Role of the Rice Hexokinases *OsHXK5* and *OsHXK6* as Glucose Sensors[1[C][W]]

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The Arabidopsis (*Arabidopsis thaliana*) hexokinase 1 (*AtHXK1*) 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 *AtHXK1*. 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 *OsHXK*s 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 insensitive2-1* 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.

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 ScHXK2, 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

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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 ScHXK2 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 ScHXK2 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 AtHXK1, 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 AtHXK1 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 *AtHXK1*

The well-characterized Glc sensor AtHXK1 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 *AtHXK1*, 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, 1MGKAAAVGTAVVVAAAVGVAVVLA24 for OsHXK5 and 1MGKGTVVGTAVVVCAAAAAAVGVAVVVS28 for OsHXK6. These analyses also indicated that both proteins contained a predicted NLS, 25RRRRRRDLE-LVEGAAAERKRK45 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 *OsHXK5ΔmTP* and *OsHXK6ΔmTP* 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

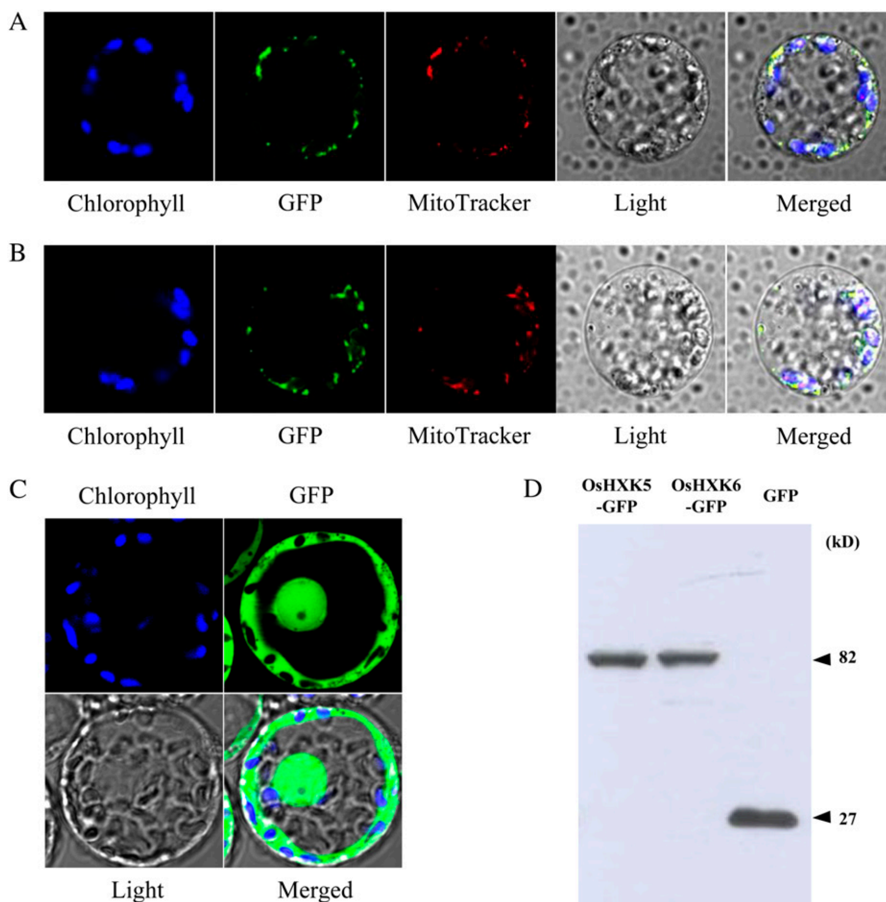


Figure 1. Subcellular localization of OsHXK5-GFP and OsHXK6-GFP fusion proteins in transfected mesophyll protoplasts of maize. A, OsHXK5-GFP. B, OsHXK6-GFP. C, GFP. Chlorophyll autofluorescence and MitoTracker were used as chloroplast and mitochondria markers, respectively. The false color (blue) was used for chlorophyll 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.

