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ORIGINAL ARTICLE

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Structure, expression, and functional analysis of the hexokinase gene family in rice (*Oryza sativa* L.)

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Abstract Hexokinase (HXK) is a dual-function enzyme that both phosphorylates hexose to form hexose 6−phosphate and plays an important role in sugar sensing and signaling. To investigate the roles of hexokinases in rice growth and development, we analyzed rice sequence databases and isolated ten rice hexokinase cDNAs, *OsHXK1* (*Oryza sativa Hexokinase 1*) through *OsHXK10*. With the exception of the single-exon gene *OsHXK1*, the *OsHXK*s all have a highly conserved genomic structure consisting of nine exons and eight introns. Gene expression profiling revealed that *OsHXK2* through *OsHXK9* are expressed ubiquitously in various organs, whereas *OsHXK10* expression is pollen-specific. Sugars induced the expression of three *OsHXKs, OsHXK2*, *OsHXK5*, and *OsHXK6*, in excised leaves, while suppressing *OsHXK7* expression in excised leaves and immature seeds. The hexokinase activity of the *OsHXKs* was confirmed by functional complementation of the hexokinase-deficient yeast strain YSH7.4-3C (*hxk1*, *hxk2*, *glk1*). OsHXK4 was able to complement this mutant only after the chloroplast-transit peptide was removed. The subcellular localization of OsHXK4 and OsHXK7, observed with green fluorescent protein (GFP) fusion constructs, indicated that OsHXK4 is a plastid-stroma-targeted hexokinase while OsHXK7 localizes to the cytosol.

Keywords Gene expression · Gene structure · Hexokinase · *Oryza* · Subcellular localization · Yeast complementation

Abbreviations cM: Kosambi values · DAF: Days after flowering · OsHXK: Rice hexokinase · *UDT1*: *Undeveloped tapetum 1* · UTR: Untranslated region

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Introduction

In higher plants, hexoses not only function as energy sources and structural components for growth and development, but also they act as regulatory molecules controlling genes involved in many essential processes such as photosynthesis, growth, senescence, and pathogen defense (Jang and Sheen 1997; Perata et al. 1997; Smeekens and Rook 1997; Wingler et al. 1998). Hexokinase catalyzes the ATP-dependent conversion of hexoses to hexose 6-phosphates. It also acts as a sugar sensor that perceives the level and phosphorylation status of sugar and transmits this information to the nucleus through a signal transduction pathway (Jang and Sheen 1997; Jang et al. 1997; Smeekens 1998). Hexose-phosphorylating enzymes are sometimes classified as hexokinases, glucokinases, and fructokinases on the basis of their hexose-substrate specificities. Although glucokinase is usually considered to be a hexokinase, it only phosphorylates glucose, whereas hexokinases are generally capable of phosphorylating a range of hexoses. Fructokinase has a much higher affinity for fructose than hexokinases and little or no activity toward glucose (Renz and Stitt 1993).

Biochemical and/or molecular studies have identified hexokinases in many plant species (Cárdenas et al. 1998; Dai et al. 1999; Guglielminetti et al. 2000; Jiang et al. 2003). *Arabidopsis* has two hexokinase genes and four hexokinase-like genes (Dai et al. 1999; Jang et al. 1997). The rice embryo expresses six hexokinase isoforms, although their molecular features have not been characterized (Guglielminetti et al. 2000). To date, only two fructokinase cDNAs from immature rice seeds have been isolated and characterized (Jiang et al. 2003).

Recent studies have demonstrated that hexokinases interact with membranes of various cellular organelles (Frommer et al. 2003), including chloroplasts, mitochondria, Golgi complexes, endoplasmic reticula, and plasma membranes. Plants contain two types of hexokinases that interact with chloroplasts: type A is a chloroplast-imported hexokinase and type B is a chloroplast membrane-bound hexokinase (Olsson et al. 2003). Type A is a novel type of inner plastidic hexokinase that differs from the well-known type B hexokinases and has been reported in only two plant species, moss (Olsson et al. 2003) and tobacco (Giese et al. 2005). Within chloroplasts, the stromal type A hexokinase phosphorylates hexose. The type B hexokinases associated with the chloroplast outer membrane phosphorylate glucose that is exported from the chloroplast by the glucose translocator (Schleucher et al. 1998; Wiese et al. 1999). Hexokinases interacting with mitochondria may be coupled to ATP production for cellular metabolism. In mammalian cells, hexokinase II inhibits mitochondria-mediated apoptotic pathways by binding to a voltage-dependent anion channel (VDAC) located in the outer mitochondrial membrane (Pastorino et al. 2002). In addition to organelle-bound hexokinases, the cytosolic hexokinase plays an important role in the removal of free hexoses from the cytosol depending on cellular demands (Moore et al. 1999). The resulting cytosolic glucose-6-phosphate is used in cytosolic metabolism or sucrose biosynthesis.

In lower and higher eukaryotes, including yeast, mammals, and plants, it has been shown that hexokinases have an important role in sugar signaling as well as in metabolism (Cárdenas et al. 1998; Harrington and Bush 2003; Moore et al. 2003; Rolland et al. 2002a). Over-expression of *AtHXK1* in tomato causes a reduction in photosynthesis, growth inhibition, and the induction of rapid senescence (Dai et al. 1999), suggesting that hexokinase has a regulatory role in photosynthetic tissues. Transgenic plants that overexpress *AtHXK* genes are hypersensitive to sugar and *AtHXK*-antisense plants are hyposensitive, supporting the hypothesis that hexokinase is a hexose sensor (Jang et al. 1997). The *AtHXK1* mutant *glucose insensitive2* (*gin2*) exhibited a glucose-insensitive phenotype in seedling development, reduced cell expansion in the adult plant, and decreased glucose-dependent gene expression (Moore et al. 2003). Transgenic plants expressing catalytically inactive *AtHXK1* mutant alleles in the *gin2* mutant background supported various signaling processes, including gene expression, cell proliferation, root and inflorescence growth, and leaf expansion and senescence (Moore et al. 2003), suggesting that the catalytic and sensory functions of AtHXK1 can be uncoupled in the *Arabidopsis* plant. For plant species other than *Arabidopsis*, however, the role of hexokinase as a sugar sensor has yet to be proven (Halford et al. 1999). For example, no sugar-sensing role was detected for hexokinases in potato plants (Veramendi et al. 2002).

As a first step toward understanding the roles of rice hexokinases, we have cloned all of the rice hexokinase cDNAs using published sequences from the rice genome. To examine the temporal and spatial expression of the rice hexokinase (*OsHXK*) genes, we performed RT-PCR analysis with various rice organs, including leaves, roots, flowers, and developing seeds. We also examined the response of the *OsHXK* genes to increased levels of the sugar glucose and fructose. To confirm the hexokinase activity of the *OsHXKs*, we performed functional complementation experiments of the hexokinase-deficient yeast strain YSH7.4-3C (*hxk1*, *hxk2*, *glk1*). In addition, we determined the subcellular localization of two OsHXK proteins, OsHXK4 and OsHXK7.

