Deficiency of rice hexokinase HXK5 impairs synthesis and utilization of starch in pollen grains and causes male sterility

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Received 20 August 2019; Editorial decision 18 September 2019; Accepted 10 October 2019

Editor: Dabing Zhang, Shanghai Jiao Tong University, China

Abstract

There is little known about the function of rice hexokinases (HXKs) in planta. We characterized hXk5-1, a Tos17 mutant of OsHXK5 that is up-regulated in maturing pollen, a stage when starch accumulates. Progeny analysis of self-pollinated heterozygotes of hXk5-1 and reciprocal crosses between the wild-type and heterozygotes revealed that loss of HXK5 causes male sterility. Homozygous hXk5-1, produced via anther culture, and additional homozygous hXk5-2, hXk5-3 and hXk5-4 lines created by CRISPR/Cas9 confirmed the male-sterile phenotype. In vitro pollen germination ability and in vivo pollen tube growth rate were significantly reduced in the hXk5 mutant pollen. Biochemical analysis of anthers with the mutant pollen revealed significantly reduced hexokinase activity and starch content, although they were sufficient to produce some viable seed. However, the mutant pollen was unable to compete successfully against wild-type pollen. Expression of the catalytically inactive OsHXK5-G113D did not rescue the hXk5 male-sterile phenotype, indicating that its catalytic function was responsible for pollen fertility, rather than its role in sugar sensing and signaling. Our results demonstrate that OsHXK5 contributes to a large portion of the hexokinase activity necessary for the starch utilization pathway during pollen germination and tube growth, as well as for starch biosynthesis during pollen maturation.

Keywords: Hexokinase, male sterility, Oryza sativa, pollen germination, pollen tube growth, starch.

Introduction

In flowering plants, pollen formation and subsequent pollination and fertilization are crucial steps for sexual reproduction. Pollen development from the microspore involves a series of coordinated cellular events, and the resulting mature pollen has a specialized function to germinate quickly, to produce a pollen tube derived from the vegetative cell that has polar growth, and to deliver two sperm cells into the embryo sac for double-fertilization (Johnson and Preuss, 2002; Lord and Russell, 2002; Dresselhaus et al., 2016). In the major cereal crop rice (Oryza sativa), sexual reproduction is the stage with the greatest sensitivity...
to environmental stress. For example, low or high temperatures can lead to defects in pollen development, germination, and tube growth, which affect spikelet fertility and grain yield (Andaya and Mackill, 2003; Jagadish et al., 2010; Coast et al., 2016).

Rice pollen loses germination activity in a relatively short period of time. For example, pollen viability drops rapidly by nearly 50% between 6 min and 20 min after anther dehiscence (Khatun and Flowers, 1995; Fu et al., 2001; Song et al., 2001; Zik and Irish, 2003). Highly efficient and rapid pollen germination and tube growth are critical for normal fertilization and spikelet fertility (Endo et al., 2009; Jagadish et al., 2010; Rang et al., 2011). Therefore, it is important to produce and maintain robust pollen grains for full fertility and complete seed set in rice. Despite its importance, little is currently known about the detailed carbohydrate metabolic pathway that is active during the late stages of pollen grain development when starch biosynthesis and degradation mainly occur.

In common with the other major cereal crops maize, wheat, and barley, rice utilizes starch as the main storage reserve in mature pollen grains. Starch is degraded as soluble sugars and used for supplying energy and carbon skeletons. In addition, it is also used for osmotic homeostasis in order to ensure pollen germination and tube growth, thus supporting proper pollination and fertilization (Dickinson, 1968; Wen and Chase, 1999; Datta et al., 2002; Hirose et al., 2010; Rounds et al., 2011; Hepler et al., 2013; Lee et al., 2016; Wu et al., 2016). Insufficient starch biosynthesis during the process of pollen development is responsible for male sterility in these crop species. For example, mutations in rice Plastidic Phosphoglucomutase and ADP-Glucose Pyrophosphorylase reduce starch content and result in male sterility (Lee et al., 2016). In addition, degradation of starch in pollen grains through the expression of α-amylase induces male sterility in maize, which provides an experimental strategy for creating male sterility (Wu et al., 2013).

