Novel Gene Encoding a Ca\textsuperscript{2+}-Binding Protein and under Hexokinase-dependent Sugar Regulation

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Received September 5, 2002; Accepted October 7, 2002

A cDNA encoding a predicted 15-kDa protein was earlier isolated from sugar-induced genes in rice embryos (\textit{Oryza sativa} L.) by cDNA microarray analysis. Here we report that this cDNA encodes a novel \textit{Ca}\textsuperscript{2+}-binding protein, named \textit{OsSURI} (for \textit{Oryza sativa} sugar-up-regulated 1). The recombinant \textit{OsSURI} protein expressed in \textit{Escherichia coli} had \textit{Ca}\textsuperscript{2+}-binding activity. Northern analysis showed that the \textit{OsSURI} gene was expressed mainly in the internodes of mature plants and in embryos at an early stage of germination. Expression of the \textit{OsSURI} gene was induced by sugars that could serve as substrates of hexokinase, but expression was not repressed by \textit{Ca}\textsuperscript{2+} signaling inhibitors, calmodulin antagonists and inhibitors of protein kinase or protein phosphatase. These results suggested that \textit{OsSURI} gene expression was stimulated by a hexokinase-dependent pathway not mediated by \textit{Ca}\textsuperscript{2+}.

Key words: EF-hand motif; hexokinase; \textit{Oryza sativa} L.; signal transduction; sugar sensing

In plants, sugars are a substrate for growth and trigger sugar-sensing systems that change gene expression. Carbohydrate depletion up-regulates genes for photosynthesis, remobilization, and export, and decreases the expression of mRNAs for storage and use of sugars.\textsuperscript{11} These signal transduction mechanisms probably have multiple sugar sensors or receptors. Hexokinase, which catalyses glycolysis and acts as a glucose sensor in yeasts, is the first sugar sensor to be detected in higher plants.\textsuperscript{2,3} Many components of a sugar signaling pathway, including a tryptophan-aspartate (WD)-40 repeats protein,\textsuperscript{4,5} a calcium-dependent protein kinase (CDPK),\textsuperscript{6} a protein phosphatase,\textsuperscript{7} sucrose non-fermenting (SNF1)-related protein kinase,\textsuperscript{8,9} and transcription factors,\textsuperscript{10,11} have been reported; the relationships among these mediators are under investigation. The results of experiments with plants expressing the \textit{Ca}\textsuperscript{2+}-specific photoprotein aequorin have suggested that cytosolic calcium levels increase in leaves when they are incubated with sugars.\textsuperscript{12,13} The sugar-inducible expression of storage protein genes such as sporamin and \textit{\textalpha} -amylase is involved, at least in part, in \textit{Ca}\textsuperscript{2+}-mediated signaling with CDPK and calmodulin, because this sugar-induced expression is inhibited by \textit{Ca}\textsuperscript{2+} channel blockers, calmodulin antagonists, and inhibitors of protein kinase and protein phosphatase.\textsuperscript{12} Similarly, these inhibitors block the accumulation of anthocyanin, which is induced by hexose, a possible substrate of hexokinase, in suspended cells of \textit{Vitis vinifera}.\textsuperscript{14} These observations suggested that sugar signal transduction mediated by hexokinase is related to the level of \textit{Ca}\textsuperscript{2+} in the cytosol.

Whether phosphorylation by hexokinase is an essential step in a sugar-response pathway can be assessed by comparison of the effects of sugar analogs that act as substrates for the enzyme with the effects of those that do not. This kind of analysis led to the hypothesis that phosphorylation of sugars by hexokinase is a key in triggering the sugar-regulated expression of certain genes.\textsuperscript{15,16} Transgenic \textit{Arabidopsis} plants expressing antisense hexokinase gene (\textit{AtHXX}) are hyposensitive to sugar, but plants expressing sense \textit{AtHXX} are hypersensitive.\textsuperscript{3} These
observations indicate that hexokinase functions as a sugar sensor in higher plants, although the signaling process is unknown.\textsuperscript{19}

We have been investigating the mechanisms underlying sugar regulation in rice plants, in which the $\alpha$-amylase gene Ramu3D is down-regulated by sugar during seed germination.\textsuperscript{19} To study this mechanism further, we used cDNA microarrays to obtain additional clones the expression of which is regulated by high- or low-sugar levels in callus-forming embryos. We identified the gene OsSUR1 (Oryza sativa sugar-up-regulated-1), which has two domains similar to the Ca$^{2+}$-binding loops of EF-hands.\textsuperscript{19} Here we report that this novel gene, which encodes a Ca$^{2+}$-binding protein, is under hexokinase-dependent sugar regulation.

