cells was loaded into each lane of SDS-polyacrylamide gels, and Western analyses of RSuS1 and RSuS2 proteins were carried out using a monoclonal antibody specifically recognizing RSuS1 in suspension-cultured cells and an RSuS2 monospecific antibody. Molecular weight markers are shown at the left margin.

in sucrose-starved cells and a gradual reduction was observed in both types of cells over 4 days of culture. The level of RSuS2 protein elevated in cells under prolonged sucrose starvation conditions and a reciprocal change was observed in the sucrose-fed cells.

Effects of glucose analogues and glucosamine on *RSus* expression

Recent studies indicate that sugar signalling in plants can occur through a number of paths including the hexokinase-dependent pathway, the hexose-dependent but hexokinase-independent pathway, and the sucrose-specific sensing and signalling pathways (Gibson 2000, Koch et al. 2000, Smeekens 2000). A number of sugar analogues have been used to characterize plant sugar responses and response pathways in a number of studies. These include glucose analogues such as L-glucose, which is not recognized by hexose transporters and is attributable to an osmotic effect, 3-*O*-methyl glucose (3-OMG) and 6-deoxyglucose (6-dG), which are taken up by cells but are poor substrates for hexokinase, and 2-deoxyglucose (2-dG) and mannose (Man), which can be phosphorylated by hexokinase but are poorly metabolized thereafter (Graham et al. 1994, Jang and Sheen 1994, Roitsch et al. 1995, Umemura et al. 1998). Figure 6 shows the effects of these non-metabolizable sugar analogues at non-toxic concentrations (Umemura et al. 1998) on the expression of the *RSus* genes. None of these glucose analogues triggered *RSus1* expression, showing that the expression of *RSus1* is independent of osmotic effects and hexose transporters and hexokinases are not involved in the sugar-sensing pathway controlling *RSus1* expression. 3-OMG and mannose at lower concentrations cannot activate *RSus1* gene expression as well, confirming that the effect of the two sugar analogues at 50 m*M* is not due to their toxicity on cells (data not shown). Glucosamine, a hexokinase inhibitor, was used for further confirmation. As shown in Fig. 7, addition of glucosamine to the media did not hamper the induction of *RSus1* expression by sucrose, glucose and fructose, but led to a

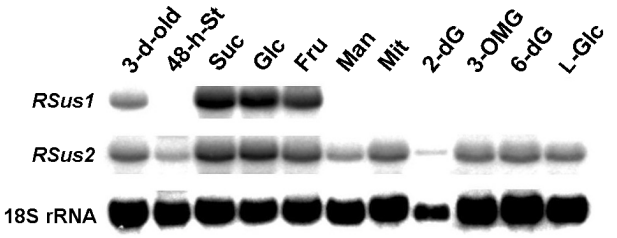

Fig. 6. Effect of sugar analogues on the expression of *RSus* genes. Cells cultured in a sucrose-containing medium for 3 days (3-d-old) were starved for 48 h (48-h-St) and then transferred to fresh media containing 50 m*M* sucrose (Suc), 50 m*M* glucose (Glc), 50 m*M* fructose (Fru), 50 m*M* mannose (Man), 50 m*M* mannitol (Mit), 0.9 m*M* 2-deoxyglucose (2-dG), 50 m*M* 3-*O*-methyl glucose (3-OMG), 9 m*M* 6-deoxyglucose (6-dG), or 50 m*M* L-glucose (L-Glc) for 24 h. Total RNA from the cells was isolated and an equal amount of RNA (15 μg) was loaded into each lane of formaldehyde agarose gels and analysed by Northern blot hybridization. Identical blots were hybridized with *RSus1*-specific, *RSus2*-specific and 18S rRNA-specific probes. The addition of 2-dG, which was shown by Umemura et al. (1998) not to have any toxic effects at 0.9 m*M* on rice embryos, resulted in an inhibition of cell growth and a degradation of rRNA.

small reduction in both *RSus1* and *RSus2* mRNA levels. Similar effects were observed when glucosamine was at a concentration lower than 50 m*M* (data not shown). The accumulation of *RSus2* mRNA was observed when cells were fed with the above non-metabolizable glucose analogues (Fig. 6), but it was not possible to reach a conclusion on whether the induction was triggered by these glucose analogues or by sugar starvation.