Materials and methods

Plant materials

Greenhouse-grown *japonica* rice (*Oryza sativa* L) of variety *Jinmi* (provided by the National Institute of Crop Sciences, Suwon, Korea) was used in all the experiments, except for the seed coat- and endosperm-specific cDNA libraries (Jun et al. 2004) which were constructed using the variety *Dongjin*. Immature seeds at different developmental stages, from 1 day after-flowering (DAF) to 15 DAF, were collected after removing the paleae and lemmas from the florets with forceps. Flower organs, pistils, stamens, and paleae/lemmas were separated from 15–22 cm panicles before anthesis. Wild-type and *udt1* (*undeveloped tapetum 1*) panicles were harvested according to their lengths. *udt1* is a rice mutant whose panicle lacks pollen in the anthers (Jung et al. 2005). The flag leaves of mature plants were used as a source organ. Roots were collected at the four-leaf seedling stage. All samples were frozen in liquid nitrogen and kept at −80°C until use.

Identification and cDNA cloning of hexokinase genes

Full-length cDNAs of the *OsHXK* genes were isolated by RT-PCR, using gene-specific primers that encompassed the translation start codon and 3′-untranslated region (UTR) of putative *OsHXK* genes, based on a search of the rice sequence database. The primer pairs were: *OsHXK1*, 5′-GGTTCAAAGCTTGTTCGATT-3′ and 5′-GCTTCTATTCATATGTGGTTTG-3′; *OsH XK2*, 5′-ATATGTACGTAAGGGCCCCATC-3′ and 5′-GAA

ATTTGTTGGAAGGACAATACT-3′; *OsHXK3*, 5′-TGGGATTCGTGGGTGGGTTT-3′ and 5′- TAGATATCAAACAATGTCCCTTTT-3′; *OsHXK4*, 5′-TAGTACGTGTAGTGAGGAGCATTT-3′ and 5′-ATGAATTTCTGGTGAACTGTAACT-3′; *OsHXK5*, 5′-CTCTCGTCCTCCTTTCTCCTAC-3′ and 5′-ATGAGATCAAACAAGAGCAATTAG-3′; *OsHXK6*, 5′-GAGGAAGGAGGAGGAGTAGGAC-3′ and 5′- TGACATACGAAAAGTGAAATTATG-3′; *OsHXK7*, 5′-CTTTGATCTTGACCACCAATCT-3′ and 5′-TCAAAATTTAAATCCGTTGATACA-3′; *OsHXK8*, 5′-CACTGAAAGGGATCAACTAAACTA-3′ and 5′-GTTTTGCAGTTCCAATTTTATTTC-3′; *OsHXK9*, 5′-GAACTCGCGTTGACCTATTGACT-3′ and 5′-CACTGAACAGATATTGCAGATAGA-3′; and *OsHXK10*, 5′-GTGGAGTGATCGATCGACTCG-3′ and 5′-GTGTCTCATGCCTACTCGGAG-3′. The cDNAs synthesized with mRNA isolated from the leaf, root, flower, and immature seed were used in the PCR reactions. All cDNAs were cloned in pGEM-T Easy vector (Promega) and sequenced automatically. To exclude PCR errors, at least five independent cDNA clones for each gene were analyzed. The cDNA sequences have been submitted to the NCBI database and their accession numbers are DQ116383–DQ116392 for *OsHXK1*–*OsHXK10*, respectively.

Sequence alignment and phylogenetic tree construction

The deduced amino acid sequences of the rice hexokinases were aligned with reported genes from other species using the CLUSTAL W program (Thompson et al. 1994). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 3.0 (Kumar et al. 2004) using the neighbor-joining method. The accession numbers of the sequences used to construct the phylogenetic tree are: *AtHXK1* (U28214), *AtHXK2* (U28215), *At3g20040*, *At1g50460*, *At4g37840*, and *At1g47840* from *Arabidopsis*; *PpHXK1* (AY260967 and AY260968) from moss; *NtHXK1a* (AY553214), *NtHXK1* (AF118133), *NbHXK1* (AY286011), *NtHXK3* (AY553216), *NtHXK2* (AY553215), and *NsHXK2* (AY664407) from tobacco; *SoHXK1* (AF118132) from spinach; *StHXK1* (AF106068) and *StHXKRP1* (AF118134) from potato; *LeHXK2* (AF208543) from tomato; *CsHXK* (AF196966) from citrus; *ScGLK1* (M24077), *ScHXK1* (M14410), and *ScHXK2* (M14411) from yeast; *AnHXK* (AJ009973) from *Aspergillus niger*; *DmHXK1-t1* and *DmHXK1-t2* (AF257603) from fruit fly; and *HsHKDC1* (NM_025130) from human.

Analysis of genomic structure and chromosomal location

The structure of each *OsHXK* gene was determined by aligning the cDNA sequences and genomic sequences of BAC/PAC clones obtained from the NCBI database. The *OsHXK* genes were located on a high-density genetic map (Wu et al. 2002) using the INtegrated rice genome Explorer (INE) database (http://www.rgp.dna.affrc.go.jp/giot/INE.html). The accession numbers of the clones used to identify structures and chromosomal locations are: AP004668 and AP004395 for *OsHXK1*; AC121365 for *OsHXK2*; AP003412 for *OsHXK3*; AP005257 and AP004379 for *OsHXK4*; AC118284 for *OsHXK5*; AP003768 for *OsHXK6*; AC093954 for *OsHXK7*; AP002743 for *OsHXK8*; AP003292 and AP003229 for *OsHXK9*; and AC108874 for *OsHXK10*.

RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was prepared from various organs using Trizol reagent (Invitrogen). The DNase-treated RNA was reverse-transcribed with an oligo-dT primer and the First Strand cDNA Synthesis Kit for RT-PCR (Roche). First-strand cDNA was used in PCR reactions with gene-specific primers and control primers for the actin gene *Act1* (McElroy et al. 1990) and *18S rRNA* (Kim et al. 2003). Gene-specific primers were designed so as to encompass at least one intron per gene to exclude any interference from genomic DNA contamination, except for the intron-less *OsHXK1*. The primers were: *OsHXK1*, 5′-AGCAGACCTACGAGAAGCTCAT-3′ and 5′-CCTCCCGATCTTCTTCAGGAT-3′; *OsHXK2*, 5′-TATACTGGGAACAGGTACTAATGC-3′ and 5′-CCATCTTTAATAGGACTCTACGAA-3′; *OsHXK3*, 5′-TACTGAGGGTTTATTCAACTTTGT-3′ and 5′-TAGTAATGCCCTAGAGCTAATGTC -3′; *OsHXK4*, 5′-GAAGTTGAGTCCATCTTGAGTGAT-3′ and 5′-TACCTCCTGTACTGAGGGTACTTC-3′; *OsHXK5*, 5′- AAAACTGTTGGAGCTAAGCTAAAG-3′ and 5′-CAACTGCTGAACTTCTTGTAATGT-3′; *OsHXK6*, 5′-GATACCTCACATGATCTGAAACAC-3′ and 5′-GTAATGCTCATAGAGACCACCATC-3′; *OsHXK7*, 5′-AGATAACCTGAAGATCACAGACAC-3′ and 5′-CATACAGAACTTCACCACATTCTA-3′; *OsHXK8*, 5′-AGCATTAGACAAGGAAAGCTTAAA-3′ and 5′-TTTTTCAGCCACTATCTTTAGGTC-3′; *OsHXK9*, 5′-TTAAACATGGCTATCAGAAGTCAA-3′ and 5′-AAAAACACACCATATTTCCAGACT-3′; *OsHXK10*, 5′-GCATTATTATGATGATGACACAGT-3′ and 5′-AAGATACACACCAGAGATCATTTT-3′; *Act1*, 5′-GGAACTGGTATGGTCAAGGC-3′ and 5′-AGTCTCATGGATACCCGCAG-3′; and *18S rRNA*, 5′-ATGATAACTCGACGGATCGC-3′ and 5′-CTTGGATGTGGTAGCCGTTT-3′. For PCR analysis of seed coat and endosperm, total cDNAs were isolated by mass in vivo excision from seed coat- and endosperm-specific libraries. These cDNA libraries were made with mRNA extracted from rice seed coats and endosperm at 6–10 DAF (Jun et al. 2004). For PCR, the amplification program consisted of an initial 94°C for 5 min, followed by 28–35 cycles of 94°C, 1 min; 56°C, 1 min; 72°C, 1 min, and a final extension of 72°C for 5 min. Each PCR was repeated at least three times with similar results.

