Role of the Rice Hexokinases OsHXK5 and OsHXK6 as Glucose Sensors

Jung-II Cho2, Nayeon Ryoo2, Joon-Seob Eom, Dae-Woo Lee, Hyun-Bi Kim, Seok-Won Jeong, Youn-Hyun Lee, Yong-Kook Kwon, Man-Ho Cho, Seong Hee Bhoo, Tae-Ryong Hahn, Youn-II Park, Ildoo Hwang, Jen Sheen, and Jong-Seong Jeon*

Plant Metabolism Research Center and Graduate School of Biotechnology, Kyung Hee University, Yongin 446–701, Korea (J.-I.C., N.R., J.-S.E., D.-W.L., H.-B.K., Y.-K.K., M.-H.C., S.H.B., T.-R.H., J.-S.J.); Department of Biology, Chungnam National University, Daejeon 305–764, Korea (S.-W.J., Y.-I.P.); Department of Horticultural Biotechnology, Kyung Hee University, Yongin 446–701, Korea (Y.-H.L.); Department of Life Sciences, Pohang University of Science and Technology, Pohang 790–784, Korea (I.H.); and Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114 (J.S.)

In higher plants, sugars are known to function as signaling molecules in addition to being a fundamental source of fuel for carbon and energy metabolism. Indeed, sugars have been shown to regulate physiological processes during the entire plant life cycle, from germination to flowering and senescence, and to function during defense responses to biotic and abiotic stresses (Jang and Sheen, 1994; Jang et al., 1997; Perata et al., 1997; Smeekens and Rook, 1997; Smeekens, 1998; Wingler et al., 1998; Rolland et al., 2001, 2006; Leon and Sheen, 2003; Gibson, 2005; Biemelt and Sonnewald, 2006; Seo et al., 2007). Therefore, to sustain normal plant growth and development, rigorous sugar sensing and signaling systems are important for coordinating and modulating many essential metabolic pathways.

Glc, one of the main products of photosynthesis, is the most widely recognized sugar molecule that regulates plant signaling pathways (Koch, 1996; Yu et al., 1996; Ho et al., 2001; Chen, 2007). Yeast (Saccharomyces cerevisiae) has several Glc sensors, including the hexokinase SchHX2, Glc transporter-like proteins Sucrose nonfermenting 3 (Snf3) and Restores glucose transport 2 (Rgt2), and G protein-coupled receptor Gpr1. These sensors have been reported to sense the internal and external Glc status as part of mechanisms controlling cell growth and gene expression (Rolland et al., 2001; Lemaire et al., 2004; Santangelo, 2006). Similarly, recent

The Arabidopsis (Arabidopsis thaliana) hexokinase 1 (AHXK1) is recognized as an important glucose (Glc) sensor. However, the function of hexokinases as Glc sensors has not been clearly demonstrated in other plant species, including rice (Oryza sativa). To investigate the functions of rice hexokinase isoforms, we characterized OsHXK5 and OsHXK6, which are evolutionarily related to AHXK1. Transient expression analyses using GFP fusion constructs revealed that OsHXK5 and OsHXK6 are associated with mitochondria. Interestingly, the OsHXK5ΔmTP-GFP and OsHXK6ΔmTP-GFP fusion proteins, which lack N-terminal mitochondrial targeting peptides, were present mainly in the nucleus with a small amount of the proteins seen in the cytosol. In addition, the OsHXK5NLS-GFP and OsHXK6NLS-GFP fusion proteins harboring nuclear localization signals were targeted predominantly in the nucleus, suggesting that these OsHXKs retain a dual-targeting ability to mitochondria and nuclei. In transient expression assays using promoter::luciferase fusion constructs, these two OsHXKs and their catalytically inactive alleles dramatically enhanced the Glc-dependent repression of the maize (Zea mays) Rubisco small subunit (RbcS) and rice α-amylase genes in mesophyll protoplasts of maize and rice. Notably, the expression of OsHXK5, OsHXK6, or their mutant alleles complemented the Arabidopsis glucose insensitive-2-1 mutant, thereby resulting in wild-type characteristics in seedling development, Glc-dependent gene expression, and plant growth. Furthermore, transgenic rice plants overexpressing OsHXK5 or OsHXK6 exhibited hypersensitive plant growth retardation and enhanced repression of the photosynthetic gene RbcS in response to Glc treatment. These results provide evidence that rice OsHXK5 and OsHXK6 can function as Glc sensors.

1 This work was supported by the Science Research Center program of the Ministry of Education, Science and Technology/Korea Science and Engineering Foundation (grant no. R11-2000-081) through the Plant Metabolism Research Center, by the Biogreen 21 Program, Rural Development Administration, by the Crop Functional Genomics Center of the 21st Century Frontier Research Program (grant no. CG2111–2), and by the Basic Research Program (grant no. R01–2007–000-20149–0) of the Korea Science and Engineering Foundation.

2 These authors contributed equally to the article.

* Corresponding author; e-mail jjeon@khu.ac.kr.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jong-Seong Jeon (jjeon@khu.ac.kr).

[C] Some figures in this article are displayed in color online but in black and white in the print edition.

[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.108.131227

studies in plants have unveiled sugar sensing and signaling systems mediated by hexokinase as a Glc sensor or G protein-coupled receptors in a hexokinase-independent way (Rolland et al., 2001, 2002, 2006; Chen et al., 2003; Moore et al., 2003; Holsbeeks et al., 2004; Cho et al., 2006b; Huang et al., 2006). In addition, plant Snf1-related protein kinase 1 (SnRK1), which is an ortholog of the yeast Snf1, plays important roles linking sugar signal, as well as stress and developmental signals, for the global regulation of plant metabolism, energy balance, growth, and survival (Baena-González et al., 2007; Lu et al., 2007; Baena-González and Sheen, 2008).