Relatively few genes involved in carbohydrate metabolism whose mutations affect pollen germination and tube growth have been functionally characterized. For example, mutations in rice Sucrose Transporter1 (SUT1) lead to male sterility. The pollen grains of the sut1 mutant normally accumulate starch during development, but do not germinate and participate in fertilization with the ovule (Hirose et al., 2010; Eom et al., 2012, 2016). Disruption of rice Sucrose Phosphate Synthase1 (SPS1) results in sterile pollen grains (Hirose et al., 2014). The sps1 mutant accumulates sufficient starch in the pollen, but its germination efficiency is reduced to half that of the wild-type, which suggests that OsSPS1 is essential in pollen germination.

Hexokinase (HXK) catalyses the first irreversible step of hexose metabolism by phosphorylating hexoses into hexose-6-phosphate. In sink organs, including pollen, this reaction directs the cleavage products of imported sucrose into the starch biosynthesis pathway. The HXK catalysis reaction also initiates all the physiologically relevant hexose utilization pathways, including glycolysis (Cho et al., 2006a, 2006b, 2009; Claeyssen and Rivoal, 2007). In rice, there are 10 HXK isofoms (Cho et al., 2006a), which raises questions about the specific roles of each one. It has been shown that different HXKs are localized to different subcellular compartments, such as the cytosol, mitochondria, and chloroplasts (Cheng et al., 2011). Few OsHXK genes have been examined in loss-of-function mutants. For example, the cytosolic hexokinase HXK7 appears to play an important role in O2-deficient germination since the hxx7 mutant shows slow germination in anoxic conditions (Kim et al., 2016). In addition, rice HXK10-RNAi lines are male-sterile, probably due to a defect in anther dehiscence (Xu et al., 2008).

The OsHXK5 protein has been detected in proteomic studies on germinating pollen grains (Dai et al., 2007), implying that it functions during pollen germination and tube growth. We have previously found that OsHXK5 is primarily localized in mitochondria, and possibly in the nucleus, with a portion functioning as a sugar sensor (Cho et al., 2009). Here, we isolated and characterized mutant alleles of OsHXK5, and genetic, phenotypic, and biochemical analysis of these mutants revealed a novel function of hexokinase that is essential for pollen development, germination, and tube growth in rice.

### Materials and methods

#### Plant material

The *Oryza sativa* subsp. *japonica* wild-type rice cultivars Dongjin (DJ) and Nipponbare (NP) together with the mutants were grown in a greenhouse under a day/night cycle of 14/10 h at 30°C with ~80% humidity. Anthers from wild-type (Dj) plants were harvested at different developmental stages for quantitative RT-PCR (qRT-PCR) analysis.

#### Isolation and production of the OsHXK5 mutants

The OsHXK5 (LOC_Os05g44760) mutant allele hxxk5-1 was isolated from the Génoplante Insertion Line Library through a screen of the Signal RiceGE (Rice Functional Genomic Express Database; http://signal.salk.edu/cgi-bin/RiceGE) (Sallaud et al., 2004). Genotypes of the hxxk5-1 mutant were determined by genomic DNA PCR using the Tbs17-specific T1 primer (5'-CCAGTCCATTGGATCGTGATGTTCTGTATATGATAC-3') and the OsHXK5 gene-specific F1 (5'-TATAAGGTTGCTCTAAGAAAGCTA-3') and R1 (5'-AAAAAGATGGTGAAGATTTCAAGG-3') primers.

The other three mutant alleles, hxxk5-2, hxxk5-3, and hxxk5-4, were generated using the CRISPR/Cas9 system. To find an effective protospacer adjacent motif (PAM) and avoid any off-target effects, we screened possible target sequences using the CRISPRdirect program (http://crispr.dbcls.jp/; Naito et al., 2015). Guide RNA (5'-GCCGGGCGATCTCGGACGCCA-3') was cloned into an entry vector, pOs-sgRNA, and then cloned into a destination vector, pH-Ubi-ca9-7, using the Gateway system (Miao et al., 2013; Lee et al., 2016). The resulting vector was transformed into the wild-type (Dj) by Agrobacterium mediation (Jeon et al., 2000). Genotypes of the hxxk5-2, hxxk5-3, and hxxk5-4 were determined by Sanger sequencing of the target PAM site. PCR amplons were generated using gene-specific primer sets (5'-GGTAAAACCCCAAGCTCAGT-3' and R2 (5'-AAAGTCACGGTGCTGTTAATGTTG-3') primers, purified, and subsequently subjected to DNA sequencing.