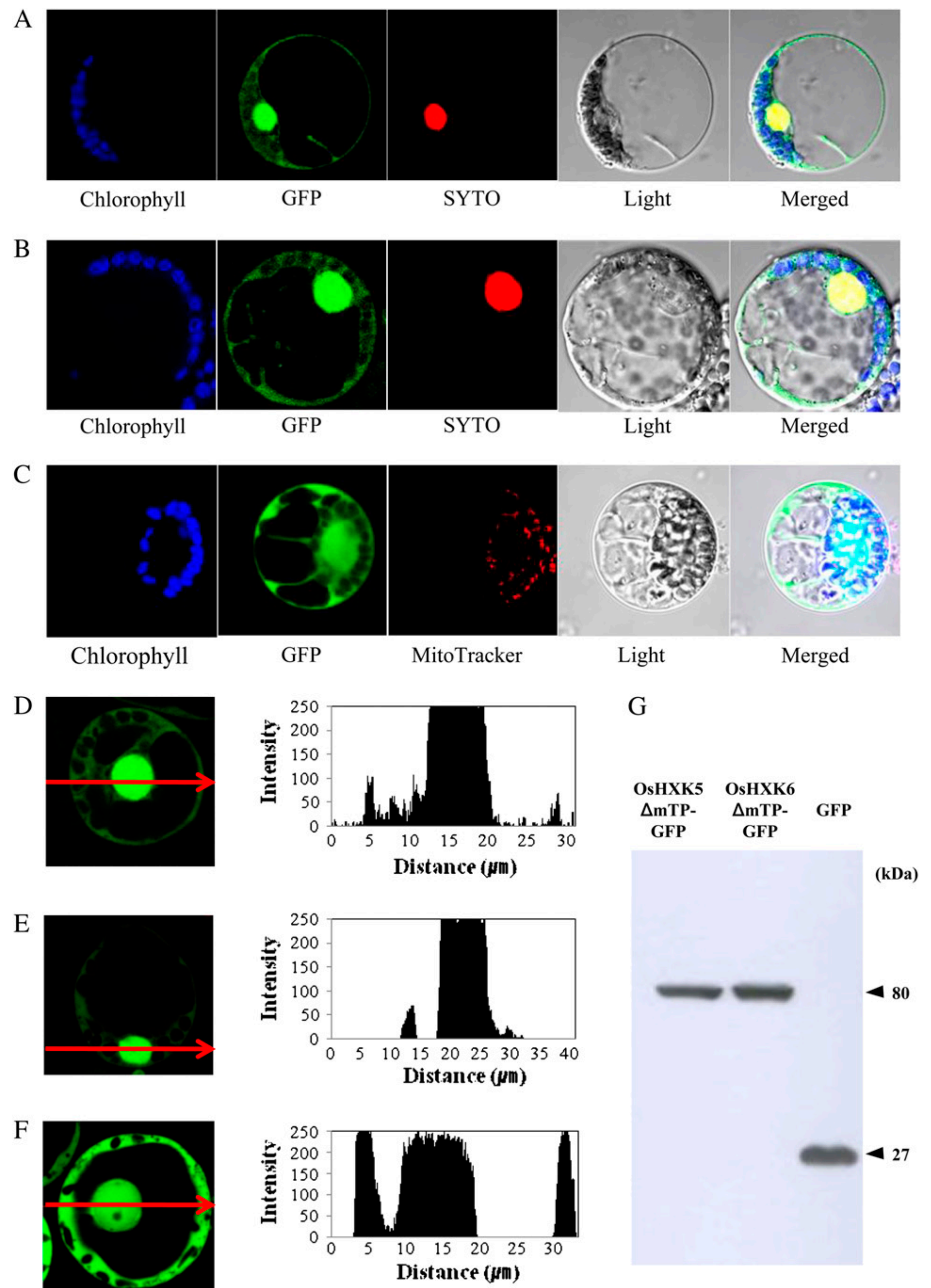


Figure 2. Subcellular localization of OsHXK5ΔmTP-GFP and OsHXK6ΔmTP-GFP fusion proteins in mesophyll protoplasts of maize. A, OsHXK5ΔmTP-GFP. B, OsHXK6ΔmTP-GFP. Chlorophyll autofluorescence and SYTO dye were used as chloroplast and nuclear markers, respectively. The false color (blue) was used for chlorophyll autofluorescence to distinguish it from the fluorescence of the SYTO dye. GFP signal is indicated in green, and the nuclear signal stained with SYTO dye is shown in red. C, OsHXK5ΔmTP-GFP. Interaction between OsHXK5ΔmTP-GFP and mitochondria was not detected. A similar result was observed for the OsHXK6 mTP-GFP fusion protein (data not shown). D to F, Localization (left) and fluorescence intensity (right) of OsHXK5ΔmTP-GFP (D), OsHXK6ΔmTP-GFP (E), and GFP (F). GFP fluorescence intensities were quantified along arrows. G, Protein gel-blot analysis for OsHXK5ΔmTP-GFP and OsHXK6ΔmTP-GFP fusion proteins with an anti-GFP antibody. GFP served as control.

constructed *OsHXK5ΔNLS-GFP* and *OsHXK6ΔNLS-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