In addition to the catalytic role of hexokinase in plants, which is to facilitate hexose phosphorylation to form hexose-6-P, the role of hexokinase as an evolutionarily conserved Glc sensor was first recognized from biochemical, genetic, and molecular studies of Arabidopsis (Arabidopsis thaliana) hexokinase 1 (AtHXK1) transgenic plants and glucose insensitive2 (gin2) mutants (Jang et al., 1997; Rolland et al., 2002; Harrington and Bush, 2003; Moore et al., 2003; Cho et al., 2006b). Transgenic plants expressing catalytically inactive AtHXK1 mutant alleles in the gin2 mutant background have provided compelling evidence that the catalytic and sensory functions of AtHXK1 are uncoupled in the Arabidopsis plant (Moore et al., 2003). Furthermore, proteomics and yeast two-hybrid interaction experiments have revealed that in the nucleus, AtHXK1 interacts with two partners, the vacuolar H+-ATPase B1 and the 19S regulatory particle of proteasome subunit, to directly control the expression of specific photosynthetic genes (Cho et al., 2006b; Chen, 2007). In these studies, the interactions between AtHXK1 and vacuolar H+-ATPase B1 or 19S regulatory particle of proteasome subunit appeared not to require the enzymatic activity of AtHXK1. In the tomato (Solanum lycopersicum) plant, AtHXK1 expression causes a reduction in photosynthesis, growth inhibition, and the induction of rapid senescence (Dai et al., 1999), which are all characteristics of sugar sensing and signaling in photosynthetic tissues. With the exception of Arabidopsis HXK1, the role of hexokinases as Glc sensors has yet to be demonstrated in other plant species (Halford et al., 1999; Veramendi et al., 2002; Rolland et al., 2006).

Hexokinases have been shown to associate with various subcellular compartments, including mitochondria, chloroplasts, Golgi complexes, endoplasmic reticula, plasma membranes, and cytosols, suggesting numerous distinct intracellular functions (Schleucher et al., 1998; Wiese et al., 1999; Frommer et al., 2003; Olsson et al., 2003; Giese et al., 2005; Cho et al., 2006a; Kandel-Kfir et al., 2006; Rezende et al., 2006; Damari-Weissler et al., 2007). In yeast, the Glc sensor SchHXK2 has a nuclear localization signal (NLS) within its N-terminal domain and resides partly in the nucleus in addition to the cytosol (Herrero et al., 1998; Randez-Gil et al., 1998). Furthermore, the nuclear localization of SchHXK2 is required for Glc repression of several genes, such as Suc2, HXK1, and GLK1 (Herrero et al., 1998; Rodríguez et al., 2001). A portion of cellular AthHXK1, which is predominantly associated with mitochondria, was also found to reside in the nucleus (Yanagisawa et al., 2003; Cho et al., 2006b). Under conditions of Glc excess, it has thus been hypothesized that nuclear AthHXK1 binds its substrate Glc, resulting in the suppression of target gene expression (Cho et al., 2006b; Chen, 2007).

We have previously isolated 10 rice (Oryza sativa) hexokinases, OsHXK1 through OsHXK10, and demonstrated that all of these subtypes possess hexokinase activity (Cho et al., 2006a). The results of this previous study showed that OsHXK4 and OsHXK7 reside in the chloroplast stroma and cytosol, respectively. Based on sequence similarity and subcellular localization, we have identified two rice hexokinases homologous to AtHXK1, OsHXK5 and OsHXK6. The subcellular localization of OsHXK5 and OsHXK6, observed with GFP fusion constructs, suggested that OsHXK5 and OsHXK6 retain a dual-targeting ability to mitochondria and nuclei. This finding prompted us to examine whether these homologues play a role in Glc sensing and signaling in rice. To address this question, we observed the function of OsHXK5 and OsHXK6 in mesophyll protoplasts of maize (Zea mays) and rice and in transgenic rice plants. In addition, we transformed the Arabidopsis gin2-1 mutant with either wild-type or catalytically inactive alleles of OsHXK5 and OsHXK6 and analyzed their sugar sensing and signaling characteristics. Finally, the conserved role of hexokinase as a Glc sensor in Arabidopsis and rice plants is discussed.

RESULTS
Identification of Rice Hexokinases Homologous to the Arabidopsis Glc Sensor AthHXK1
The well-characterized Glc sensor AthHXK1 is predominantly associated with mitochondria but also has detectable localization in the nucleus, where it binds to Glc and acts in conjunction with partner proteins as a transcriptional repressor (Cho et al., 2006b). To isolate rice hexokinases homologous to AthHXK1, we first predicted the subcellular localization of OsHXKs using the TargetP program (Emanuelsson et al., 2000, 2007; http://www.cbs.dtu.dk/services/TargetP) for determination of the presence of any N-terminal presequences, including putative mitochondrial targeting peptides (mTPs), and the predictNLS program for determination of NLSs (Cokol et al., 2000; http://cubic.bioc.columbia.edu/services/predictNLS). These analyses revealed that of the 10 OsHXKs, OsHXK5 and OsHXK6 had a predicted N-terminal mTP, 1MGKAAAVGTVVAAAAGVAVVL24 for OsHXK5 and 1MGKGTIVGTVVCAAAAAAVGVAVVSS28 for OsHXK6. These analyses also indicated that both proteins contained a predicted NLS, 25RRRRDRLELVEGAAERK45 for OsHXK5 and 29RRRRSKR-
EAEEERRRR44 for OsHXK6, within their N-terminal domains. Together with our previous phylogenetic analyses of rice HXKs (Cho et al., 2006a), these data suggest that OsHXK5 and OsHXK6 are evolutionarily closely related to the Arabidopsis Glc sensor AtHXK1.

To determine the subcellular localization of these two rice homologues of AtHXK1, we generated GFP fusion constructs for OsHXK5 and OsHXK6 under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Supplemental Fig. S1). Results of subcellular localization experiments showed that signals of OsHXK5-GFP and OsHXK6-GFP fusion proteins were primarily colocalized with the mitochondrial dye MitoTracker in maize protoplasts (Fig. 1, A and B) and also in Arabidopsis protoplasts (data not shown), demonstrating that both hexokinases are associated with mitochondria. Protein-gel blot analysis using an anti-GFP antibody confirmed production of the predicted GFP fusion proteins, 81.6 kD and 82.1 kD for OsHXK5-GFP and OsHXK6-GFP, respectively (Fig. 1D).