Materials and Methods

\textit{Preparation of rice embryos.} Rice seeds (Oryza sativa L. cv. Notohikari) were sown in Petri dishes containing liquid Murashige-Skoog salt mixture and 2 mg/l 2,4-dichlorophenoxyacetic acid. Seeds that germinated on this medium had an enlarged scutellar side, which allowed treatment with a 3% gellan gum plate containing the sugars listed below (in Fig. 4B) at the concentration of 5 or 00 mM. All procedures were done as described by Umemura et al.\textsuperscript{19} Suspension cultures derived from embryos were prepared as reported by Shimamoto et al.\textsuperscript{20}

\textit{Microarray analysis.} Preparation of full-insert cDNA microarray of rice: The 1265 unique cDNA clones used for the probes of our microarrays were provided by the Rice Genome Research Program (RGP: http://rgp.dna.afrc.go.jp/). Preparation of the full-insert cDNAs from the clones and of the microarray was described by Yazaki et al.\textsuperscript{21}

\textit{Expression of the OsSUR1 fusion protein.} The open reading frame of the OsSUR1 cDNA clone obtained from the Rice Genome Research Program was ligated into a His-tagged vector, pET-32a$^+ (Novagen, Darmstadt, Germany). This construct was introduced into E. coli BL21(DE3)LysS, and protein expression was induced with 1 mM isopropyl-$\beta$-D-thiogalactopyranoside at 37°C for 2 h. Total E. coli protein was extracted in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 100 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride by freezing and thawing, and purified with TALON metal-affinity resin (Clontech Laboratories, Palo Alto, CA) by the manufacturer's instructions.

\textit{Location of OsSUR1 in vivo.} In an investigation of the subcellular location of OsSUR1 protein, the cDNA was fused in-frame to sGFP(S65T).\textsuperscript{22} The resulting construct was introduced into young leaf sheaths of rice by particle bombardment with a Biolistic gene gun (PDS-1000/He, Bio-Rad Laboratories, Hercules, CA) by the manufacturer's protocol. The bombarded sheath was incubated on a Murashige-Skoog salt mixture plate containing 3% gellan gum for 8 h at 25°C. The transformed cells were mounted on a glass slide and examined with confocal laser scanning microscope (MRC 1024, Bio-Rad), with excitation at 488 nm and a standard fluorescein isothiocyanate filter set.\textsuperscript{23}

\textit{Northern blotting.} Total RNA was isolated by the aurantricarboxylic acid method of Skadsen.\textsuperscript{20} The amount of RNA put in the gel was checked by rRNA ethidium bromide staining. Blotted membranes were examined with a BAS2000 Bio-Imaging analyzer (Fujifilm, Tokyo, Japan).

\textit{Ca$^{2+}$-binding assay.} Samples were put through SDS-PAGE. The gels were stained with coomassie brilliant blue or electroblotted onto a nitrocellulose membrane in the $^{45}$Ca$^{2+}$-binding assay.\textsuperscript{25} The nitrocellulose membrane was washed with buffer A (10 mM imidazole-HCl (pH 6.8) containing 60 mM KCl, 5 mM MgCl$_2$, and 0.05% (v/v) Tween 20), and incubated in buffer B (15 mM sodium acetate (pH 5.2), 60 mM KCl, 5 mM MgCl$_2$, and 0.05% (v/v) Tween 20) for 5 min. $^{45}$CaCl$_2$ (37 kBq/ml; Du Pont) was added, and then 1 ml Tris base solution was added to the pH of 8.5. After incubation of membrane for 1 h at room temperature, it was washed with distilled water for 10 s, dried, and then examined with a BAS2000 Bio-Imaging analyzer.\textsuperscript{26}

\textit{Ca$^{2+}$ electrophoretic mobility shift assay.} Samples were put through SDS-PAGE containing 5 mM ethylene glycol-(beta-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA).\textsuperscript{27} The gels were stained with coomassie brilliant blue.

\textit{Pharmacological analysis.} Suspended cells derived from embryos were cultured in a medium lacking sugars for 24 h before one of various inhibitors was added. After 1 h of treatment with an inhibitor, glucose was added to the final concentration of 90 mM and the cells were incubated for 12 h. CaCl$_2$, EGTA, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), chlorpromazine, and ruthenium red were dissolved in water; 6-dimethylaminopurine (6-DMAP), cantharidin, LaCl$_3$, and cycloheximide in dimethyl sulfoxide.