Discussion

Sugar-modulated expression of *RSus1* and *RSus2* are differentially regulated

In maize, the genes encoding for SuS, *Sus1* and *Sh1*, show contrasting carbohydrate responses; that is, *Sus1* is upregulated while *Sh1* is downregulated in the presence

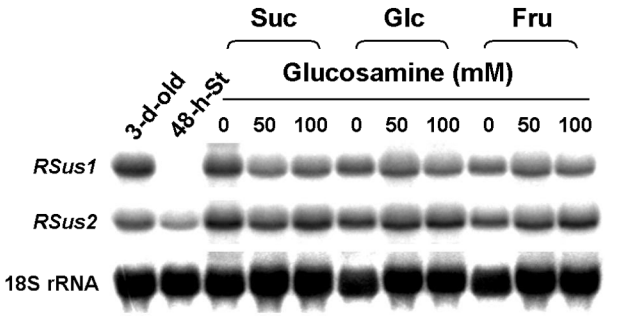

Fig. 7. Effect of glucosamine on *RSus* expression in the presence of sugars. Cells cultured in a sucrose-containing medium for 3 days (3-d-old) were starved for 48 h (48-h-St) and then transferred to media containing 50 m*M* sucrose (Suc), glucose (Glc) or fructose (Fru) in the presence or absence of glucosamine for 24 h. Total RNA from the cells was isolated and an equal amount of RNA (15 μg) was loaded into each lane of formaldehyde agarose gels and analysed by Northern blot hybridization. Identical blots were hybridized with *RSus1*-specific, *RSus2*-specific and 18S rRNA-specific probes.

of abundant carbohydrate supplies (Koch 1996). In the suspension-cultured cells used in this study, we demonstrated that the *Sus1*-type gene in rice, *RSus1*, is also upregulated by sugars. However, *RSus2*, the *Sh1*-type gene, is sugar as well as starvation upregulated (Fig. 1B, and Fig. 4C,D). Possession by the same gene of a similar response to the two contrasting carbohydrate levels is unusual, and, to our knowledge, this is the first case among *Sus* genes.

Sucrose-modulated expression of *RSus1* was regulated at the transcriptional level as revealed from that the transcription activity of *RSus1* significantly increased in the presence of sucrose (Fig. 3), but the stability of the transcribed *RSus1* mRNA was not affected by sucrose abundance (Fig. 2). However, the small difference between the protein level of RSuS1 in sucrose-fed cells and that in sucrose-depleted cells (Fig. 5) indicated that there are other mechanisms involved in regulating *RSus1* expression. In the sucrose-starved cells, the *RSus1* mRNA could hardly be detected after 48 h of sucrose starvation (Fig. 1A,B) but the RSuS1 proteins were still present in cells even after prolonged sucrose starvation (Fig. 5). A possible explanation is that the RSuS1 proteins are extremely stable in sucrose-starved cells. In a previous study, we reported that the gene products of *RSus1* and *RSus2* formed homo- and hetero-tetrameric RSuS isoforms in etiolated rice seedlings (Huang and Wang 1998). RSuS1 might be stabilized through the formation of heterotetramers with RSuS2 since the *RSus2* gene was expressed under sucrose starvation conditions, and the RSuS2 protein level was even higher in sucrose-starved cells than in sucrose-fed cells. Regulation of *RSus2* was different from that of *RSus1*. First, the stability of *RSus2* mRNA was higher in sucrose-starved cells, suggesting that post-transcriptional regulation of *RSus2* occurred. Second, although the transcriptional activity of *RSus2* in sucrose-fed and sucrose-starved cells was similar, the transcription and/or the stability of *RSus2* mRNA, was negatively affected by CHX. The results showed that newly synthesized proteins induced by sucrose or by sucrose starvation were involved in the regulation of *RSus2* expression at transcriptional and/or post-transcriptional levels. Furthermore, the changes in mRNA level and those in protein level did not show marked differences, except that the level of RSuS2 protein was higher under prolonged sucrose starvation conditions. However, it may attribute to the higher stability of the *RSus2* mRNA in sucrose-starved cells.