Sugar treatment

Seedlings at the four-leaf stage were maintained for 48 h under dark conditions to deplete endogenous sugars before treatment with various sugars (Cho et al. 2005; Dian et al. 2003). Leaves were excised, cut into 1-cm pieces, and treated in the dark at 28°C with MS media containing 175 mM glucose, fructose, or mannitol. One-2 DAF caryopses also were treated with 175 mM glucose, fructose, or mannitol. The samples were harvested after 12 or 24 h of treatment.

RNA gel-blot analysis

Total RNA (20 μg) isolated from sugar-treated excised leaves or immature seeds using Trizol reagent was separated in 1.3 % agarose gels containing formaldehyde, and transferred onto the Hybond-N^+ nylon membrane (Amersham Biosciences). Hybridization was carried out with [α-^{32}P] dCTP-labeled gene-specific probes according to standard procedures under high-stringency hybridization conditions (Jeon et al. 2000b). The blot was hybridized in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, and 7% (w/v) SDS for 20 h at 60°C. After washing, the hybridization signals were recorded with a phosphoimager (Typhoon, Amersham Biosciences). The *18S rRNA* probe (Kim et al. 2003) was used as loading control. Image Master 2D Elite Ver 4.1 software (Amersham Bioscience) was used to analyze the intensity of hybridization signals.

Yeast complementation assays

Full-length cDNAs of the *OsHXK* genes were subcloned into the yeast shuttle vector pDR196 (Wipf et al. 2003). The hexokinase-deficient yeast triple mutant YSH7.4-3C (*hxk1*, *hxk2*, *glk1*; De Winde et al. 1996) was used for genetic complementation experiments. The yeast mutant was grown on YPgal medium (2% bacto-peptone, 1% yeast extract, 2% galactose). The medium for the selection of transformed colonies contained 0.67% yeast nitrogen base (Difco) and 2% of a carbon source (D-glucose, D-fructose, or galactose), supplemented with the appropriate amino acids and lacking uracil. As a control, the YSH7.4-3C mutant strain was transformed with the pDR196 vector alone.

Subcellular localization of the OsHXK::GFP fusion protein

To examine the subcellular localization of the OsHXK4 and OsHXK7 proteins, each cDNA fragment containing the entire open reading frame of *OsHXK4* and *OsHXK7* was cloned between the *35S* promoter and *sGFP* (Chiu et al. 1996) of the JJ461 vector that contains the *hygromycin phosphotransferase* (*hpt*) gene as a selectable marker. The *OsHXK*::*GFP* fusion constructs were transformed using the *Agrobacterium*-mediated co-cultivation method described by Jeon et al. (2000a). Transgenic calli and plants were examined using the confocal microscope (LSM 510 META, Carl Zeiss, Jena, Germany). Chlorophyll autofluorescence was used as a chloroplast marker.

Results

cDNA cloning of rice hexokinase genes

Systematic blast searches of the rice genome [International Rice Genome Sequencing Project (IRGSP) 2005], using nucleotide and amino acid sequences of *Arabidopsis HXK1* and *HXK2*, identified ten putative hexokinase genes (Table 1). For each of the TBLASTN searches with *AtHXK1* and *AtHXK2*, a score greater than 100 and/or an *E* value lower than 10^{-20} was considered a significant match. Full-length cDNAs of nine of the *OsHXK* genes were isolated by RT-PCR using gene-specific primers upstream of the translation start codon and downstream of the stop codon (Supplementary Fig. 1). RT-PCR experiments did not detect an endogenous transcript for *OsHXK1* in the various samples from leaves, roots, flowers, immature seeds, or sugar-treated or rice-blast-infected leaves (data not shown), indicating that the *OsHXK1* gene is inactive in the examined tissues. It is also possible that *OsHXK1* was transcriptionally active in an untested tissue. Since *OsHXK1* is a unique single-exon gene, its genomic sequence, including the putative open reading frame, was used in place of the cDNA in subsequent experiments.

The crystal structures of several hexokinases have been determined from various organisms including yeast (Kuser et al. 2000) and *E. coli* (Lunin et al. 2004). Hexokinases generally have conserved amino acid residues for binding hexose and ATP (Bork et al. 1992, 1993; Kuser et al. 2000). Alignment of deduced amino acid sequences from the *OsHXK* cDNAs confirmed that all of them possessed the hexose and ATP-binding sites (Supplementary Fig. 1). Bork et al. (1992) found three additional conserved regions in hexokinases: connect 1 and connect 2, involved in putative hinge motion, and a helical domain. In rice hexokinases, the connect 1 and helical domains, respectively, are located in front of the conserved phosphate 2 and adenosine interaction regions. The C-terminal end of OsHXKs, adjacent to the adenosine interaction region A, corresponds to the connect 2 domain (Supplementary Fig. 1).

Molecular evolutionary analysis

We determined the phylogenetic relationship between rice hexokinases and those from different species in an effort to find a possible link between function and evolution among these enzymes. The rooted phyloge-

Table 1 Results of TBLASTN searches for rice hexokinase genes in the draft sequence of the rice genome

Gene name	Matched BAC/PAC clone	Chromosomal location (cM)	*AtHXK1*			*AtHXK2*		
			Identity (%)	Score (Bits)	*E* value	Identity (%)	Score (Bits)	*E* value
OsHXK1	AP004668	7 (~52)	53	496	5e-140	52	475	2e-133
OsHXK2	AC121365	5 (108.5)	69	132	2e-30	69	134	5e-31
OsHXK3	AP003412	1 (164.1)	52	118	5e-26	50	114	9e-25
OsHXK4	AP005257	7 (41.7)	54	136	1e-31	50	132	2e-30
OsHXK5	AC118284	5 (107.4)	66	136	8e-38	61	134	1e-37
OsHXK6	AP003768	1 (127.3)	65	160	1e-38	61	152	2e-36
OsHXK7	AC093954	5 (37.2)	62	133	1e-35	62	132	3e-35
OsHXK8	AP002743	1 (25.8)	63	135	3e-31	61	123	1e-27
OsHXK9	AP003292	1 (124.8)	60	153	1e-36	58	145	2e-34
OsHXK10	AC108874	5 (~70)	47	100	1e-20	45	91.3	2e-21

Values for identity (%) between sequences were determined by sequence alignment analysis using the CLUSTAL W program (Thompson et al. 1994)

netic tree, constructed using the neighbor-joining method, had several interesting features (Fig. 1). Plant hexokinases formed a major group, which was separate from yeast and animal hexokinases and divided into four subgroups. Group I was further subdivided into the dicot group I-a and the monocot group I-b. However, groups I and II, including an *Arabidopsis* hexokinase-like gene *At4g37840*, may be regarded as a single isolated group since their bootstrap value is relatively low. *AtHXK1* and *AtHXK2* were included in group I and were very similar to *OsHXK2* (Table 1, Fig. 1). *OsHXK4* fell into group III with the tobacco hexokinase *NtHXK2*, which is targeted to the chloroplast (Giese et al. 2005), suggesting that OsHXK4 may be a chloroplast stromal enzyme. In contrast to the other hexokinases, *OsHXK3* and *OsHXK10* have a unique insertion domain composed of about ten amino acids and belonging to group IV (Fig. 1, Supplementary Fig. 1). It seems that each of two hexokinase genes from rice and *Arabidopsis* that belong to the group IV was duplicated after the monocot–dicot divergence. The intron-less *OsHXK1* forms a branched terminal, instead of the basal group of one clade. Therefore, it appears that *OsHXK1* is a relatively recent gene resulting from a retrotransposition, and is not the ancestor of the other hexokinases. Notably, the hexokinase genes from various kingdoms, including plant, yeast, and animal, make up lineage-specific groups, suggesting that the rice and *Arabidopsis* hexokinase genes descend from a common ancestral hexokinase (Cárdenas et al. 1998).