Results

\textit{Sequence analysis and genomic structure of OsSUR1 gene.} Using a cDNA microarray, we previously identified several clones expression of which is regulated by
high- or low-sugar levels in callus-forming embryos from cDNAs containing 1265 expressed sequence tags of rice. One of them corresponded to a novel sugar-up-regulated cDNA, OsSURI, obtained from the Japanese Rice Genome Program (accession number C98373). Sequence analysis showed that OsSURI cDNA contained a 414-bp open reading frame encoding a protein of 137 amino acid residues with a predicted molecular mass of 14,974 and two EF-hand motifs (Fig. 1). The amino acid sequence identified by OsSURI was compared with protein sequences deposited in the public databases with the FASTA program, we found to show 48.9% identity to a cDNA clone AT2g44310 from Arabidopsis thaliana (accession number AY114617), the function of which has not been published. Southern analysis showed that the OsSURI full-length cDNA probe detected strongly hybridizing DNA fragments as well as weakly hybridizing bands under high-stringency conditions (not shown). This finding indicated that there is at least one other, distantly related gene in the rice genome.

\[ {^{60}}Ca^{2+}\text{-binding activity of OsSURI proteins} \]

Many proteins that bind to Ca\(^{2+}\) contain EF-hand motifs. The presence of a highly conserved EF-hand motif in the predicted amino acid sequence of OsSURI suggested that the protein product might have Ca\(^{2+}\)-binding activity; therefore, the Ca\(^{2+}\)-binding activity of a recombinant OsSURI protein was tested. Figure 2 shows the Ca\(^{2+}\)-binding activity of recombinant OsSURI. A His-tagged OsSURI protein bound to \({^{45}}Ca^{2+}\) (lanes 8 and 9), but a His-tagged peptide and endogenous E. coli proteins (lane 6 and 7) did not, indicating that the OsSURI protein could bind with Ca\(^{2+}\). We did not observe any mobility shift in the protein in an electrophoretic mobility shift assay? (not shown).

\[ \text{Tissue-specific expression of OsSURI gene} \]

The tissue-specific expression of OsSURI is shown in Fig. 3A. OsSURI mRNA was not detected in embryos and endosperms from dry and seeds soaked for 3 days (lanes 1–4), shoots and roots from greening seedlings after 5 days of growth (lanes 5 and 6), or shoots from etiolated seedlings after 3, 6, or 9 days of growth (lanes 7–9). The original OsSURI clone was isolated from a cDNA library derived from embryonic callus, but gene expression was not observed in embryos from dry seeds or seeds soaked for 3 days. When Northern blotting was repeated over time started, however, OsSURI mRNA was detected transiently in the embryos from 6 h after soaking up to 24 h (Fig. 3B). Northern blotting of mature plant tissues showed that much OsSURI mRNA accumulated in the internodes (Fig. 3C; lane 2), but not in other tissues such as roots (lane 1), leaf blades, leaf sheaths and rachis (lanes 3–5). Northern blotting of mature and young internodes that had not fully elongated is shown in Fig. 3D. OsSURI mRNA was detected to almost the same extent in all internodes at different positions in mature plants (lanes 2–6); in contrast, there was little or no RNA in the young internodes (lane 1). These results suggested that the OsSURI gene was expressed in the connecting tissues between the source organs (endosperms and leaf blades) and sink organs (very young shoots and roots, flowers, and grains).

\[ \text{Sugar regulation of OsSURI gene expression} \]

Northern blotting of callus-forming embryos of rice (Fig. 4A) showed that expression of the OsSURI gene was repressed in a time-dependent way by sugar starvation and completely repressed by 24 h. Expression was promoted again within 30 min by 90 mM
glucose, which increased transcription for up to 24 h.

In contrast, RAMy3D, a rice α-amylase gene used as a marker for sugar down-regulation in the rice embryo,\(^\text{29}\) had the opposite expression being repressed by sugar feeding and derepressed by sugar starvation, suggesting that the OsSURI gene was up-regulated by sugar.