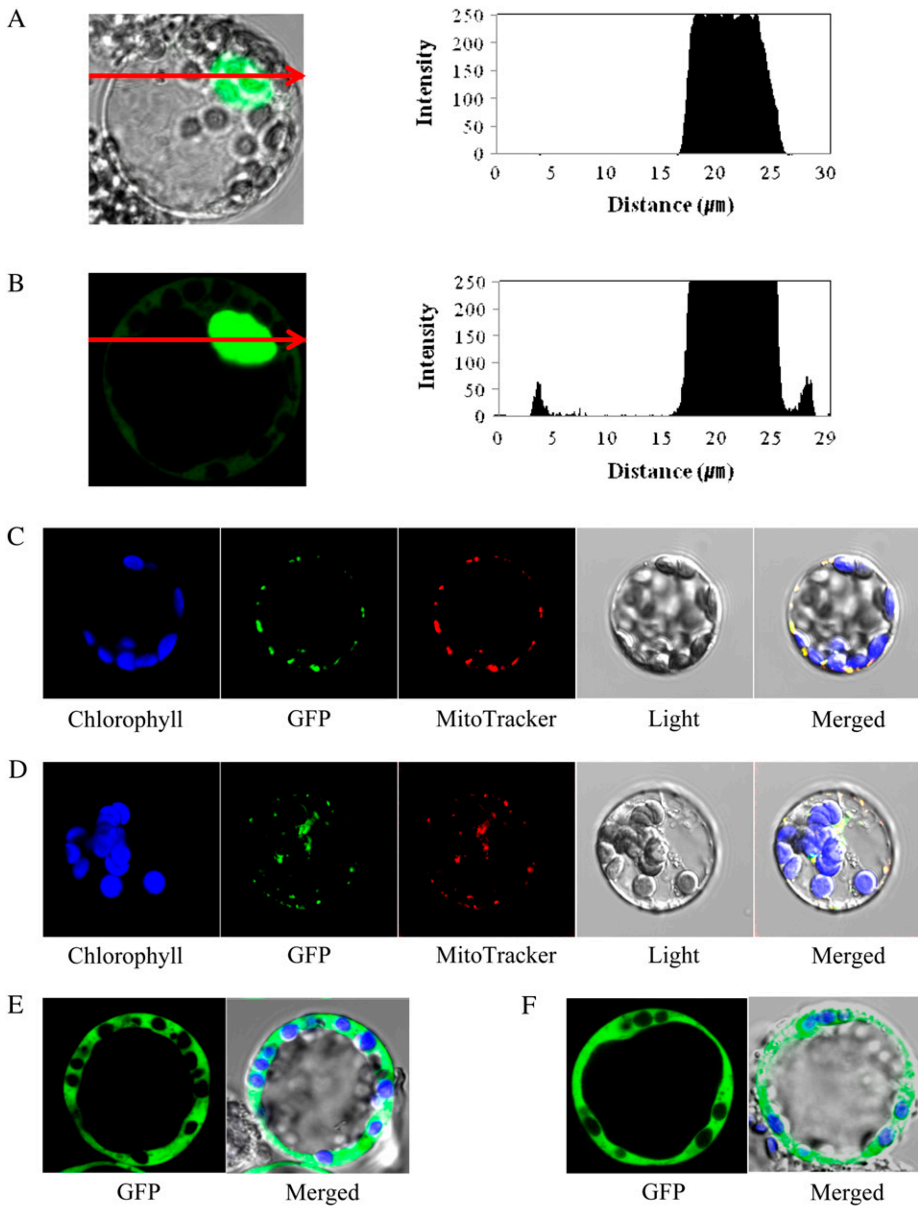


Figure 3. Subcellular localization of OsHXK-GFP fusion proteins in mesophyll protoplasts of maize. A and B, Localization (left) and fluorescence intensity (right) of OsHXK5NLS-GFP (A) and OsHXK6NLS-GFP (B). GFP fluorescence intensities were quantified along arrows. C, OsHXK5ΔNLS-GFP. D, OsHXK6ΔNLS-GFP. Chlorophyll autofluorescence and MitoTracker were used as chloroplast and mitochondria markers, respectively. E, OsHXK5ΔmTPΔNLS-GFP. F, OsHXK6ΔmTPΔNLS-GFP. The merged images of chlorophyll autofluorescence, GFP, and light-field are shown.

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 *OsHXK*s 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

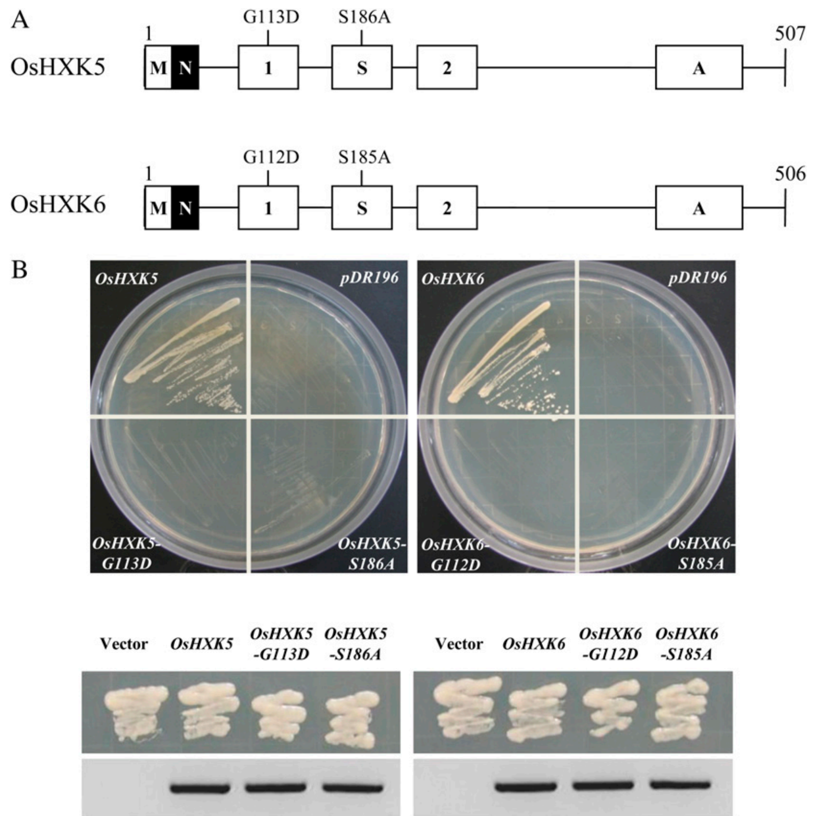


Figure 4. Transformation of catalytically inactive mutants for *OsHXK5* and *OsHXK6* into a yeast hexokinase mutant. A, Schematic representation of *OsHXK5* and *OsHXK6* and their catalytically inactive mutation sites. Mitochondrial targeting signals and NLSs are indicated as white (M) and black (N) 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 YSH7.4-3C (*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 YSH7.4-3C mutant strain transformed with the pDR196 vector was used as a control. As control experiment, YSH7.4-3C 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.]