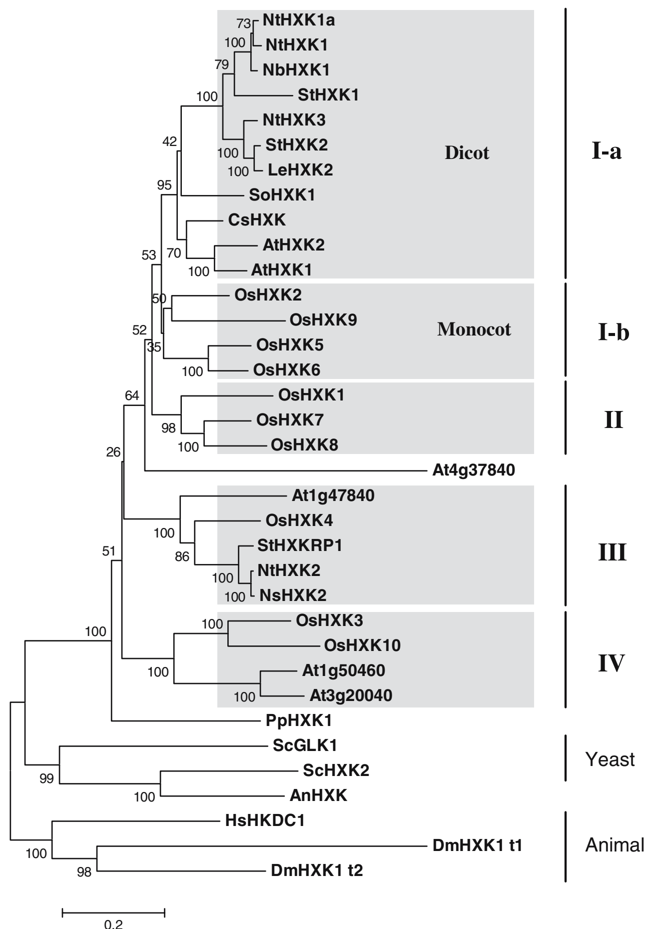

Fig. 1 Phylogenetic tree of hexokinase genes from various sources. The tree was constructed using MEGA version 3.0 (Kumar et al. 2004). The scale bar corresponds to a distance of 20 changes per 100 amino acid positions. Accession numbers are described in Materials and methods

Genomic structure and chromosomal location of *OsHXK* genes

The inferred genomic structures of the *OsHXK* genes were determined by aligning the cDNA sequences and the genomic sequences of the BAC/PAC clones obtained from the NCBI database (Fig. 2). The isolated full-length cDNA clones from the variety *Jinmi* perfectly matched the genomic sequences of BAC/PAC clones of the cultivar *Nipponbare*, except for several base-pair substitutions in the UTR. With the exception of the

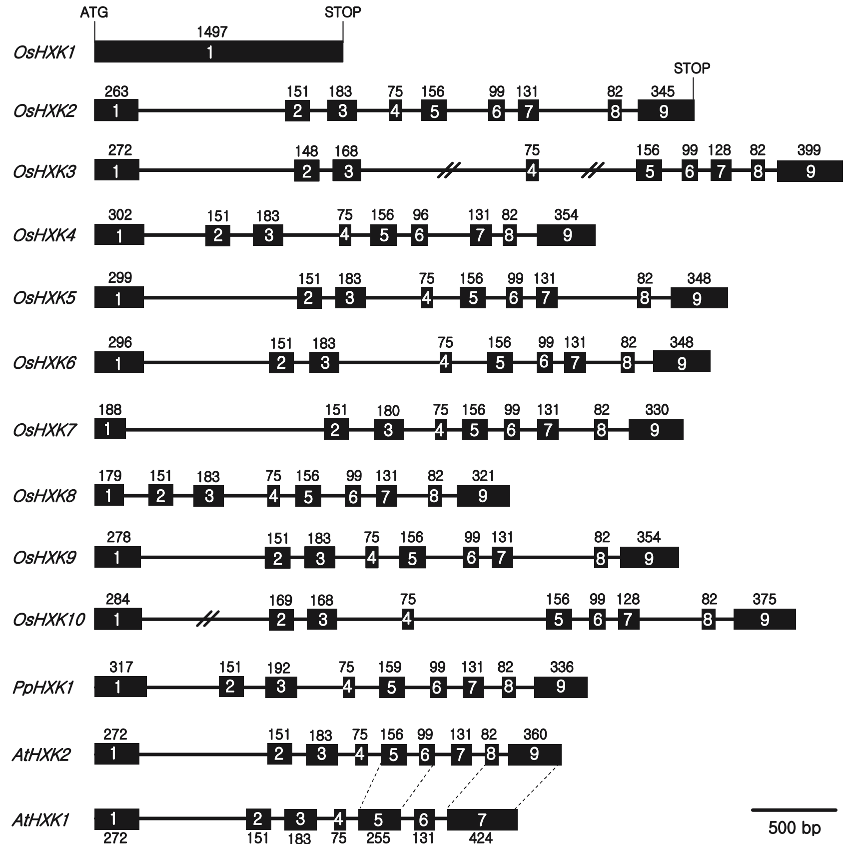

Fig. 2 Genomic structure of the *OsHXK* gene family. Exons are indicated by *black rectangles* and introns by *lines*. *Numbers* indicate the exon size in nucleotides. Exons are numbered within the *rectangles*. Accession numbers of genomic DNAs used to determine the structure of the *OsHXK* gene family are described in Materials and methods

intron-less *OsHXK1*, all of the *OsHXK* genes have a highly conserved structure composed of nine exons interrupted by eight introns. The genes possess nearly identical exon lengths, with the exception of the first and last exons. The exon/intron distribution of the rice hexokinase genes was the same as moss *PpHXK1* and *Arabidopsis AtHXK2*, whereas *AtHXK1* is composed of seven exons and six introns.

INE, a database integrating sequencing information and genetic and physical maps of the rice genome (Sakata et al. 2000), was used to determine the chromosomal locations of *OsHXK* genes. All of the genes mapped to one of three chromosomes: *OsHXK3, OsHXK6, OsHXK8*, and *OsHXK9* to chromosome 1; *OsHXK2*, *OsHXK5*, *OsHXK7*, and *OsHXK10* to chromosome 5; and OsHXK1 and *OsHXK4* to chromosome 7 (Table 1).