To test whether both OsHXKs could localize to both mitochondria and nuclei, we generated the OsHXK mutants OsHXK5DmTP and OsHXK6DmTP fused to GFP by deleting predicted mTPs (Supplemental Fig. S1). Interestingly, signals of OsHXK5ΔmTP-GFP and OsHXK6ΔmTP-GFP were detected strongly in nuclei and weakly in cytosols, as confirmed by colocalization studies with the SYTO nuclear dye, but were not localized to mitochondria (Fig. 2, A–C). The quantitative analysis of GFP fluorescence intensity supported that GFP signals were mostly present in nuclei of maize protoplasts expressing OsHXK5ΔmTP-GFP or OsHXK6ΔmTP-GFP (Fig. 2, D and E). We confirmed that OsHXK5ΔmTP-GFP (79.0 kD) and OsHXK6ΔmTP-GFP (80.1 kD) fusion proteins were effectively produced in vivo using protein-gel blot analysis with an anti-GFP antibody (Fig. 2G). In control experiments, signals in maize protoplasts expressing only GFP were observed strongly both in the cytosol and in the nucleus (Figs. 1C and 2F).

To further examine function of the predicted NLSs, we fused the NLSs of OsHXK5 and OsHXK6 to GFP, respectively, thereby generating OsHXK5NLS-GFP and OsHXK6NLS-GFP (Supplemental Fig. S1). In transient expression assay using maize protoplasts, signals of the GFP fusion products were predominantly localized in nuclei (Fig. 3, A and B), indicating that the NLSs of OsHXK5 and OsHXK6 are functional nuclear targeting sequences in vivo. The quantitative analysis of GFP fluorescence intensity again supported that GFP signals were mostly detected in nuclei of maize protoplasts expressing OsHXK5NLS-GFP or OsHXK6NLS-GFP (Fig. 3, A and B). To confirm this result, we

---

**Figure 1.** Subcellular localization of OsHXK5-GFP and OsHXK6-GFP fusion proteins in transfected mesophyll protoplasts of maize. A, Chlorophyll autofluorescence and MitoTracker were used as chloroplast and mitochondria markers, respectively. The false color (blue) was assigned to chloroplast autofluorescence to distinguish it from the fluorescence of MitoTracker. GFP signal is indicated in green, and the mitochondrial signal stained with MitoTracker is shown in red. The merged images of chlorophyll autofluorescence, GFP, and MitoTracker as well as light-field images are shown. D, Protein gel-blot analysis for OsHXK5-GFP and OsHXK6-GFP fusion proteins with an anti-GFP antibody. GFP served as control.
constructed OsHXK5ΔmTP-GFP and OsHXK6ΔmTP-GFP by deleting the NLSs of OsHXK5 and OsHXK6 (Supplemental Fig. S1). Consistently, transient expression assays revealed that both GFP fusion products were primarily localized to mitochondria (Fig. 3, C and D). By deleting both mTP and NLS of the two OsHXKs, we generated OsHXK5ΔmTPΔNLS-GFP and OsHXK6ΔmTPΔNLS-GFP (Supplemental Fig. S1). These two GFP fusion products were mainly detected in cytosols (Fig. 3, E and F). Our results suggest that these OsHXKs are targeted to mitochondria and also possibly to nuclei, raising the possibility that OsHXK5 and OsHXK6 are functional homologues of the Arabidopsis Glc sensor AtHXK1.

Expression of OsHXK5, OsHXK6, and Their Mutant Alleles in Maize and Rice Mesophyll Protoplasts

It has been reported in Arabidopsis that the sugar sensing and signaling functions of AtHXK1 do not depend on its Glc phosphorylation activity (Moore et al., 2003; Cho et al., 2006b). To uncouple the sugar sensing and signaling activities from Glc phosphorylation, we employed a targeted mutagenesis experiment to generate catalytically inactive mutants of the candidate rice Glc sensors OsHXK5 and OsHXK6. In the mutant alleles, ATP binding was eliminated by mutating the conserved Gly (G) in the phosphate
1 domain of the ATP-binding site to Asp (D) and phosphoryl transfer was prevented by mutating the conserved Ser (S) in the sugar-binding domain to Ala (A; Kraakman et al., 1999; Moore et al., 2003; Cho et al., 2006a). These mutant alleles were referred to as OsHXK5-G113D, OsHXK5-S186A, OsHXK6-G112D, and OsHXK6-S185A, according to their mutation sites (Fig. 4A). To determine whether enzyme catalytic activity was abolished in the mutant alleles, the individual cDNA clones were tested to complement the yeast triple mutant YSH7.4-3C (hxk1, hxk2, glk1), which lacks endogenous hexokinase activity. While yeast cells transformed with wild-type cDNAs of OsHXK5 and OsHXK6 were able to grow on selection medium containing Glc as the sole carbon source (Cho et al., 2006a), yeast cells transformed with the OsHXK mutant alleles or the empty pDR196 vector did not grow on the selection medium (Fig. 4B, top). In the control experiment, all transformed yeast cells grew on the Gal-containing medium (Fig. 4B, middle). In addition, expressions of HXK5, HXK6, and their catalytically inactive mutant alleles were confirmed by reverse transcription (RT)-PCR analysis (Fig. 4B, bottom). These findings demonstrate that the mutant OsHXKs lacked catalytic activity.