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 *α-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 *OsHXK*s 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

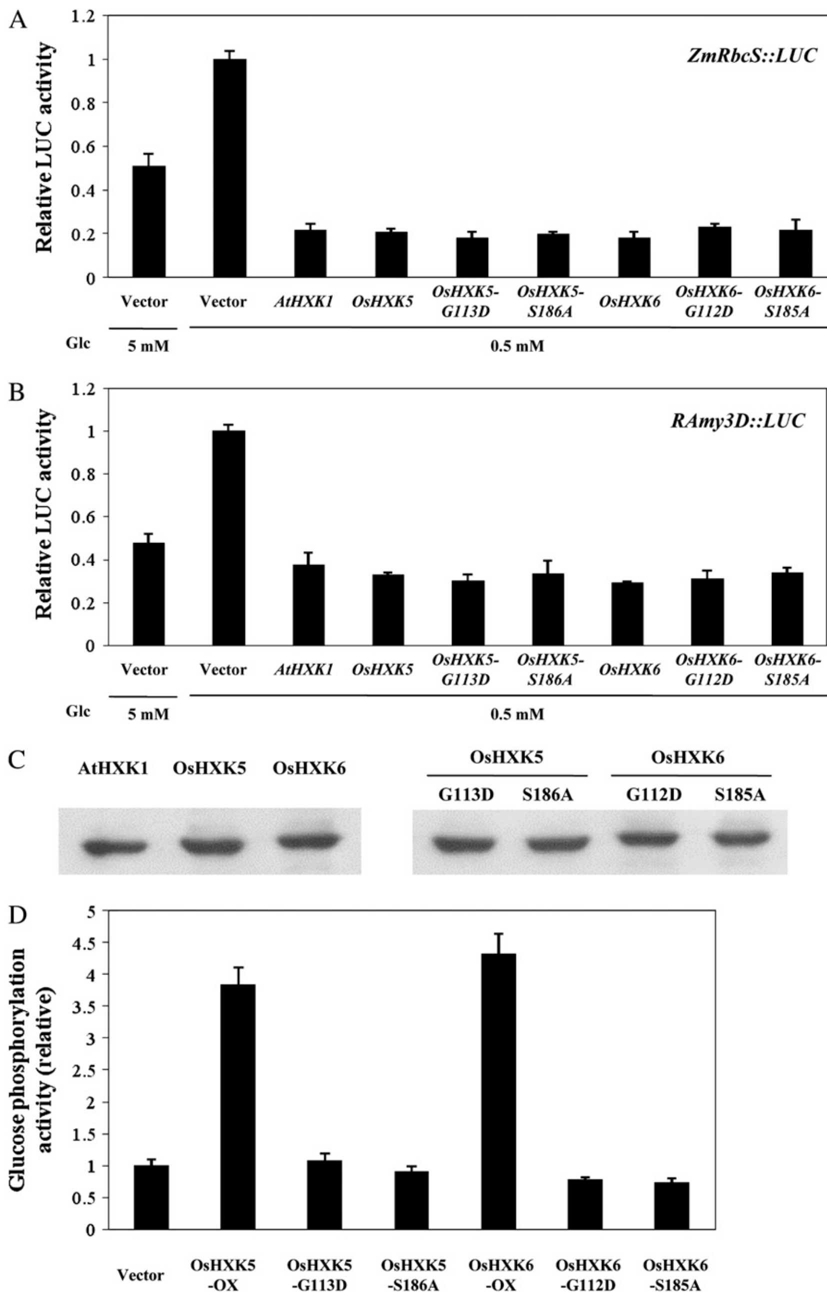


Figure 5. Expression of Glc responsive genes *ZmRbcS* (A) and *RAmy3D* (B) in maize mesophyll protoplasts transfected with the effectors *AtHXK1*, *OsHXK5*, *OsHXK6*, or *OsHXK* mutant alleles under the control of the *CaMV35S* promoter in response to Glc treatment. *ZmUBQ*::*GUS* was included in each sample as an internal control, and control protoplasts were transfected with empty vector. Promoter activities of Glc responsive reporter constructs are represented as relative LUC/GUS activity. All transient expression experiments were repeated three times with similar results. C, The steady expression of effector proteins was detected by protein-blot analysis using an anti-Myc antibody. D, Relative Glc phosphorylation activity in control protoplast (empty vector) and in protoplasts expressing *OsHXK5*, *OsHXK6*, or their catalytically inactive mutant alleles. Glc phosphorylation activity in control protoplast (empty vector) was arbitrarily considered as 1. Each data point represents the mean ± SD from three separate experiments.

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 *OsHXK*s on high Glc (6%)-containing, half-strength Murashige and Skoog (MS) media. Results indicated that the growth of all of these *OsHXK*s 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 *OsHXK*s could suppress expression of the target genes in a similar way, we measured mRNA levels of *CAB*, *SBP*, 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 homozygous 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