Analysis of *OsHXK* gene expression

The expression pattern of the *OsHXK* genes was examined by semi-quantitative RT-PCR using gene-specific primers, with the rice actin gene, *act1* transcript serving as the control. All *OsHXK* genes, except *OsHXK1* and *OsHXK10*, were expressed in all tested organs. *OsHXK10* was expressed only in flowers (Fig. 3a). Sucrose is unloaded in the sink rice seeds where it serves as a storage material, carbohydrate backbone, and energy source (Borisjuk et al. 2004; Lim et al. 2006). Since hexokinases are thought to play an important role in utilizing hexoses produced from sucrose hydrolysis during seed development (Koch 2004), the eight genes, *OsHXK2* through *OsHXK9*, expressed in immature seeds were analyzed further. Transcripts of four genes, *OsHXK2*, *OsHXK4*, *OsHXK5*, *OsHXK6*, and *OsHXK8*, were abundant in endosperms, whereas *OsHXK3* and *OsHXK7* were expressed preferentially in seed coats (Fig. 3b), suggesting that *OsHXK3* and *OsHXK7* have distinct roles during longitudinal growth of the seed coat and endosperm development. Weak *OsHXK9* expression was detected in both endosperms and seed coats (Fig. 3b).

To investigate the temporal expression of *OsHXKs* during seed development, RT-PCR analysis was performed using ovaries prior to pollination and seeds collected 1–15 DAF, with *18s rRNA* as the control (Fig. 3c). The transcript levels of *OsHXK2*, *OsHXK3*, *OsHXK4*, *OsHXK5*, *OsHXK6*, and *OsHXK9* increased gradually from the ovaries prior to pollination up to 5–6 DAF, following which it decreased. The transcript levels of *OsHXK3* and *OsHXK4* decreased dramatically at 9–10 DAF. In contrast, *OsHXK8* transcripts levels remained high during the starch-filling phase (9–15 DAF) and *OsHXK7* transcript levels were relatively constant at all stages.

Fig. 3 **a**–**c** Analysis of *OsHXK* gene expression by semi-quantitative RT-PCR. Transcript levels of a single gene were comparable in different tissue samples; however, transcript levels among the different genes were not comparable in the RT-PCR reactions. **a** Expression of *OsHXK* genes in rice leaf, root, flower, and immature seeds from 1–12-DAF caryopses. **b** Expression of *OsHXK* genes in endosperm and seed coat. The control transcript was rice actin, *Act1*. **c** Expression levels of *OsHXK* genes during rice seed development. Rice seeds were harvested over a time course of 1–15 DAF and from the ovaries prior to pollination. The control transcript was *18S rRNA*

To further examine the spatial distribution of the *OsHXK10* gene in flowers, RT-PCR analysis was performed using pistils, stamens, and paleae/lemmas dissected from mature flowers. The *OsHXK10* transcript was detected only in the stamens (Fig. 4a). The transcript was not detectable in *udt1* mutant flowers lacking mature pollen (Jung et al. 2005), suggesting that *OsHXK10* expression is pollen-specific (Fig. 4b).

Fig. 4 **a, b** *OsHXK10* gene expression in rice flowers. **a** Expression of the *OsHXK10* gene in various flower parts. **b** *OsHXK10* gene expression in wild-type and *udt1* mutant plants. The *udt1* knockout plant lacks pollen in the anther locules. Panicles were harvested in lengths of 0–2, 2–8, 8–15, 19–20.5, and 20.5–22 cm. The RT-PCR products were separated on an agarose gel, blotted to membranes, and hybridized with gene-specific probes

Effects of sugars on *OsHXK* gene expression

To examine the effects of soluble sugars on the expression of *OsHXK* genes, excised leaves with depleted endogenous sugars were treated with MS media containing 175 mM glucose, fructose, or mannitol (Fig. 5a). Treatment with mannitol did not affect *OsHXK* gene expression, indicating that the sugar effects were not due to osmotic stress. We included the mannitol treatment as a control in order to eliminate the usual induction caused by osmotic stress. RNA gel-blot analyses indicated that of the eight *OsHXK* genes expressed in leaves, the transcript levels of three—*OsHXK2*, *OsHXK5*, and *OsHXK6*—were increased markedly by the treatment with glucose or fructose, for 12 and 24 h (Fig. 5a, Supplementary Fig. 2). In contrast, *OsHXK7* was downregulated by these sugars, suggesting that this gene may have a distinct role (Fig. 5a, Supplementary Fig. 2). The remaining *OsHXK* genes did not respond significantly to glucose or fructose treatment.

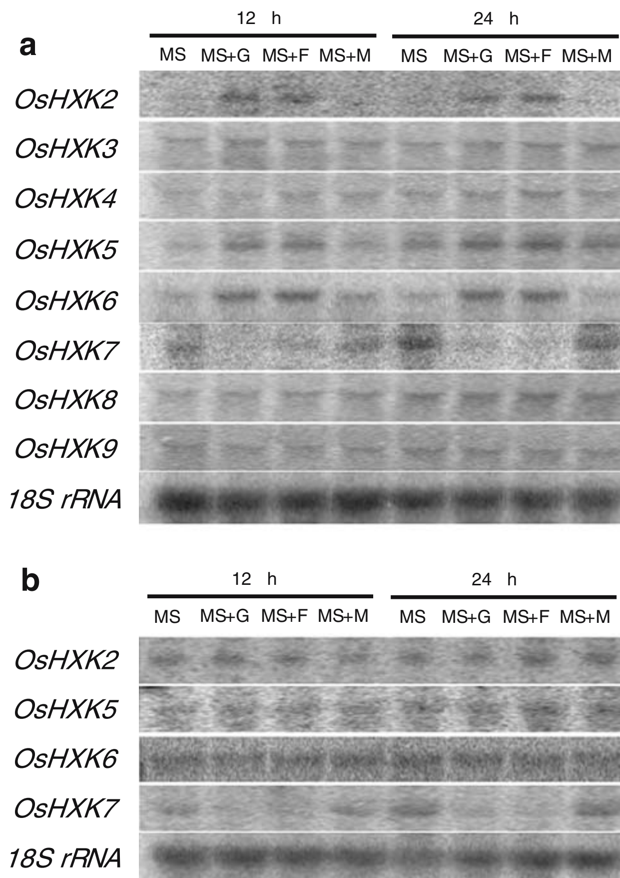

Fig. 5 a, **b** Effect of sugars on *OsHXK* gene expression in rice leaves and caryopses. **a** RNA gel-blot analysis of excised leaves after 12 and 24 h on MS media with or without glucose, fructose, or mannitol. **b** RNA gel-blot analysis of 1–2 DAF caryopses after 12 and 24 h on MS media with or without glucose, fructose, or mannitol. MS, MS media; MS + G, MS media + 175 mM glucose; MS + F, MS media + 175 mM fructose; MS + M, MS media + 175 mM mannitol. The control transcript was *18S rRNA*

The effects of soluble sugars on *OsHXK* gene expression were examined in immature seeds of 1–2 DAF plants (Fig. 5b). In this experiment, we examined the expression of the four *OsHXK* genes, *OsHXK2*, *OsHXK5*, *OsHXK6*, and *OsHXK7*, which were altered in rice leaves in response to glucose or fructose treatment. Twelve and 24 h after the treatment with glucose or fructose, the expression of *OsHXK7* was reduced significantly in immature seeds (Fig. 5b, Supplementary Fig. 2). However, the other *OsHXK* genes tested, *OsHXK2*, *OsHXK5*, and *OsHXK6*, were not modulated by these sugars (Fig. 5b, Supplementary Fig. 2), although they were significantly induced in leaves in response to glucose and fructose (Fig. 5a, Supplementary Fig. 2). It is likely that the high concentration of endogenous sugars in the immature seed prevented exogenous sugar induction of *OsHXK* gene expression. However, it is also possible that *OsHXK* gene expression is differentially regulated by sugars in leaves and seeds.