Using a Glc repression assay in mesophyll protoplasts of maize and rice (Sheen, 2001), we tested whether the wild-type and catalytically inactive OsHXKs possessed Glc sensing and signaling functions in the monocot plant species. In this experiment, the reporter constructs consisted of the promoter of a well-known Glc-repressible gene, the maize Rubisco
small subunit of maize (ZmRbcS), linked to the reporter gene luciferase (LUC; Jang and Sheen, 1994). It has been established that expression of the rice \( \alpha \)-amylase 3D (RAmy3D) gene is repressed rapidly in response to Glc treatment (Yu et al., 1996; Umemura et al., 1998; Ho et al., 2001). Thus, we generated the RAmy3D promoter::LUC fusion as an additional reporter construct. First, we confirmed that high Glc (5 mM) conditions reduce the expression of reporter genes following the ZmRbcS or RAmy3D promoter in mesophyll protoplasts of maize and rice, while a low Glc concentration (0.5 mM) does not (Fig. 5, A and B; Supplemental Fig. S2, A and B). These results support previous experiments showing that the transient gene expression assay using mesophyll protoplasts is efficient for analyses of sugar sensing and signaling (Sheen, 2001; Moore et al., 2003). Next, we found that expression of OsHXK5 or OsHXK6 dramatically reduced LUC expression driven by either the ZmRbcS or RAmy3D promoter in response to 0.5-mM Glc treatment (Fig. 5, A and B; Supplemental Fig. S2, A and B), indicating enhancement of Glc-dependent repression of these genes in mesophyll protoplasts of both maize and rice. Furthermore, expression of the catalytically inactive OsHXK alleles for OsHXK5 and OsHXK6 suppressed reporter gene expression in response to Glc treatment (Fig. 5, A and B; Supplemental Fig. S2, A and B). Protein gel-blot analyses using CaMV35S::OsHXK-Myc fusion constructs indicated that OsHXK5, OsHXK6, and their mutant alleles were expressed at similar levels in mesophyll protoplasts (Fig. 5C). We also confirmed that OsHXK mutant alleles lack Glc phosphorylation activity in their transfected maize protoplasts, demonstrating that these are catalytically inactive in vivo. In contrast, expression of wild-type OsHXKs increased Glc phosphorylation activity in maize protoplasts (Fig. 5D). This result is consistent with the data of the yeast complementation assay (Fig. 4). In addition, these Myc fusion constructs were found to enhance a similar suppression of Glc-dependent LUC expression driven by either the ZmRbcS or the RAmy3D promoter (data not shown). These results strongly suggest that OsHXK5 and OsHXK6 function as conserved Glc sensors in maize and rice.

**Analysis of Transgenic gin2-1 Plants Expressing OsHXK5, OsHXK6, or Their Mutant Alleles**

To examine a possible role for the two rice hexokinase isoforms OsHXK5 and OsHXK6 as Glc sensors, we tested whether either OsHXK could complement the Arabidopsis gin2-1. To individually express OsHXK5, OsHXK6, and the catalytically inactive mutant alleles OsHXK5-G113D, OsHXK5-S186A, OsHXK6-G112D, and OsHXK6-S185A, each cDNA was placed under the control of the CaMV35S promoter. The resulting constructs were transformed into the gin2-1 mutant by the floral-dip method (Clough and Bent, 1998). More than 10 independent transgenic lines for

---

**Figure 4.** Transformation of catalytically inactive mutants for OsHXK5 and OsHXK6 into a yeast hexokinase mutant. A, Schematic representation of OsHXK5 and OsHXK6 and their catalytically inactive mutation sites. Mitochondrial targeting signals and NLSs are indicated as white (M) and black (N) rectangles. 1, 2, and A indicate the conserved phosphate 1, 2, and adenosine interaction regions within the ATP-binding site, respectively. The region S indicates the conserved sugar-binding domain (Cho et al., 2006a). B, Complementation of the hexokinase-deficient yeast triple mutant YSH74.4-3C (hxk1, hxk2, glk1) with OsHXK5, OsHXK6, and their catalytically inactive mutant alleles. The transformed colonies were streaked on the SD-Ura medium (synthetic defined minimal medium lacking uracil) containing 2% d-Glc as a sole carbon source and grown for 3 d at 30°C (top). The YSH74.4-3C mutant strain transformed with the pDR196 vector was used as a control. As control experiment, YSH74.4-3C mutant strains transformed with pDR196, OsHXK5, OsHXK6, and their catalytically inactive mutant alleles were streaked on the SD-Ura medium containing 2% d-Gal (middle). Expression levels of HXK5, HXK6, and their mutant alleles in these strains were measured by RT-PCR analysis (bottom). [See online article for color version of this figure.]
each construct were selected on the basis of hygromycin resistance. Expression levels of transgenes in the transformed plants were measured by RNA gel-blot analysis (data not shown). As a result, homozygous lines of two independent transgenic plants for each OsHXK with relatively high transgene expression were used in subsequent analyses.

To test whether OsHXK5, OsHXK6, and mutant alleles restore a Glc-sensitive response in the gin2-1 background, we sowed progeny of all selected transgenic gin2-1 plants with OsHXKs on high Glc (6%)-containing, half-strength Murashige and Skoog (MS) media. Results indicated that the growth of all of these OsHXKs transgenic plants was drastically suppressed in response to 6% Glc with short hypocotyl lengths and anthocyanin accumulation (Fig. 6; Supplemental Fig. S3). All tested transgenic plants did not show any differences in 6% mannitol or in Glc-free conditions (Fig. 6; Supplemental Figs. S3 and S4), indicating that the high Glc effects in transgenic gin2-1 plants expressing OsHXK5, OsHXK6, or mutant alleles are not due to osmotic stress.