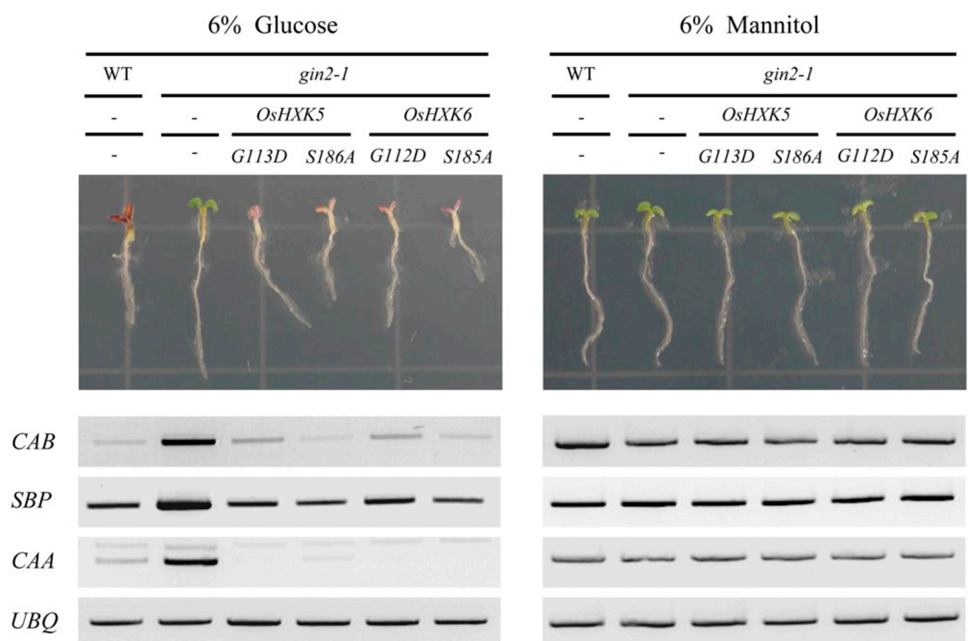


Figure 6. Complementation of the Arabidopsis *gin2-1* mutant by expression of catalytically inactive *OsHXK5* and *OsHXK6* mutant alleles. Top, Seedlings homozygous for the transgene, and *gin2-1* and wild-type (WT) seedlings grown on 1/2 MS medium with 6% Glc or mannitol for 6 d. Bottom, Expression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

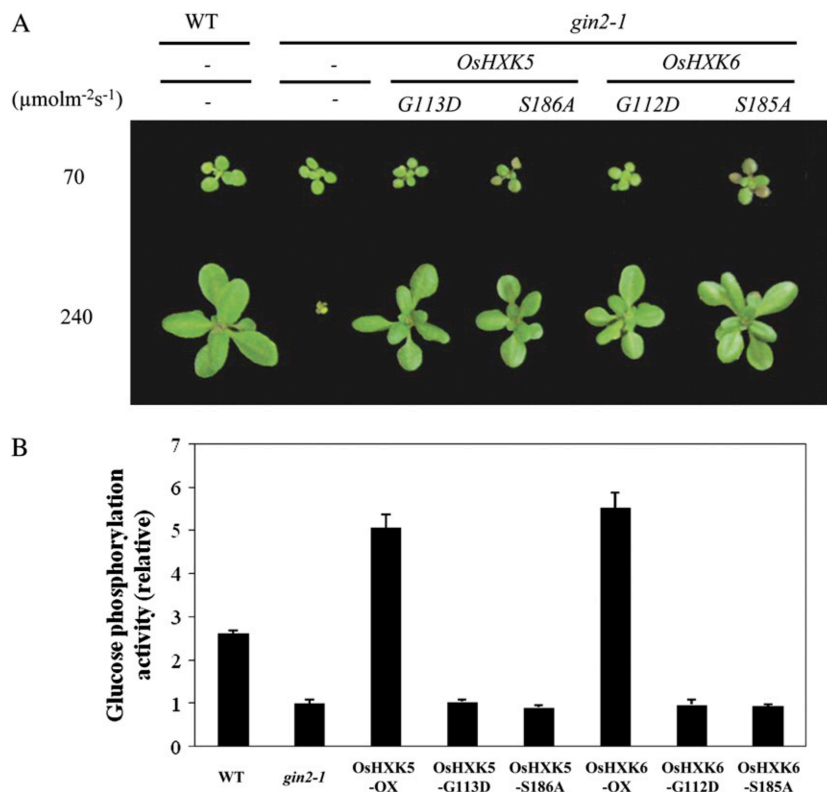


Figure 7. Complementation of the growth defect phenotype of the Arabidopsis *gin2-1* by the overexpression of catalytically inactive alleles of *OsHXK5* and *OsHXK6* in the *gin2-1* background. A, Growth phenotypes of wild-type (WT), *gin2-1*, and transgenic plants under low (70 μmol m^{-2} s^{-1}) or high (240 μmol m^{-2} s^{-1}) light condition. B, Relative Glc phosphorylation activity in wild-type (WT), *gin2-1*, and transgenic Arabidopsis plants expressing *OsHXK5*, *OsHXK6*, or their catalytically inactive mutant alleles. Glc phosphorylation activity in *gin2-1* was arbitrarily considered as 1. Each data point represents the mean ± SD from three separate experiments.

as Glc sensors in rice plants as well as in the Arabidopsis *gin2-1* mutant background.