Complementation of hexokinase-deficient yeast

To examine hexokinase activity, the individual cDNA clones were used to complement the yeast triple mutant YSH7.4-3C (*hxk1*, *hxk2*, *glk1*) that lacks endogenous hexokinase activity. All of the yeast cells transformed with full-length *OsHXK* cDNAs, except for those transformed with *OsHXK4*, were able to grow on a selection medium containing glucose or fructose as the sole carbon source (Fig. 6). Yeast cells transformed with the empty pDR196 vector did not grow on this selection medium.

In the phylogenetic analysis, *OsHXK4* appeared to be very similar to *NtHXK2* that encodes a stromal hexokinase (Fig. 1). TargetP program (Emanuelsson et al. 2000) analysis predicted that the OsHXK4 protein was localized to the chloroplast, with the highest reliability class value 1 (data not shown). Therefore, the failure of the full-length *OsHXK4* cDNA to complement the yeast mutant could have been due to the presence of an N-terminus putative chloroplast-transit peptide. To determine whether the N-terminal 46-amino acid peptide interfered with OsHXK4 hexokinase activity, the yeast mutant was transformed with a cDNA corresponding to the mature OsHXK4 protein lacking the predicted chloroplast transit peptide (*OsHXK4-TP*). These transformants had hexokinase activity (Fig. 6), suggesting that OsHXK4 is a chloroplast-imported hexokinase. Therefore, all of the cloned *OsHXK* cDNAs encode hexokinases.

Subcellular localization of *OsHXK4* and *OsHXK7*

The differential subcellular localization of hexokinases may help to mediate their distinct, putative roles. Here, the subcellular localization of two *OsHXK*s, *OsHXK4* and *OsHXK7*, was examined in transgenic calli and

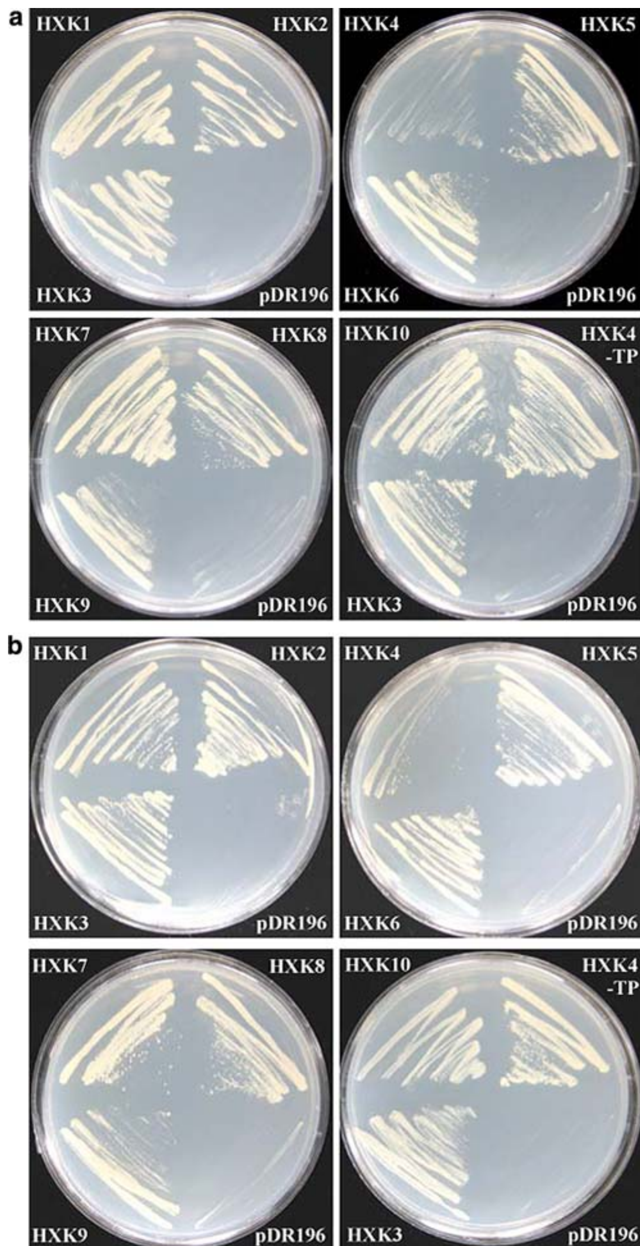

Fig. 6 a, **b** Complementation of the hexokinase-deficient yeast triple mutant YSH7.4-3C (*hxk1*, *hxk2*, *glk1*; De Winde et al. 1996) with *OsHXK* cDNAs. The transformed colonies were streaked and grown on a medium containing 2% D-glucose (**a**), D-fructose (**b**), or galactose as the sole carbon source and grown for 3 days at 30°C. The YSH7.4-3C mutant strain was transformed with the empty vector pDR196. HXK4-TP lacked N-terminal which encodes a putative transit peptide in *OsHXK4*. SD/-ura, synthetic defined minimal medium lacking uracil

plants expressing *GFP* fusion constructs (Fig. 7). *OsHXK4* localization was examined to determine whether it is expressed preferentially in chloroplast stroma, as predicted from the phylogenetic analysis, its N-terminal structure, and the yeast complementation experiment. The results indicated that the GFP fusion protein was indeed targeted to chloroplast stroma in transgenic calli and leaves (Fig. 7a, b), indicating that *OsHXK4* is a chloroplast-imported hexokinase.

Reduced expression of *OsHXK7* in response to sugar treatment in leaves and immature seeds was distinct from all other *OsHXKs* which were either induced or unaffected (Fig. 5). Thus, to elucidate its possible role, the subcellular localization of OsHXK7 was determined. This revealed that OsHXK7::GFP proteins were localized exclusively in the cytosol of transgenic calli, leaves, and roots (Fig. 7c–e). Thus, it is suggested that in rice plants, *OsHXK7* functions in the utilization of free cytosolic hexoses.

Discussion

Rice hexokinase gene family

We here cloned ten cDNAs belonging to the rice hexokinase gene family and verified that all of them encode proteins with hexokinase activity. Plant hexokinase genes are usually isolated by functional complementation of a yeast mutant. Previously, two *Arabidopsis* hexokinase cDNAs were isolated by complementation of a yeast triple mutant (*hxk1*, *hxk2*, *glk1*) (Dai et al. 1995). Similarly, Jang et al. (1997) identified two hexokinase genes, *AtHXK1* and *AtHXK2*, from an expression library in the yeast *hxk1*, *hxk2* double mutant DBY2219. In the present study, we demonstrated that the isolated *OsHXK* cDNAs function as hexokinases in the yeast triple mutant YSH7.4-3C (*hxk1*, *hxk2*, *glk1*). Glucokinases, which usually belong to hexokinase genes in many higher plants, phosphorylate only glucose. The deduced amino acid sequences of the OsHXK proteins differ significantly from rice fructokinase genes (Jiang et al. 2003). In addition to the OsHXK proteins' ability to confer fructose and glucose utilization in the yeast *hxk1*, *hxk2*, *glk1* triple mutant, comparative analysis of their amino acid sequences indicates that they are hexokinases rather than fructokinases or glucokinases. It has been reported that *Arabidopsis* has no more than six hexokinase genes, including four uncharacterized hexokinase-like genes (Dai et al. 1999; Jang et al. 1997). Thus, it appears that the rice hexokinase gene family is larger than that of *Arabidopsis*.