In rice, glucose analogs such as 6-deoxyglucose and 3-O-methylglucose, which are taken up by plant cells but not phosphorylated by hexokinase, did not repress the RAMy3D gene, but 2-deoxyglucose, which can be phosphorylated but is not metabolized further, down-regulates RAMy3D promoter activity; this finding indicates that hexokinase has a role in the sugar-sensing mechanism that triggers repression of the RAMy3D gene.\(^\text{18}\) Here, the effects of sugars on the expression of both OsSURI and RAMy3D genes were examined further with embryos fed various sugars and glucose analogs adjusted to 5 or 90 mM for 12 h after day of sugar starvation. The transcription of OsSURI was promoted by 90 mM glucose, fructose, sucrose or mannose (Fig. 4B; lanes 4, 6, 8, and 15), but not by 5 mM concentration of these hexoses (lanes 3, 5, 7, and 14). Sucrose promoted OsSURI gene expression, probably by being its hydrolyzed to hexose. A 5 mM concentration of 2-deoxyglucose derepressed OsSURI expression (lane 9). OsSURI gene expression was suppressed with 3-O-methylglucose, 6-deoxyglucose, or mannitol (lanes 10-13, 16, and 17). 3-O-methylglucose and 6-deoxyglucose, which are taken up by plant cells, and mannitol, which is an osmotic control, are not phosphorylated by hexokinase. The observed repression and derepression of OsSURI gene expression was completely different from that observed for the RAMy3D gene. These results indicate that both derepression of OsSURI and repression of RAMy3D depend on hexose phosphorylation by hexokinase. The hexokinase-regulated gene expression of OsSURI and RAMy3D might be a useful marker with which to investigate the sugar-sensor mechanism of hexokinase signal transduction.

Figure 4C shows that, in contrast to cells starved of sugar (lanes 2 and 4), OsSURI mRNA accumulated when culture was with added glucose addition, dimethyl sulfoxide treatment, added CaCl\(_2\) (lanes 1, 3, 5, and 6, respectively); EGTA treatment (lane 7), which causes Ca\(^{2+}\) depletion; ruthenium red (lane 8), which blocks endomembrane Ca\(^{2+}\) channels such as vacuolar voltage-dependent channels;\(^\text{29}\) two calmodulin antagonists, W7 and chlorpromazine (lanes 9 and 10, respectively); the Ca\(^{2+}\) channel blocker LaCl\(_3\) (lane 11); the protein phosphatase inhibitor cantharidin (lane 12); a protein kinase inhibitor, 6-DMAP (lane 13); and an inhibitor of cytosolic translation, cycloheximide (lanes 13 and 14). These results suggest that components of the general Ca\(^{2+}\) signal transduction pathway, such as Ca\(^{2+}\), calmodulin, protein kinases, and phosphatases, were not involved in the induction of OsSURI gene expression. The response of OsSURI gene expression was rapid after the addition of sugars. Cycloheximide itself did not have any effects on the expression of OsSURI with glucose (Fig. 4C; lanes 14 and 15) or without glucose (not shown). These finding suggest that intercellular transcription factors that regulate OsSURI expression were depending on stimulated hexokinase signal transduction and not depending on Ca\(^{2+}\) signaling. The possibility could not be ruled out that the sugar regulation of OsSURI expression was mainly due to the stability of its mRNA molecule.

**Discussion**

**Sugar- but not Ca\(^{2+}\)-mediated signaling for OsSURI expression**

Sugars act as signaling molecules, as well as being intermediates in metabolic processes and in the synthesis of biological compounds.\(^\text{30}\) The signal transduction pathways in which sugars function are complex, with multiple mechanisms of regulation.\(^\text{31,32}\) In an attempt to investigate the sugar response in plants, we isolated from rice embryos cDNAs that are either up- or down-regulated by sugars using cDNA microarray technology. We characterized a novel cDNA, OsSURI, that is up-regulated by sugar and...
Fig. 4. Expression of OsSUR1 and RAmy3D Genes in Callus-forming Embryos and Suspension Cultured Cells.

Total RNA (10 μg) was isolated from callus-forming embryos treated with various sugars and chemicals for the indicated times, separated in a 1.2% formaldehyde-agarose gel, and transferred onto a nylon membrane.

A: Callus-forming rice embryos were starved of sugar for 1 day, treated with 90 mM glucose for the indicated times up to 24 h, and then harvested so that total RNA could be isolated. The blot was hybridized with 32P-labeled full-length OsSUR1 or RAmy3D cDNAs.

B: Effects of various sugars on expression of the OsSUR1 and RAmy3D genes. Lane 1, callus-forming rice embryos immediately excised from endosperm; lane 2, embryos treated by sugar starvation for 1 day, and then treated for 12 h with glucose at the concentration of 5 mM (lane 3) or 90 mM (lane 4); fructose at 5 mM (lane 5) or 90 mM (lane 6); sucrose at 5 mM (lane 7) or 90 mM (lane 8); 2-deoxyglucose at 5 mM (lane 9); 3-O-methylglucose at 5 mM (lane 10) or 90 mM (lane 11); 6-deoxyglucose at 5 mM (lane 12) or 90 mM (lane 13); mannose at 5 mM (lane 14) or 90 mM (lane 15); mannitol at 5 mM (lane 16) or 90 mM (lane 17). The embryos were then harvested for total RNA isolation.