#### RNA isolation and RT-PCR analysis

Total RNA was prepared from leaves, pollen grains, and anthers using Trizol reagent, and was reverse-transcribed using the ReverTra Ace qPCR RT Master Mix cDNA Synthesis kit (Toyobo, Tokyo, Japan). To evaluate the expression patterns of OsHXKs, we prepared samples from anthers at four different developmental stages. First-strand cDNA was used in PCR reactions with gene-specific and control primers. All primers for qRT-PCR are listed in Supplementary Table S1 at JXB online.
Anther culture

Anthers were collected from spikelets at a stage when the distance between the flag leaf and the penultimate leaf was 3–5 cm. The anthers were then cultured on modified N\textsubscript{0} medium to induce calli and subsequently plants, as previously described (Eom et al., 2016).

Flow cytometry analysis

Samples of ~20 mg of leaves were finely chopped with a clean razor blade in 1 ml of ice-cold Tris-MgCl\textsubscript{2} buffer (0.2 M Tris, 4 mM MgCl\textsubscript{2}, 0.5% Triton X-100, pH 7.5) in a glass Petri dish on ice (Pfoesser et al., 1995). Nuclei were stained in 50 µg l\textsuperscript{-1} propidium iodide solution with 50 µg l\textsuperscript{-1} RNase, filtered through a 40-µm cell strainer, and kept on ice. Flow cytometry was performed with a medium flow rate in a FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA, USA), and the data acquired were analysed using the BD FACSDiva software. An FL2 detector was used to measure fluorescence, and phycoerythrin, peridin chlorophyll protein, forward scatter, and side scatter parameters were used for analysis according to the manufacturer’s instructions.

Pollen staining and in vitro germination assays

Mature pollen grains were placed directly into 10% Lugol’s solution for starch staining. Pollen germination was performed on artificial media containing 20% sucrose, 0.32 mM H\textsubscript{2}BO\textsubscript{3}, 1 mM CaCl\textsubscript{2}, and 1% agarose under high humidity conditions (Kariya, 1989; Hirose et al., 2014). After 30 min, germinated pollen grains were observed using a BX61 microscope (Olympus), and were stained with 10% Lugol’s solution.

In vivo pollen-tube growth assays

Spikelets were collected at various times after the panicles were fully pollinated, and fixed overnight with 3:1 (v/v) mixture of absolute ethanol and glacial acetic acid. The spikelets were then washed in a vial of distilled water. Dissected ovaries were transferred to 2 N NaOH, treated over-night to clear the tissue, and then stained for 5 h with 0.2% aniline blue and glacial acetic acid. The spikelets were then washed in a vial of distilled water. Dissected ovaries were transferred to 2 N NaOH, treated overnight to clear the tissue, and then stained for 5 h with 0.2% aniline blue and glacial acetic acid. The spikelets were then washed in a vial of distilled water. Dissected ovaries were transferred to 2 N NaOH, treated overnight to clear the tissue, and then stained for 5 h with 0.2% aniline blue and glacial acetic acid.

Measurement of hexokinase activity

Samples of ~3 mg of anthers containing mature pollen grains were resuspended in 100 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.6), 5% (v/v) glycerol, 5 mM EDTA, 5 mM DTT, 1× proteinase inhibitors, and 1 mM PMSF. The samples were collected at the bottom of a tube by centrifugation at 1000 g for 5 min at 4 °C, and then ground on ice using a pestle in a 1.5-ml tube. Soluble proteins were obtained by centrifugation at 15 000 g for 10 min at 4 °C and used for the enzyme assay. Hexokinase activity was measured using a NAD(P)H-coupled enzymatic method (Cho et al., 2009).