Hexose uptake per se and the phosphorylation of hexoses by hexokinase are not involved in the sugar-sensing pathway controlling *RSus1* expression

In suspension-cultured cells of *Chenopodium rubrum*, the addition of either glucose or 6-dG induced the expression of the *Sus* gene, suggesting that the non-phosphorylated glucose is the signal for sugar-induced gene expression (Godt et al. 1995). In this study, it was found that hexose uptake per se and the phosphorylation of hexoses by hexokinase were not involved in the sugar-sensing pathway controlling *RSus1* expression since 3-OMG, 6-dG and mannose did not induce *RSus1* (Fig. 6). However, addition of glucosamine resulted in a reduction in the *RSus1* mRNA level (Fig. 7), suggesting that the catalytic function of hexokinase in cells was required to maintain the maximal expression of *RSus1*.

It is unclear how hexoses and sucrose modulate the induction of *RSus* and whether the expressions of *RSus1* and *RSus2* are mediated by the same signal transduction pathway. It was found that cell wall invertase activity was high in sucrose-fed suspension-cultured cells of rice but low in glucose- or fructose-fed cells (Y.-H. Lee 1997. Thesis, National Taiwan University, Taipei, Taiwan). Therefore, in addition to being taken up via sucrose transporters, sucrose may be hydrolysed by cell wall invertases and the resulting hexoses are transported into cells by hexose transporters. The sucrose that is hydrolysed by cell wall invertases and the hexoses that are directly fed to cells may affect the expression of *RSus1* gene via the same pathway. It has been shown that sucrose synthesis occurs rapidly in vivo when hexoses are the sole carbon source in the unloading medium (Geigenberger and Stitt 1993, Viola 1996). Rapid turnover of sucrose, the futile cycle, has also been observed in a number of plant species (Wendler et al. 1990, Geigenberger and Stitt 1991, Nguyen-Quoc and Foyer 2001). For example, in tomato fruits, the unloaded sucrose is broken down and rapidly re-synthesized within 2 h (N'tchobo et al. 1999). Sucrose synthesis in plants is preferentially catalysed by sucrose phosphate synthase (SPS; EC 2.4.1.14), but it has been suggested that SuS is also able to catalyse the synthesis of sucrose (Gross and Pharr 1982, Tupy and Primot 1982, Geigenberger and Stitt 1993, Viola 1996). Recent studies indicate that SuS may be associated with the plasma membrane and the actin cytoskeleton in addition to being a soluble enzyme in the cytosol (Amor et al. 1995, Winter et al. 1997, 1998). We postulate that the re-synthesized sucrose either from the transported hexoses or from the unloaded sucrose may be the signal molecule for the induction of *RSus* expression. In this aspect, the membrane-associated SuS is a better candidate, being the signal producer, than the soluble SuS and SPS because the reaction catalysed by it can be distinguished from the main stream of sucrose metabolism and synthesis that occurs in the cytosol. Furthermore, the constant presence of RSuS1 and RSuS2 proteins in sucrose-starved cells and sucrose-fed cells can allow the cells to respond to sugar availability immediately. Further investigation is needed to elucidate whether our hypothesis is valid and whether other signals such as the influx of sucrose into cells, sugar metabolites, etc., are required for the sugar-modulated expression of the *RSus* genes.

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