It is widely known that the Glc sensor AtHXK1 suppresses the expression of the RbcS gene, chlorophyll a/b-binding protein 2 (CAB2), sedoheptulose-biphosphatase (SBP), and carbonic anhydrase (CAA)
in response to high Glc treatment (Jang et al., 1997; Rolland et al., 2002; Moore et al., 2003; Cho et al., 2006b). To examine whether the rice OsHXKs could suppress expression of the target genes in a similar way, we measured mRNA levels of CAB, SBF, and CAA genes in transgenic gin2-1 plants. Results indicated that both wild-type and all transgenic plants expressing OsHXK5, OsHXK6, or mutant alleles significantly suppressed expression of these photosynthetic genes in response to high Glc treatment. To examine whether the rice OsHXKs could suppress expression of the target genes in a similar way, we measured mRNA levels of CAB, SBF, and CAA genes in transgenic gin2-1 plants. Results indicated that both wild-type and all transgenic plants expressing OsHXK5, OsHXK6, or mutant alleles significantly suppressed expression of these photosynthetic genes in response to high Glc treatment. In contrast, wild-type and all transgenic plants did not alter the gene expressions in 6% mannitol or Glc-free conditions. gin2-1 mutants did not exhibit suppression of Glc-dependent gene expression (Fig. 6; Supplemental Figs. S3 and S4). These results indicate that any of these transgenes restored suppression of Glc-dependent gene expression in the gin2-1 background.

It has also been observed that AtHXK1 has a role in growth promotion as indicated by the observed growth defect phenotype under high light conditions (Moore et al., 2003). To see whether the overexpression of rice hexokinases can compensate for the growth defect phenotype of gin2-1, we grew the transgenic gin2-1 plants expressing OsHXK5, OsHXK6, or mutant alleles under low (70 μmol m⁻² s⁻¹) and high (240 μmol m⁻² s⁻¹) light conditions. Under the low light condition, wild-type, gin2-1, and transgenic plants did not display significant differences in their growth (Fig. 7A; Supplemental Fig. S5). In contrast, whereas gin2-1 plants retained the severe growth defect phenotype under high light conditions, transgenic plants for OsHXK5, OsHXK6, and their mutant alleles were able to restore plant growth and leaf expansion to the same degree as wild-type plants (Fig. 7A; Supplemental Fig. S5). In addition, we confirmed that expression of the catalytically inactive HXK mutant alleles did not alter Glc phosphorylation activity in transgenic gin2-1 plants expressing these mutant alleles (Fig. 7B). These findings indicate that OsHXK5 and OsHXK6 can recapitulate the role of AtHXK1 in growth promotion in Arabidopsis.

Analysis of Transgenic Rice Plants Expressing OsHXK5 or OsHXK6

To further investigate the function of OsHXK5 and OsHXK6 as Glc sensors in rice plants, we produced transgenic rice plants expressing CaMV35S::OsHXK5 or CaMV35S::OsHXK6. Two independent transgenic rice lines for each OsHXK gene were selected for further analyses based on high expression of the transgenes (data not shown). Individuals from homzygous plants of the selected lines were germinated on water agar media containing 30 mM Glc. The growth of transgenic rice seedling plants expressing OsHXK5 and OsHXK6 was more severely inhibited on the Glc-containing media than was observed for wild-type rice plants (Fig. 8A). Transgenic rice plants displayed an enhanced Glc-dependent growth inhibition, including reduced plant height, compared with wild-type controls (Fig. 8, A and B). In support of these phenotypes, we also observed that expression of the rice RbcS gene was more sensitively suppressed in transgenic than in wild-type rice plants in response to Glc treatment (Fig. 8C). We included sorbitol treatment as a control to eliminate the usual effects caused by osmotic stress. Under these conditions, no significant plant growth inhibition or repression of RbcS gene expression was observed in rice plants, indicating that the results obtained by Glc treatment were not due to osmotic stress. These Glc repression experiments further support the concept that OsHXK5 and OsHXK6 function

Figure 6. Complementation of the Arabidopsis gin2-1 mutant by expression of catalytically inactive OsHXK5 and OsHXK6 mutant alleles. Top, Seedlings homozygous for the transgene, and gin2-1 and wild-type (WT) seedlings grown on 1/2 MS medium with 6% Glc or mannitol for 6 d. Bottom, Expression levels of CAB, SBF, and CAA measured by RT-PCR analysis in transgenic, gin2-1, and wild-type plants. UBQ was used as control.
as Glc sensors in rice plants as well as in the Arabidopsis gin2-1 mutant background.

DISCUSSION
OsHXK5 and OsHXK6 Possess a Dual-Targeting Ability to Mitochondria and Nuclei

In plants, localization of hexokinase isoforms to different subcellular compartments is probably involved with their distinct functions during growth and development (Frommer et al., 2003; Cho et al., 2006a; Claeyssen and Rivoal, 2007). For example, OsHXK4, a rice hexokinase that we have previously shown to be targeted to the chloroplast stroma, is hypothesized to be involved in starch and fatty acid synthesis and in the pentose-P pathway in the chloroplast when energy supplies are limited, such as during the night and in sink organs (Olsson et al., 2003; Cho et al., 2006a). Although some functions remain to be determined, it has been proposed that the cytosolic hexokinases, including the rice isoform OsHXK7, are mainly involved in glycolysis or cytosolic metabolism (for example, Suc biosynthesis) through the removal of free hexoses in the cytosol (Da-Silva et al., 2001; Cho et al., 2006a). In particular, the Arabidopsis hexokinase AtHXK1 is present in mitochondria and nuclei and is involved in sugar signaling and sensing as well as in sugar metabolism (Jang et al., 1997; Moore et al., 2003; Cho et al., 2006b).

Rice has a large hexokinase gene family consisting of 10 genes (Cho et al., 2006a). To gain evidence indicative of isoform function, we have further determined the subcellular localization of rice hexokinase isoforms. In this study, we found that two rice hexokinases, OsHXK5 and OsHXK6, are predominantly localized in mitochondria. Interestingly, our localization experiments revealed that deletion of N-terminal mTP sequences limits their localization to mainly nuclei with a small amount of the proteins seen in cytosols (Figs. 1 and 2). We also demonstrated that both OsHXK5 and OsHXK6 harbor functional NLS motifs (Fig. 3). These data suggest that both OsHXK isoforms retain a dual targeting ability to mitochondria and nuclei, which is consistent, in part, with observations from AtHXK1 (Cho et al., 2006b). Thus, it is likely that OsHXK5 and OsHXK6 are the rice orthologous hexokinases of the Arabidopsis Glc sensor AtHXK1, raising the possibility that OsHXK5 and OsHXK6 may be involved in sugar sensing and signaling in rice.