Determination of starch and soluble sugars

Samples of ~3 mg of anthers containing mature pollen grains were harvested at the end of the light period from mature panicles. The soluble sugars glucose, fructose, sucrose, and insoluble starch were measured using NAD(P)H-coupled enzymatic tests in the ethanol soluble and insoluble fractions as described by Lee et al. (2008). The measured metabolite contents were normalized to the anther fresh weight.

Production of transgenic rice expressing catalytically inactive OxHXK5

To introduce a catalytically inactive mutant form of OsHXK5 into the hxs5 null-mutant, we cloned the OsHXK5–G113D mutant form (Cho et al., 2009) between the maize Ubiquitin1 promoter and four Myc tag sequences, followed by the Nos terminator of the Gateway binary vector, pJJ4385, which contains the BASTA resistance gene as a selection marker. The resulting vector was transformed into hxs5-1 by Agrobacterium mediation (Jeon et al., 2000). To detect the OsHXK5–G113D–Myc protein in leaves and in anthers with mature pollen, protein gel-blot analysis was performed using the Myc antibody (Rabbit anti-c–myc antibody; A190-105A, Bethyl Laboratories, Montgomery, TX, USA) (Lee et al., 2008). Six lines were selected and were designated as OsHXK5\textsuperscript{G113D}.

Results

Preferential expression of HXK5 during late stages of pollen development

We have previously identified 10 rice hexokinases and shown that they are expressed in various tissues, including leaves, roots, seed, and flowers (Cho et al., 2006a). To identify HXKs that are expressed during late stages of pollen development and germination, we examined six Affymetrix rice micro-array data series prepared from developing anthers and pollen grains in the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) (Nguyen et al., 2016) (Supplementary Fig. S1). The analysis showed that OsHXK5 (LOC_Os05g44760) is expressed throughout all stages but is specifically expressed at high levels during the mature pollen and pollen germination stages. In contrast, expression of OsHXK2 (LOC_Os05g44590) was detectable only until the tricellular pollen stage, and expression of the other HXKs was barely detected during anther and pollen development. We were not able to examine expression of OsHXK6 (LOC_Os01g53930), a close homolog gene of OsHXK5, because the OsHXK6 probe was unavailable in the public array data (Supplementary Fig. S1).

To confirm expression of HXK5, we performed qRT-PCR analysis on anthers collected at different developmental stages from meiosis to mature pollen (Fig. 1A). As the anthers matured, expression of HXK5 gradually increased and was highest at the mature pollen stage, which was consistent with the micro-array data. In our analysis, HXK6 appeared to be expressed at relatively low levels during all stages. In contrast, HXK2 and HXK10 were expressed weakly at the mature stage, and the other HXKs were not expressed at any of the stages. Further analysis in germinating pollen grains indicated relatively high expression of HXK5 and relatively low expression of HXK6 (Fig. 1B). This suggested that HXK5 plays an important role during the pollen development and germination stages in rice.

Isolation and progeny analysis of the OsHXK5 mutant

To understand the in vivo function of OsHXK5, we isolated hxs5-1 from the Génoplante Insertion Line Library (Sallaud et al., 2004). The mutant allele harbored a Tos17 retrotransposon in the ninth exon of OsHXK5 (Fig. 2A). We performed genomic DNA PCR analyses using gene- and Tos17-specific primers to isolate homozygotes from the segregating progeny of self-pollinated heterozygous hxs5-1 mutant plants. This showed no homozygous Tos17 insertion mutant line (hxs5-1/hxs5-1); all appeared to be either wild-type (NP) or heterozygote (HXS5/hxs5-1; He) (Fig. 2B). The progeny segregation ratios from self-pollinated heterozygous plants were nearly 1:1:0 for the wild-type, heterozygote, and homozygote plants,
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respectively (47:45:0; Table 1). This clearly suggested a defect in either the male or female gametophyte of the hxk5-1 mutant allele during the process of genetic inheritance.