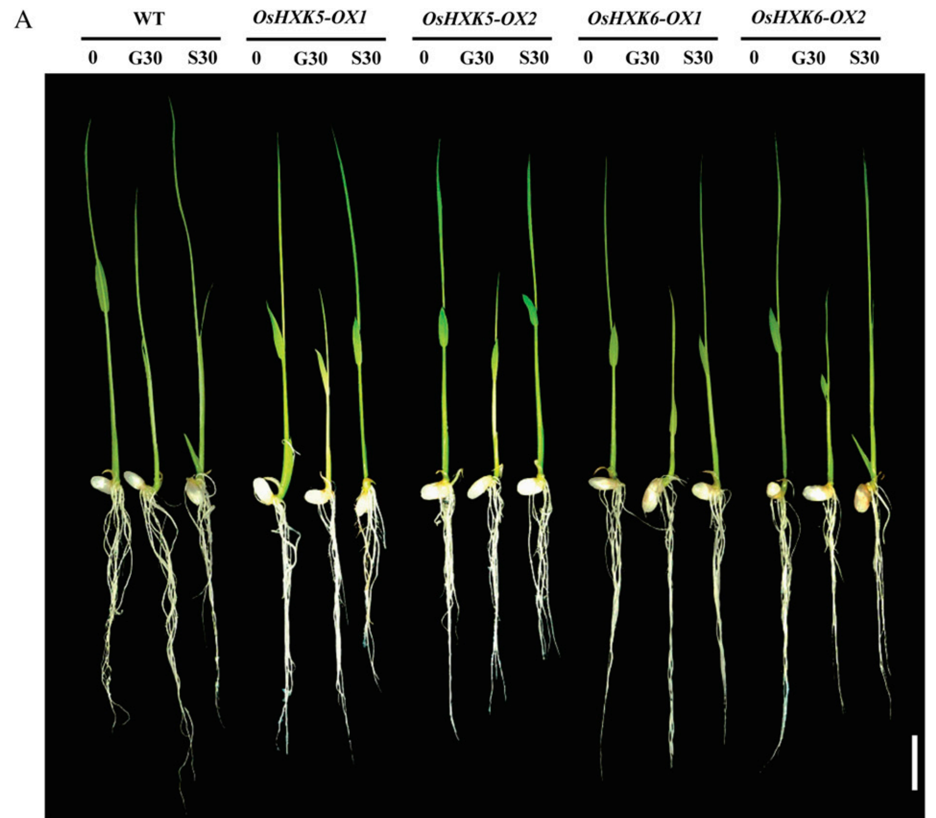
DISCUSSION

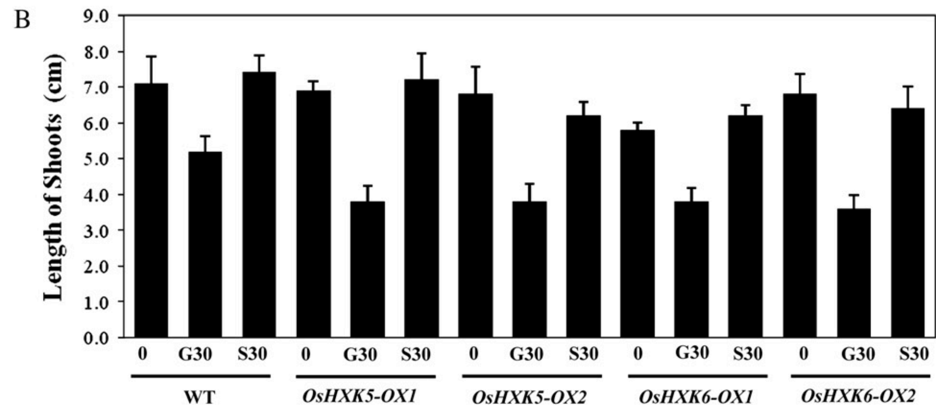
OsHXK5 and OsHXK6 Possess a Dual-Targeting Ability to Mitochondria and Nuclei

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





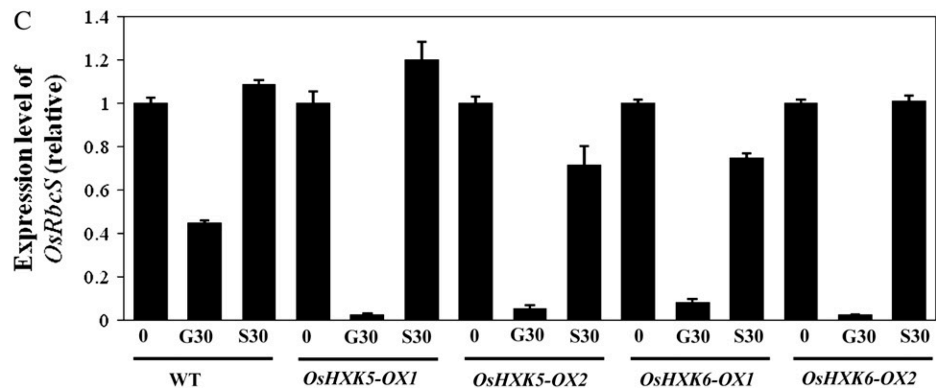


Figure 8. Growth phenotype of wild-type (WT) and transgenic rice seedlings expressing *OsHXK5* or *OsHXK6* in response to Glc treatment. A, Growth phenotype of seedling plants grown on water agar media containing Glc-free (0), 30 mM Glc (G30), and 30 mM sorbitol (S30). Bar = 1 cm. B, Shoot lengths of 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 ScHXK2 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; Rodríguez et al., 2001; Ahuatzi et al., 2004). The nuclear localization of ScHXK2 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 *RAmy3D*, 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 *RAmy3D* 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 *RAmy3D* expression under sugar starvation, while Glc and Suc induced the repression of *RAmy3D* (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 *RAmy3D*. Our current transient expression experiments using the *RAmy3D* promoter further support previous studies reporting that the sugar-dependent repression of *RAmy3D* 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 *RAmy3D* expression under Glc starvation (Lu et al., 2007). It would be interesting to see whether OsHXK5- and OsHXK6-dependent sugar repression of the *RAmy3D* 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, was 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.biosci.ohio-state.edu/~plantbio/Facilities/abrc/), and transgenic plants were grown on soil at 22°C under a 16-h-light/8-h-dark photoperiod. Wild-type rice (*Oryza sativa*) cv Dongjin and transgenic plants were grown in a greenhouse at 30°C during the day and at 20°C at night in a light/dark cycle of 14/10 h during several generations. For protoplast isolation, wild-type maize (*Zea mays*) cv Yeonnong and rice plants were grown at 25°C in a growth chamber under the dark conditions during 7 d and 8 to 10 d, respectively, and their young leaves were used in analyses.