It is worthwhile to note that although the majority of AtHXK1-GFP is associated with mitochondria, a minute amount of AtHXK1 is also present in nuclei in vivo and functions as a corepressor in a transcriptional complex identified from leaf extracts of Arabidopsis (Cho et al., 2006b). Thus, the predominant association of OsHXK5-GFP and OsHXK6-GFP with mitochondria does not exclude the possibility that a portion of OsHXK5 and OsHXK6 is localized to nuclei in vivo. It will be interesting to investigate whether OsHXK5 and
Figure 8. Growth phenotype of wild-type (WT) and transgenic rice seedlings expressing OsHXK5 or OsHXK6 in response to Glc treatment. A, Growth phenotype of seedling plants grown on water agar media containing Glc-free (0), 30 mM Glc (G30), and 30 mM sorbitol (S30). Bar = 1 cm. B, Shoot lengths of wild-type and transgenic rice plants grown on the different media. C, Relative expression of the rice RbcS gene in second and third leaves of wild-type and transgenic rice seedlings overexpressing OsHXK5 or OsHXK6 grown on the different media. The expression value in seedlings grown on Glc-free water agar plate for each line was arbitrarily considered as 1. Each data point represents the mean ± sd from three separate experiments.
OsHXK6 are targeted to nuclei in vivo upon high Glc or other treatments and also whether a cleavage of mTPs of OsHXK5 and OsHXK6 occurs for their nuclear localization.

The SchHXK2 NLS is required both for Glc-dependent nuclear localization and for interaction with Mig1, a transcriptional repressor responsible for Glc repression of several genes, including SUC2, HXK1, and GLK (Herrero et al., 1998; Rodriguez et al., 2001; Ahuatzi et al., 2004). The nuclear localization of SchHXK2 is involved in the formation of regulatory DNA-protein complexes with the cis-acting elements of these hexokinase-dependent, Glc-repressible genes (Herrero et al., 1998). From our observations, it is likely that presence of NLS peptides facilitates the nuclear localization of OsHXK5 and OsHXK6. Therefore, investigation of the connection between the NLS peptides of OsHXK5 and OsHXK6 and sugar signaling in rice will help us to elucidate their functional mechanism. Isolating interacting proteins with OsHXK5 or OsHXK6 can also aid the understanding of sugar sensing and signaling mechanisms in rice.

**OsHXK5 and OsHXK6 Retain a Role as Glc Sensors**

In this study, we have shown several lines of evidence that OsHXK5 and OsHXK6 function as Glc sensors. First, AtHXK1, OsHXK5, OsHXK6, and their catalytically inactive alleles exhibited similar Glc sensing and signaling functions in maize and rice protoplasts. They all significantly enhanced Glc-dependent repression of two sugar responsive genes, *RbcS* and *R Amy3D*, in mesophyll protoplasts of maize and rice (Fig. 5; Supplemental Fig. S2). Second, overexpression of OsHXK5, OsHXK6, or their catalytically inactive mutant alleles recovered a Glc-sensitive seedling phenotype in the Arabidopsis *gin2-1* background on high Glc media (Fig. 6; Supplemental Fig. S3). All transgenic *gin2-1* plants that overexpress OsHXK5, OsHXK6, or mutant alleles suppressed photosynthetic gene expression when they were grown on high Glc-containing media. When the transgenic plants were grown under high light conditions, overexpression of each wild-type or mutant *OsHXK* alleles promoted the growth and leaf expansion of *gin2-1* mutant plants (Fig. 7; Supplemental Fig. S5). Third, the transgenic rice plants overexpressing OsHXK5 or OsHXK6 displayed a hypersensitive response that caused both seedling growth retardation and repression of the *RbcS* gene in response to Glc treatment (Fig. 8). Collectively, these results support that at least two rice hexokinases, OsHXK5 and OsHXK6, function as Glc sensors, suggesting an evolutionarily conserved role for hexokinases as Glc sensors in plant species.

Rice hexokinases have been implicated in Glc sensing and signaling, in that the treatment with the hexokinase-specific competitive inhibitor glucosamine relieved sugar-dependent repression of *R Amy3D* in rice embryos (Umemura et al., 1998). In addition, in rice suspension cells, the Glc analogs 3-O-methyl-Glc and 6-deoxy-Glc, which are taken up by cells but not phosphorylated by hexokinase, did not block *R Amy3D* expression under sugar starvation, while Glc and Suc induced the repression of *R Amy3D* (Ho et al., 2001). In these experiments, another Glc analog, Man, which is phosphorylated but is slowly processed by plant cells, suppressed the expression of *R Amy3D*. Our current transient expression experiments using the *R Amy3D* promoter further support previous studies reporting that the sugar-dependent repression of *R Amy3D* occurs in a HXK-dependent manner. The Snf1 protein kinase is required for the derepression of Glc-repressible genes in yeast (Rolland et al., 2006). Similarly, rice SnRK1A appeared to be necessary for the activation of *R Amy3D* expression under Glc starvation (Lu et al., 2007). It would be interesting to see whether OsHXK5- and OsHXK6-dependent sugar repression of the *R Amy3D* gene was connected with SnRK1A-mediated sugar signaling in rice.

It is worthwhile to note that expression of the OsHXK5 and OsHXK6 hexokinases, which function as Glc sensors, is up-regulated in rice leaves by the treatment of hexose sugars, Glc and Fru (Cho et al., 2006a). These findings suggest that the increased expression of OsHXK5 and OsHXK6 may facilitate the suppression of target gene expression under high sugar conditions. It has also been reported that OsHXK5 and OsHXK6 are expressed in all plant tissues, such as the leaf, root, and flower, and in immature seeds. Expression was high in the early stages of endosperm development during the longitudinal growth of rice seeds (Cho et al., 2006a). These data may suggest that both HXKs function as Glc sensors in the source and sink tissues of rice plants in addition to their role in sugar metabolism as glycolytic enzymes. In this context, whether OsHXK5 and OsHXK6 play a similar role as Glc sensors in rice sink organs such as embryos and endosperms will be a valuable question to address in future investigations.