C: Pharmacological analysis of the expression of the OsSUR1 gene. Lane 1, suspension cultured cells in a medium containing 3% sucrose; lanes 2–15, cells cultured in a medium without sugar for 24 h (lane 2), then treated with an inhibitor or antagonist at the indicated concentration for 1 h, and then with glucose at 90 mM for 12 h: lane 3, glucose only (90 mM); lane 4, no glucose added; lane 5, dimethyl sulfoxide (1% v/v); lane 6, CaCl2 (5 mM), lane 7, EGTA (2 mM); lane 8, ruthenium red (100 μM); lane 9, W7 (50 μM); lane 10, chlorpromazine (50 μM); lane 11, LaCl3 (500 μM); lane 12, cantharidin (5 μM); lane 13, 6-DMPA (500 μM); lane 14, cycloheximide (2 μM); lane 15, cycloheximide (20 μM). The amount of RNA put on the gel was checked by rRNA ethidium bromide staining.

encodes a Ca2⁺-binding protein. CDPK and calmodulin are involved in Ca2⁺-mediated signaling that regulates the sugar-inducible expression of storage protein genes. In this study, we found that the OsSUR1 protein had EF-hand motifs and a Ca2⁺-binding activity similar to Ca2⁺-binding ability of CDPK and calmodulin. The OsSUR1 open reading frame fused to sGFP(S65T) protein was uniformly distributed in the cytosol of young leaf sheath cells, together with something similar to the control protein sGFP(S65T) (not shown). The sucrose-inducible protein CDPK is associated with the plasma membrane,

Role of hexokinase-dependent signaling in sugar sensing

Analysis of plants expressing either the sense or antisense hexokinase gene has shown that hexokinase acts in ethylene signal transduction and in plant growth and senescence. In the transduction of sugar signals, hexokinase can act as a sensor for both sugar-repressible and sugar-inducible gene expression. Sugar-inducible, down-regulation mediated by hexokinase occurs for genes linked to a role of sugar in plant functions such as photosynthesis, and starch degradation, and adaptation in leaf cells in the dark. Sugar-inducible gene with up regulation mediated by hexokinase include those encoding nitrate reductase, phospholipid D, involved in leaf senescence, and enhanced response to abscisic acid (ERA) which is involved in abscisic acid signal transduction and meristem cell-cycle control. OsSUR1 was a hexokinase-dependent sugar-inducible gene that encoded a Ca2⁺-binding protein. This finding suggests that hexokinase-dependent signaling relates to a Ca2⁺-mediated plant function. Because OsSUR1 transcription is not inhibited by cycloheximide, it is possible that the transcription factor that regulates OsSUR1 expression may be linked directly to the hexokinase-mediated signaling pathway.

Physiological aspects of OsSUR1

Most of the genes up regulation by sugars are generally expressed in sink tissues. However, the OsSUR1 gene accumulates in sugar translocation tissues, such as the internodes of mature plants and embryos of soaked seeds. Internodes and embryos are important in the transport of carbohydrates to sink tissues, such as rachis, young roots, and shoots. The expression of OsSUR1 induced by the transported sugar may itself be involved in sugar transport in carbohydrate translocation tissues. The induction of sugar up-regulated genes and the accumulation of anthocyanin are necessary not only for sugar- and hexokinase-mediated signaling but also for Ca2⁺ signal transduction. Although the OsSUR1 gene had Ca2⁺-binding ability, Ca2⁺ signaling did not seem to be involved in the expression of the gene. This finding suggests that the expression of OsSUR1 may be regulated directly by hexokinase signaling. It is possible, therefore, that the OsSUR1 gene has a role in the later steps of Ca2⁺ signaling mediated by sugar and hexokinase. This novel gene, OsSUR1, encoding a
Ca$^{2+}$-binding protein, provides several new insights into the regulation of genes involved in hexokinase and Ca$^{2+}$-mediated plant function.

Acknowledgments

We thank Dr. T. Yuasa for valuable advice on the $4{^{2}}$Ca$^{2+}$-binding assay and Dr. Y. Niwa for the green fluorescent protein cassette gSFP(S65T). This work was supported by a MAFF grant (Rice Genome Project, GS-2218) and the JSPS (Japan Society for Promotion of Science) "Research for the Future" Program.

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