Systematic searches of the rice genome annotation database (http://www.ricegaas.dna.affrc.go.jp/rgadb/) revealed that rice most likely has three fructokinase genes, *OsFKI* (AF429948), *OsFKII* (AF429947) and a predicted cDNA (LOC_Os06g12600) (data not shown). Two fructokinases, OsFK1 and OsFKII, were found to utilize fructose as substrates (Jiang et al. 2003). In the phylogenetic analysis, OsFKs appeared to form a major group that is independent of the rice hexokinase family, supporting the hypothesis that the hexokinase and fructokinase groups are quite distinctive in terms of evolution as well as in substrate-specificity of enzyme (Bork et al. 1993).

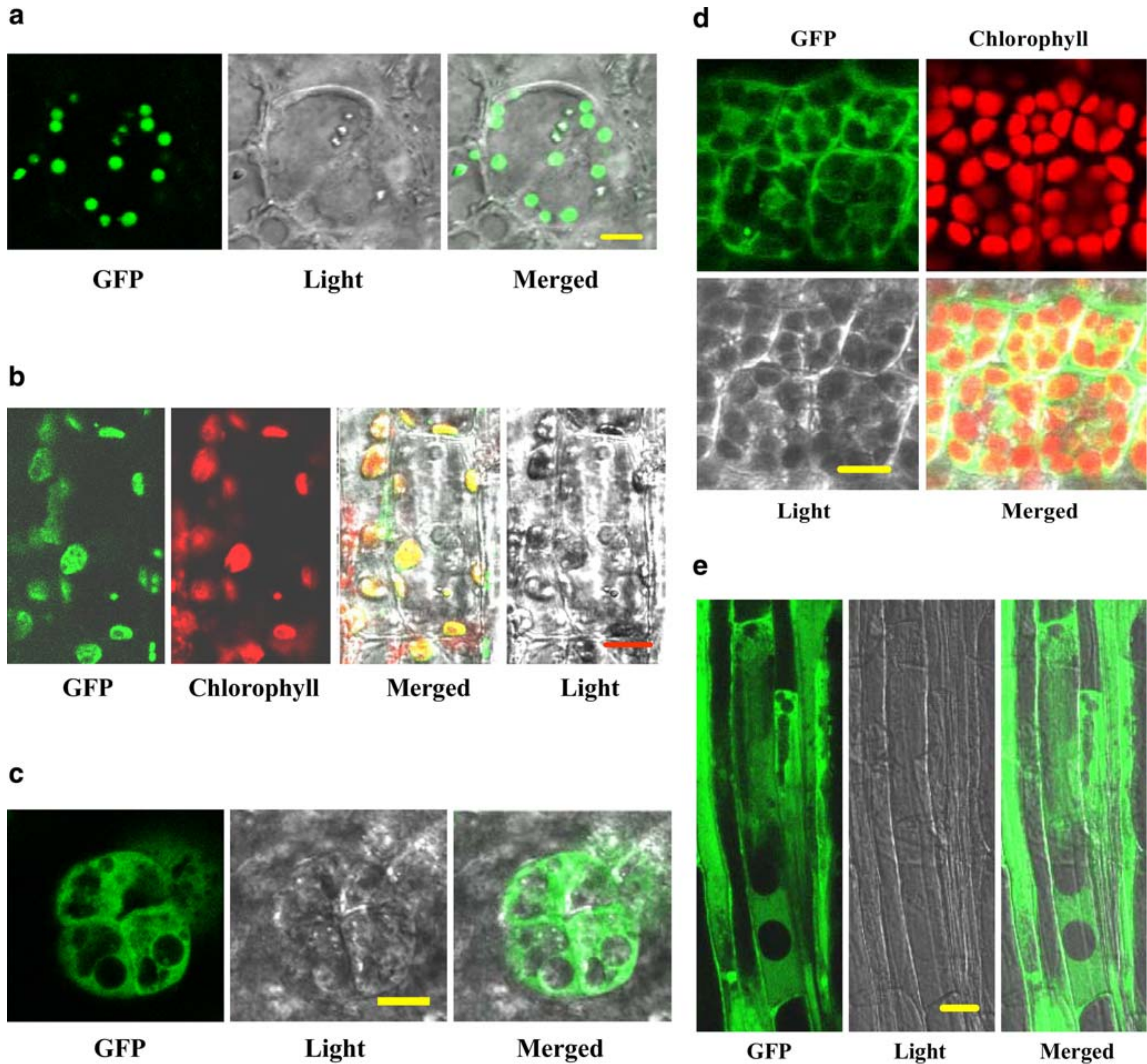

Fig. 7 a–e Subcellular location of the OsHXK::GFP fusion proteins in calli, leaves and roots of transgenic rice plants. **a** HXK4::GFP in transgenic callus. **b** HXK4::GFP in leaf epidermal cells of transgenic rice plants. **c** HXK7::GFP in transgenic callus. **d**, **e** HXK7::GFP in leaf epidermal cells **d** and roots e of transgenic rice plants. Fluorescent signals of GFP and chlorophyll were examined under a confocal microscope (LSM 510 META, Carl Zeiss, Jena, Germany). Chlorophyll autofluorescence in leaf epidermal cells was used as a chloroplast marker. GFP signal is indicated in *green*, and chlorophyll autofluorescence is shown in *red*. GFP fluorescence merged with light image and/or autofluorescence image. *Bars* 5 μm in **a** and 10 μm in **b**, **c**, **d** and **e**

Expression of *OsHXKs* in rice organs

With the exception of *OsHXK1* and *OsHXK10*, transcripts of all rice *OsHXK* genes, were detectable in rice leaf, root, flower, and immature seed, suggesting that each OsHXK has either a unique or redundant function in various rice organs. It is likely that OsHXKs have different subcellular localizations, and thereby play distinct roles. Interestingly, most *OsHXK* genes, with the exception of *OsHXK8*, are highly expressed in early seed development. Previously, high levels of invertase activity and a high hexose-to-sucrose ratio were found in young cereal seeds, including maize (Cheng and Chourey 1999), rice (Hirose et al. 2002), and barley (Borisjuk et al. 2004). Notably, in developing seeds, the shift from cell division to storage product accumulation is coincident with the change from invertase to sucrose synthase-mediated sucrose metabolism and from high hexose-to-sucrose to high sucrose-to-hexose (Borisjuk et al. 2004; Koch 2004; Lim et al. 2006). Thus, our observations suggest that OsHXKs, except for OsHXK8, play a role in early embryo and endosperm development during the

longitudinal growth of rice seeds. In contrast, OsHXK8 may function in the endosperm in the filling phase of rice seed development. In support of this theory, *OsHXK8* transcripts were much more abundant in the endosperm than in the seed coat.

Interestingly, *OsHXK10* expression appeared to be pollen-specific. Pollen development is a complex process that requires carbohydrate import from the apoplast during maturation, germination, and pollen growth. Pollen-specific genes involved in carbon metabolism have been identified in various plant species (Goetz et al. 2001). Goetz et al. (2001) reported that the tobacco cell-wall invertase, Nin88, has a critical role in pollen development and that the expression of a *Nin88* antisense construct induces male sterility. Sugar transporters are involved in the uptake of carbohydrates during pollen development (Williams et al. 2000). We have recently identified a pollen-preferential cell-wall invertase and a putative sugar transporter in rice (data not shown). It will be interesting to investigate whether the coordinated expression of these pollen-specific genes is essential for carbon metabolism during rice pollen development.