Vector Construction

To examine the subcellular localization of OsHXK5 and OsHXK6, each full-length cDNA fragment, excluding stop codon and 3′-untranslated region, was amplified with the addition of *Xba*I and *Xho*I sites and then cloned between the *CaMV35S* promoter and *sGFP* (Chiu et al., 1996) of the pJJ1450 vector. *OsHXK5ΔmTP* (amino acids 25–507; GenBank accession no. DQ116387) and *OsHXK6ΔmTP* (amino acids 29–506; GenBank accession no. DQ116388) were made by deletion of the mTPs in their N termini. *OsHXK5NLS* (amino acids 24–47) and *OsHXK6NLS* (amino acids 28–46) were amplified with the addition of *Xba*I and *Xho*I sites. *OsHXK5ΔmTPΔNLS* (amino acids 47–507) and *OsHXK6ΔmTPΔNLS* (amino acids 45–506) were made by deletion of both mTP and NLS in their N termini. The primer pairs used for the PCR amplification were: for *OsHXK5*, 5′-GCTCTAGAAGGGAAGGCGGAG-CAGCGGTG-3′ and 5′-CCCTCGAGAGTCGATCTCGGCATACTGGGA-3′; for *OsHXK6*, 5′-GCTCTAGAGGAAGGAGGAGGAGTAGGACGC-3′ and 5′-CCCTCGAGACTCGACGCTAGCATACTGGGA-3′; for *OsHXK5ΔmTP*, 5′-GCTCTAGAATGCGGAGGCGGAGGAGGAGGGAC-3′ and the reverse primer of *OsHXK5*; for *OsHXK6ΔmTP*, 5′-GCTCTAGAATGCGGAGGAG-GAGGAGCAAGCGG-3′ and the reverse primer of *OsHXK6*; for *OsHXK5NLS*, 5′-GCTCTAGAATGGCGCGGAGGCGGAGGAGG-3′ and 5′-CCCTCGA-GACGCCACCTTCCTCTTCCTCTC-3′; for *OsHXK6NLS*, 5′-GCTCTAGA-ATGTCGCGGAGGAGGAGGAGCAA-3′ and 5′-CCCTCGAGAGGCGG-CCCTCCTCCGCCGCTC-3′; for *OsHXK5ΔmTPΔNLS*, 5′-GCTCTAGAATG-GCGGCGGTGATCGAGGACGTG-3′ and the reverse primer of *OsHXK5*; and for *OsHXK6ΔmTPΔNLS*, 5′-GCTCTAGAATGGCCGCCGCTGTGATCGAG-GAG-3′ and the reverse primer of *OsHXK6*. *OsHXK5ΔNLS* and *OsHXK6ΔNLS* were generated by an internal deletion of NLS motifs (amino acids 25–45 for OsHXK5 and 29–44 for OsHXK6) using a PCR-mediated fusion strategy with each four primers (Sieburth and Meyerowitz, 1997): for *OsHXK5ΔNLS*, the forward primer of *OsHXK5*, 5′-CACCGCCGCCACCGCCAGCACCACCGC-CAC-3′, 5′-GTGGTGCTGGCGGTGGCGGCGGTGATCGAG-3′, and the reverse primer of *OsHXK5*, and for *OsHXK6ΔNLS*, the forward primer of *OsHXK6*, 5′-CACAGCGGCGGCCGACACCACCACCGCCAC-3′, 5′-GTG-GTGGTGTCGGCCGCCGCTGTGATCGAG-3′, and the reverse primer of *OsHXK6*. These PCR-amplified products digested with *Xba*I and *Xho*I were subcloned into the pJJ1450 vector.

To overexpress *OsHXK5* and *OsHXK6* in Arabidopsis and rice, individual cDNAs were placed under the control of the *CaMV35S* promoter using the pPZP2Ha3(+) vector (Fuse et al., 2001). To generate catalytically inactive mutants of *OsHXK5* and *OsHXK6*, the conserved Gly (G) in the ATP-binding site and Ser (S) in the phosphoryl transfer site were mutated to Asp (D) and Ala (A), respectively, by PCR-mediated targeted mutagenesis. The primer pairs used for the PCR amplification were: 5′-GGAAGTTGGTGTCTCCAA-GATCCAATGCAT-3′ and 5′-GATCTTGGAGACACCAACTTCCGCGTCCTG-3′ for *OsHXK5-G113D*; 5′-CACTGGGAAGGCAAAGGTGAAGCCCAGCTC-3′ and 5′-GCTTCACCTTTGCCTTCCCAGTGAGCCAGA-3′ for *OsHXK5-S186A*; 5′-GGAAATTGGTGTCCCCAAGATCGAGAGCAT-3′ and 5′-CGATCTTGGG-GACACCAATTTCCGTGTTAT-3′ for *OsHXK6-G112D*; and 5′-CACTGGGA-AAGCAAAGGTGAAGCCTAACTC-3′and 5′-GCTTCACCTTTGCTTTCCCA-GTGCACCAAA-3′ for *OsHXK6-S185A*. These amplified mutant alleles digested with *Xba*I and *Xho*I were cloned into the pPZP2Ha3(+) vector, and the resulting constructs were named as *OsHXK5-G113D*, *OsHXK5-S186A*, *OsHXK6-G112D*, and *OsHXK6-S185A*.