In this study, we have not clearly determined whether nuclear localization of OsHXK5 and OsHXK6 was necessary for sugar sensing and signaling in rice plants, although it is likely that a portion of the pool of both hexokinases present in nuclei contributes to sugar-mediated signaling. Recently, it was reported in Arabidopsis that mitochondrial-bound AtHXK1 interacts with F-actin (Balasubramanian et al., 2007). As an alternative regulatory mechanism of sugar sensing and signaling, this study suggested that the actin cytoskeleton possibly functions in plant growth along with AtHXK1-dependent Glc signaling. Thus, it will be interesting to further investigate whether OsHXK5 or OsHXK6 equipped with a nuclear exporting signal (NES) loses its sugar sensing and signaling functions. Finally, loss-of-function mutants or RNAi transgenic rice plants for both OsHXK5 and OsHXK6 will be valuable for more detailed characterization of function of these hexokinases in sugar sensing and signaling in rice, an agronomically important crop species.
MATERIALS AND METHODS

Plant Materials and Growth

Arabidopsis (Arabidopsis thaliana) wild-type (Landsberg erecta ecotype) and gin2-1 plants, supplied by the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH; www.arabidopsis.org), were grown in a growth chamber under the dark conditions during the winter months. Mesophyll protoplasts were isolated from etiolated leaves by a modification of the method of Facelli and Boller (1984) and transgenic plants were grown on soil at 22°C under a 16-h-light/8-h-dark photoperiod.

To generate reporter vectors, the promoters of RAmmy3D were amplified by PCR using primers 5'-CGCGGATCCGATCTTCCAACCGTGTTACCAAGC-3' and 5'-TGACGGTACCTGAGAGGGGCTGTTTCA-3'. The amplified products digested with BamHI and NcoI were ligated into the firefly LUC gene to generate RAmmy3D::LUC. The maize Rice5 promoter::LUC construct (ZmRice5::LUC) derived from ZmRice5::CAT was used as additional reporter molecule (Hwang and Sheen, 2001). The maize Ubiquitin promoter derived from pGA1613 binary vector (Kim et al., 2003) digested with HindIII and BamHI was fused to a β-glucuronidase (GUS) gene linked to the terminator of the Nopaline synthase gene to create the internal control reporter construct, ZmUBQ::GUS. OsHXK-Myc fusion constructs were generated by the ligation of the Myc sequence to the C-termini of OsHXK5, OsHXK6, and mutant alleles. Full-length cDNAs of the OsHXK5, OsHXK6, and their catalytically inactive mutant alleles were amplified with added XhoI and Xhol sites by PCR and subcloned into the pSp and Xhol sites of the yeast (Saccharomyces cerevisiae) shuttle vector pDPR196 (Wispf et al., 2003).

Subcellular Localization of OsHXK-GFP Proteins

The GFP fusion constructs were delivered into maize and Arabidopsis mesophyll protoplasts using a polyethylene glycol-calcium mediated method (Hwang and Sheen, 2001; Cho et al., 2004) followed by 12- to 24-h incubation to allow transient expression. Mitochondria were visualized by staining with MitoTracker Orange CMTMRos (Molecular Probes), and nuclei were stained with the SYTO dye (Molecular Probes). Chlorophyll autofluorescence was used as a chloroplast marker. Expression of these fusion constructs was monitored using a confocal microscope (LSM 510 META, Carl Zeiss). GFP fluorescence intensity was quantified with Carl Zeiss LSM 510 META software (version 3.2 SP3). Expression of these GFP fusion proteins was detected by protein gel blot analysis according to Lee et al. (2007). Total proteins extracted from maize protoplasts (1 × 10⁶ cells/sample) transfected with GFP fusion constructs were electrophoresed on a 12% SDS-PAGE gel and immunoblotted with an anti-GFP antibody (B-2; nc-9996, Santa Cruz Biotechnology).

Yeast Complementation Assay

The hexokinase-deficient triple mutant YEPF 4-3C (hsk1, hsk2, glk1; De Winde et al., 1996) was used for transformation with full-length cDNAs of OsHXK5, OsHXK6, and their catalytically inactive mutant alleles. The procedures for yeast complementation assays were described previously (Cho et al., 2006a).

Hexokinase Activity Assay

Transfected maize protoplasts (1–2 × 10⁶ cells/reaction) were extracted by vortexing in a protoplast lysis buffer containing 25 mM Tris-P, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), 10% glycerol, 1% Triton X-100. After centrifugation at 13,000 g for 1 min, the resulting supernatant was used in hexokinase activity assays. For hexokinase activity measurements in wild-type and transgenic Arabidopsis plants, 1 g of plant material was ground to a fine powder in liquid nitrogen and then solubilized in 5 mL AT buffer (50 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100) according to the method previously described (Wiese et al., 1999). Insoluble debris was removed by centrifugation at 13,000g for 1 min, and the resulting supernatant was used in hexokinase activity assays.

Hexokinase activity was determined by monitoring the formation of NAD+ (Wiese et al., 1999). The formation of NAD+ was measured by absorbance change at 340 nm using a Cary 300 Bio UV/Vis spectrophotometer (Varian). The reaction mixture contained 100 mM imidazole-HCl, pH 6.9, 1.5 mM MgCl₂, 0.5 mM NADP, 1.1 mM ATP, 2 units Glc–6-ph dehydrogenase, and 5 mM Glc.