Regulation of *OsHXK* expression by sugar

OsHXK2, *OsHXK5*, and *OsHXK6* expression appeared to be significantly upregulated in rice leaves by glucose and fructose. Similar results were obtained in leaves and immature seeds treated with sucrose (data not shown). Global transcription profiling in *Arabidopsis* revealed that glucose regulates a broad range of genes which are associated with carbohydrate metabolism, signal transduction, and metabolite transport (Price et al. 2004). In that study, expression of two hexokinases (*AtHXK1* and *AtHXK2*) was highly induced by glucose and this effect was eliminated by the protein synthesis inhibitor cycloheximide (CHX). This suggests that the induction of hexokinase genes by glucose is a multistep response requiring de novo protein synthesis, presumably caused by the change of metabolic activities.

In the present study, *OsHXK7* gene expression appeared to be reduced by sugars in leaves and immature seeds. In yeast, two sugar kinases, *ScHXK1* (yeast hexokinase1) and *ScGLK1* (yeast glucokinase1), were repressed by glucose (Herrero et al. 1995). The glucose-repression mechanism in yeast is hypothesized to be regulated by the general glucose repressor Mig1, which is activated via the inhibition of Snf1 kinase activity. The Mig1 complex exerts the repression of diverse gene families and their family-specific transcriptional inducer genes, which in turn leads to a repression of downstream genes (Rolland et al. 2002b). In *Arabidopsis*, many transcription factors were repressed by glucose (Price et al. 2004), suggesting that a similar glucose-repression mechanism may be present in plants.

Several other plant hexokinases have been implicated in glucose sensing and signaling that mediate the repression of genes involved in photosynthesis, the glyoxylate cycle and synthesis of α-amylase. The repression is blocked by the addition of hexokinase-specific competitive inhibitors (Jang et al. 1997; Rolland et al. 2002a). In the rice embryo, transcription of the *RAmy3D* gene was tightly repressed by sugar, and treatment with glucosamine, a hexokinase inhibitor, relieved these sugar-suppressive effects (Umemura et al. 1998). Similarly, also in rice embryos, two hexokinase isoforms were shown to be inhibited by hexokinase inhibitors mannoheptulose and glucosamine (Guglielminetti et al. 2000). These combined observations suggest that hexokinases are involved in the sugar-sensing process in rice embryos. To address whether a rice hexokinase gene(s) has a conserved function in sugar sensing and signaling, it will be valuable to determine whether *gin2*, a null mutant of *AtHXK1*, is complemented by either wild-type OsHXKs or modified OsHXKs that lack hexokinase catalytic activity.

Sugar-induced gene promoters have been functionally dissected (Rolland et al. 2002a; Rushton et al. 1995; Sun et al. 2003). This revealed that a WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by its binding to the sugar-responsive element (SURE) and the W-box of the *iso1* (encoding isoamylase1) promoter (Sun et al. 2003). Similarly, in cereals, WRKY proteins have been isolated that bind the promoters of α-*Amy2* genes (Rushton et al. 1995). We inspected the 1.5-kb region upstream of the translation start codon for *OsHXK* genes (data not shown). This revealed that four *OsHXKs*, *OsHXK2*, *OsHXK5*, *OsHXK6*, and *OsHXK7*, whose expression was altered by sugars, carry an average of four W-boxes. Whether these elements contribute to the regulation of *OsHXKs* is an issue that remains to be addressed.

Subcellular localization of OsHXKs and their possible roles

We identified a chloroplast stromal-type hexokinase, OsHXK4, the first of this type to be recognized in a monocot. It has been suggested in the moss *Physcomitrella patens* that the stromal hexokinase converts hexose to hexose phosphate in the chloroplast for use in starch and fatty acid syntheses and in the pentose phosphate pathway, when the energy supply is limited such as during the night and in sink organs (Olsson et al. 2003). Within the plastid, hexoses are derived from hydrolytic starch breakdown or imported from the cytosol. The plastidic glucose translocator (pGlcT) is thought to import extra-plastidial hexoses into plastids in heterotrophic tissues, as well as to export hexoses produced from starch degradation in the dark (Fischer and Weber 2002). A rice *pGlcT* was observed to be mostly expressed in rice seeds but lesser in leaves (Toyota et al. 2006), suggesting the demand for stromal hexokinase activity when utilizing hexose in the plastid of sink organs.

It also has been suggested that a chloroplast stromal hexokinase is required for stomatal opening (Ritte and Raschke 2003). Studies indicate that stomatal opening in *Vicia faba* is brought about by elevated turgor due to the accumulation of organic osmotica including hexose phosphates that are caused primarily by starch breakdown in guard cell chloroplasts (Ritte et al. 1999; Ritte and Raschke 2003). The tobacco stromal hexokinase *NtHXK2* is highly expressed in guard cells, in root tips, and cells of the vascular starch sheath and xylem parenchyma (Giese et al. 2005). These data suggest that the stromal hexokinase is more likely involved in starch degradation than in starch synthesis. Further investigations are underway to clarify the role of the chloroplast-imported hexokinase, OsHXK4.

OsHXK7 appeared to be a cytosolic hexokinase. Cytoslic hexoses are thought to be generated from sucrose by neutral invertase or sucrose synthase (Koch 2004) and vacuolar or apoplastic hexoses produced by sucrose hydrolysis are also transported to the cytosol (Moore et al. 1999). These ultimately are phosphorylated by a cytosolic hexokinase. Recent studies in *Arabidopsis* have shown that maltose is the major form of carbon exported from the chloroplast at night (Sharkey et al. 2004; Weise et al. 2004). Glucose derived from the hydrolytic starch breakdown via maltose also requires cytosolic hexokinase activity (Sharkey et al. 2004).

In maize seedling roots, hexokinase inhibitors preferentially inhibited the membrane-bound hexokinases but not the cytosolic hexokinases (Da-Silva et al. 2001). The authors proposed that the cytosolic hexokinase activity is involved mainly in the glycolysis process, since a regulatory role for hexokinase in plant sugar sensing is blocked by hexokinase inhibitors (Rolland et al. 2002a). Notably, cytosolic expression of a yeast-derived invertase in transgenic tobacco increases sugar levels but does not suppress the expression of photosynthetic genes, suggesting that the sugar signal is sensed at a membrane location rather than in the cytosol (Herbers et al. 1996). Thus, it is likely that OsHXK7 participates in cytosolic metabolism or sucrose biosynthesis through the removal of free hexoses in the cytosol. Nevertheless, this does not exclude the possibility that its role is in sugar sensing. To clarify its function in rice plants, characterization of a null mutant of *OsHXK7* is underway.

Amino acid sequence alignment of OsHXKs and other known HXKs revealed that OsHXK3 and OsHXK10 possess an N-terminal membrane anchor region that is shared in the spinach SoHXK1 localized to the outer envelope membrane of plastids (Olsson et al. 2003; Wiese et al. 1999). Therefore, OsHXK3 and OsHXK10 are likely membrane-bound proteins, although this has not been conclusively demonstrated. Determining the subcellular localization of the remaining OsHXKs will help to elucidate their function(s).

In this study, we have isolated and characterized all of the rice hexokinase genes. Transgenic rice plants or those with knocked-out rice hexokinase genes should prove invaluable for more detailed characterization of hexokinase functions in carbon metabolism and sugar sensing.

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