As effector vectors for transient gene expression assay, *OsHXK5*, *OsHXK6*, and the respective catalytically inactive mutant alleles of the rice hexokinases were placed under the control of the *CaMV35S* promoter of the pJJ1549 vector. To generate reporter vectors, the promoters of *RAmy3D* were amplified by PCR using primers 5′-CGGGATCCGATCTTCAACCACCTGTGCTAGCT-3′ and 5′-TGCCATGGATCTGTGTAAGCTGAAACCGTGTT-3′. The amplified products digested with *Bam*HI and *Nco*I were fused to the firefly *LUC* gene to generate *RAmy3D*::*LUC*. The maize *RbcS* promoter::*LUC* construct (*ZmRbcS*::*LUC*) derived from *ZmRbcS::CAT* was used as additional reporter molecule (Hwang and Sheen, 2001). The maize *Ubiquitin* promoter derived from pGA1611 binary vector (Kim et al., 2003) digested with *Hin*dIII and *Bam*HI 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 linkage 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 *Xba*I and *Xho*I sites by PCR and subcloned into the *Spe*I and *Xho*I sites of the yeast (*Saccharomyces cerevisiae*) shuttle vector pDR196 (Wipf 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 SP2). 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 (1×10^6 cells/sample) transfected with *GFP* fusion constructs were electrophoresed on a 12% SDS-PAGE gel and immunoblotted with an anti-GFP antibody (B-2: sc-9996, Santa Cruz Biotechnology).

Yeast Complementation Assay

The hexokinase-deficient yeast triple mutant YSH7.4-3C (*hxk1*, *hxk2*, *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 ($1–2 \times 10^6$ 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 $MgCl_2$, 1 mM EDTA, 1% Triton X-100) according to the method previously described (Wiese et al., 1999). Insoluble debris was removed by centrifugation at 13,000*g* 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 $MgCl_2$, 0.5 mM NADP, 1.1 mM ATP, 2 units Glc-6-P dehydrogenase, and 5 mM Glc.

Transient Expression Assay Using Maize and Rice Mesophyll Protoplasts

Maize mesophyll protoplasts ($1–2 \times 10^5$ 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 ($3–6 \times 10^5$ 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 Glc responsive reporter constructs and effector constructs using a polyethylene glycol-calcium-mediated method

(Hwang and Sheen, 2001; Cho et al., 2004). *ZmUBQ::GUS* was included in each sample as an internal control and was not affected by Glc treatment. Transfected protoplasts were incubated for 6 h with 0.5 mM or 5 mM Glc and then harvested. The harvested protoplasts were resuspened in lysis buffer and used for LUC and GUS assays. LUC assays were performed using the LUC assay system (Promega), and GUS assays were performed by previously described methods (Jefferson et al., 1987). The fluorescence generated by LUC and GUS activity was measured by the VICTOR2 1420 multilabel counter (PerkinElmer Life Sciences). In each sample, the measured LUC activity was divided by the GUS activity to normalize the data for variation in experimental conditions, and all transient expression experiments were repeated three times with similar results. In maize protoplast transfected with *OsHXK5*, *OsHXK6*, or their catalytically inactive mutant alleles, expression of these effector proteins was confirmed by protein-blot analysis using an anti-Myc antibody (Clone A46, Upstate Biotechnology) according to Lee et al. (2007).

Arabidopsis Transformation

To produce transgenic plants overexpressing *OsHXK5*, *OsHXK6*, or their catalytically inactive mutant alleles, the *Agrobacterium tumefaciens* GV3101 strains harboring each of the vector constructs were grown to stationary phase in Luria-Bertani liquid culture with 25 mg L^{-1} kanamycin at 28°C, 250 rpm. *gin2-1* plants were transformed by the floral deep method as previously described (Clough and Bent, 1998). All transgenic plants were selected on Gamborg B5 medium containing 25 mg L^{-1} hygromycin.

Rice Transformation

To produce transgenic rice plants overexpressing *OsHXK5* and *OsHXK6*, the *A. tumefaciens* LBA4404 strains harboring individual vector constructs were grown on AB media with 25 mg L^{-1} kanamycin for 3 d at 28°C, and rice transformation was performed by the *Agrobacterium*-mediated co-cultivation method as described previously (Jeon et al., 2000). 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 mM Glc, and 30 mM sorbitol, respectively. For imbibitions 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′-ATGGCCACTTCAGCAATCCAA-3′ and 5′-CACAACTTGACACGCC-CATAT-3′ for *CAB* (At3g27690), 5′-ATGGAGACCAGCATCGCGTG-3′ and 5′-CTTCCACTGGACCTCCCAT-3′ for *SBP* (At3g55800), 5′-TGAATAC-GCTGTCTTGCACC-3′ and 5′-TGTGATGGTGGTGGTAGCGA-3′ for *CAA* (At5g14740), and 5′-GTGGTGCTAAGAAGAGGAAGA-3′ and 5′-TCAAGC-TTCAACTTCTTCTTT-3′ for *ubiquitin4* (*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 quantitative real-time PCR were as follows: for *RbcS*, RbcS-forward 5′-AGC-AATGGCGGCAGGAT-3′, RbcS-reverse 5′-GAACTTCTTGATGCCCTCA-ATCG-3′ and RbcS-probe FAM-CACACCTGCATGCACC-NFQ; and for *ubiquitin5* (*UBQ5*), UBQ5-forward 5′-CCGCCTCCGCAAGGA-3′, UBQ5-reverse 5′-AAGTGGTTGGCCATGAAGGT-3′ and UBQ5-probeFAM-CCAA-CGCCGAGTGCG-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 *OsHKX-GFP* fusion constructs used in subcellular localization experiments. A, *OsHKX5-GFP* fusion constructs. B, *OsHKX6-GFP* fusion constructs.

Supplemental Figure S2. Expression of Glc responsive genes *ZmRbcS* (A) and *RAmy3D* (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) seedling 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.

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