Transient Expression Assay Using Maize and Rice Mesophyll Protoplasts

Maize mesophyll protoplasts (1–2 × 10⁶ cells/sample) were isolated from the second leaves of etiolated plants according to the method of Sheen (2001) (http://genetics.mgh.harvard.edu/sheenweb). Rice protoplasts (3–6 × 10⁶ cells/sample) were isolated from etiolated leaves by a modification of the method used in Chen et al. (2006). For transient expression assays, isolated protoplasts were cotransfected with Gc responsive reporter constructs and effector constructs using a polyethylene glycol-calcium-mediated method.
were germinated on water agar media containing Glc-free, 30 mM Glc, and 30 mM mannitol, respectively, and washed with sterile distilled water. Surface-sterilized seeds were sterilized with 70% ethanol for 10 min and with 0.8% NaOCl for 30 min, and then they were overlaid on Gamborg B5 medium containing Glc-free, 6% Glc, and 6% mannitol, respectively, for 6 d under 100 μmol m⁻² s⁻¹ light conditions. To examine growth phenotype, the transgenic rice plants were regenerated from the transformed calli on selection media containing 50 mg L⁻¹ hygromycin and 250 mg L⁻¹ cefotaxime. To produce homozygous transgenic rice plants with OsHXK5 or OsHXK6, transgenic plants were grown in the greenhouse during several generations.

Glc Repression Assay
For Glc-repression assays in Arabidopsis, seedlings were grown on 1/2 MS medium containing Glc-free, 6% Glc, and 6% mannitol, respectively, for 6 d under 100 μmol m⁻² s⁻¹ light. To examine growth phenotype, the transgenic plants with OsHXK5, OsHXK6, or their catalytically inactive mutant alleles were grown on soil for 18 d under low (70 μmol m⁻² s⁻¹) and high (240 μmol m⁻² s⁻¹) light conditions. In rice, dehulled seeds of wild-type and transgenic rice plants were sterilized with 70% ethanol for 10 min and with 0.8% NaOCl for 30 min, respectively, and washed with sterile distilled water. Surface-sterilized seeds were germinated on water agar media containing Glc-free, 30 μM Glc, and 30 mM sorbitol, respectively. For repetitions of sterilized seeds, petri dishes were placed at 37°C in the dark for 24 h and then placed in a growth chamber under constant light conditions for 7 to 10 d at 25°C. Water agar media was used in place of MS media to exclude the interference of sugar signaling responses by nitrogen sources. To investigate the repression of RbcS gene, second and third leaves of seedlings were harvested for RNA preparation.

RNA Isolation and PCR Analysis
Total RNA was prepared from seedlings using Trizol reagent and reverse-transcribed with oligo(dT) primer and the First-Strand cDNA Synthesis kit for RT-PCR (Roche). In Arabidopsis plants, PCR was performed using primers 5′-ATGCGGACTCCAGCAATCCAA-3′ and 5′-CACAACTTGAACGCCTCATA-3′ for CAB (At5g27690), 5′-ATGAGAACACGCTCTCGGTG-3′ and 5′-CTCTACTGACTCCCTCAG-3′ for SFP (At3g5880), 5′-TGAATAC-GCTTCTCTGACC-3′ and 5′-TGTATGCTGCTGGTGTGCAG-3′ for CAA (At1g14740), and 5′-GTCGTGCTAAGAGAGGAAGA-3′ and 5′-TCAAGC-TTCAACTTCTTCT-3′ for ubiquitin1 (Ubq, At5g20620) as an internal control, according to Moore et al. (2003). For quantitative real-time PCR, gene-specific PCR primers and fluorogenic probes for the TaqMan assay were designed by the Assays-by-Design Service (Applied Biosystems). Gene expressions were analyzed by using the TaqMan Universal PCR Master Mix and an ABI PRISM 7000 sequence detector (Applied Biosystems) according to the manufacturer's instructions. In the analysis of rice plants, the gene-specific primers and probes used for quantification of real-time PCR were as follows: for RbcS, RbcS-forward 5′-AGC-AAATGGCCGCGACGATG-3′, RbcS-reverse 5′-GAACCTTCTGAGTGGCCTCA-A TTCG-3′ and RbcS-probe PAM-CACACCCGACAGCACC-NFQ, and for ubiquitin5 (UBq5), UBq5-forward 5′-CAGGCTTCCGCAAGAA-3′, UBq5-reverse 5′-AAGTGGTGGCCATGAGG-3′ and UBq5-probeFAM-CCACCGAGTCGC-NFQ. Ubq5 gene expression was used for normalization of real-time PCR results (Jain et al., 2006).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Schematic diagrams of all OsHXK-GFP fusion constructs used in subcellular localization experiments. A, OsHXK5-GFP fusion constructs. B, OsHXK6-GFP fusion constructs.

Supplemental Figure S2. Expression of Glc responsive genes ZmRbcS (A) and R Amy3D (B) in rice mesophyll protoplasts transfected with the effectors AtHXK1, OsHXK5, OsHXK6, or OsHXK mutant alleles under the control of the CaMV35S promoter in response to Glc treatment.

Supplemental Figure S3. Complementation of the Arabidopsis gin2-1 mutant by expression of OsHXK5 or OsHXK6.

Supplemental Figure S4. Growth phenotype of transgenic, gin2-1, and wild-type (WT) seedlings plants grown on Glc-free 1/2 MS medium.

Supplemental Figure S5. Complementation of the growth defect phenotype of the Arabidopsis gin2-1 by the overexpression of OsHXK5 or OsHXK6 in the gin2-1 background.

ACKNOWLEDGMENTS
We thank Dr. Wolf B. Frommer (Carnegie Institution) for the yeast shuttle vector pDR196, Dr. Joris Windericks (PlantKunde en Microbiologie, Belgium) for providing the hexokinase-deficient yeast strain YSH74-3C, and Dr. Sang-Dong Yoo (Sungkyunkwan University, Korea) for helpful discussions.

Received October 16, 2008; accepted November 10, 2008; published November 14, 2008.

LITERATURE CITED
gyrase is involved in chloroplast nuclear partitioning. Plant Cell 16: 2665–2682


Role of OsHXK5 and OsHXK6 as Glucose Sensors