**Genetic transmission analysis of the hxk5-1 mutant allele**

To determine whether the gametophytic defect was caused by the male or female component, we performed reciprocal crosses between wild-type and hxk5-1 heterozygotes (Table 2). When the hxk5-1 heterozygote was used as a pollen donor, none of the 45 F1 plants yielded the heterozygous genotype; all were wild-type. However, when the heterozygous line was used as a female donor, the genotypes of 44 F1 plants were nearly 1:1 for the wild-type (23) and the hxk5-1 heterozygote (21). This clearly indicated that the hxk5-1 mutant pollen was sterile and suggested a crucial role of OsHXK5 in male fertility.
Production and characterization of the hox5-1 homozygous mutant

To further examine the functions of HXK5 during rice pollen development, pollination, and fertilization, we produced homozygous plants via anther culture of the hox5-1 heterozygote. Unsurprisingly, approximately half of the regenerated plants were wild-type and the other half were hox5-1 homozygous mutants. The regenerated hox5-1 homozygous plants were confirmed by genomic DNA PCR (Fig. 3A). In the RT-PCR analysis of the homozygous mutants, the HXK5 transcript was not detected in leaves or in anthers harboring pollen, whereas the transcript was abundant in both these tissues in the wild-type plants (Fig. 3B). Homozygous plants produced via anther culture grew normally and were similar to the wild-type during vegetative growth (Fig. 3C). In flow cytometry analysis, the hox5-1 homozygous plants showed the same diploid levels as the wild-type plants, representing the 2C DNA content (Supplementary Fig. S2A). However, the homozygous hox5-1 plants bore a limited number of seeds, resulting in fertility of ~5–10% with a mean of 7.4% in our greenhouse growth conditions (Fig. 4A, B). Notably, the self-pollinated seeds produced from the hox5-1 homozygous mutant were confirmed to be homozygotes (Supplementary Fig. S2B), which excluded cross-pollination with the wild-type.

Table 1. Segregation in self-pollinated rice hox5-1 heterozygous mutant plants

| Parent plant          | Percentage of observed/expected genotypes of progeny (actual numbers recorded) |
|-----------------------|---------------------------------------------------------------------------------|
| HXK5/hox5-1           | Wild-type 51.1/25 (47/92) HXK5/hox5-1 48.9/50 (45/92) hox5-1/hox5-1 0/25 (0/92) |

Genotypes of progeny plants were determined by PCR using gene- and Tos17-specific primers.

Table 2. Results of reciprocal crosses between the rice wild-type and hox5-1 heterozygous plants

| Genetic cross          | Percentage of observed/expected genotypes of progeny (actual numbers recorded) |
|------------------------|--------------------------------------------------------------------------------|
| Paternal               | Wild-type HXK5/hox5-1 HXK5/hox5-1 Wild-type 100/50 (45/45) 0/50 (0/45) |
| Maternal               | Wild type HXK5/hox5-1 52/50 (23/44) 48/50 (21/44) |

Genotypes of F1 plants were determined by PCR using gene- and Tos17-specific primers.

Fig. 3. Molecular and phenotypic analysis of rice hox5-1 mutants produced via anther culture. (A) Genotype analysis of the hox5-1 homozygous mutant. An F1/R1 primer set was used for the wild-type (WT) HXK5 allele (top), and an F1/T1 primer set was used for the hox5-1 allele (bottom). (B) Expression analysis of HXK5 in leaf tissues and anthers harboring mature WT and hox5-1 pollen grains. OsUBQ5 was used as the internal PCR control. (C) Mature WT and hox5-1 plants grown in soil for 8 weeks.
were stained relatively weakly compared to the wild-type (Fig. 5A). Based on Hoechst 33342 nuclear staining, all pollen grains from the wild-type (cv. NP as a representative) and the hxk5-1 and hxk5-2 mutant plants contained one vegetative nucleus and two generative nuclei (Fig. 5A, insets). This indicated that the hxk5-1 and hxk5-2 mutant pollen contained less starch, but that it underwent nuclear division normally. We also found similar results in pollen grains from hxk5-3 and hxk5-4 (data not shown).

We then compared pollen germination and tube growth between the wild-type and hxk5-1 and hxk5-2 using in vitro and in vivo germination assays. For the in vitro analysis, we tested mature pollen grains collected immediately prior to pollination. This revealed that wild-type grains germinated with an

![Fig. 4.](https://academic.oup.com/jxb/article/71/1/116/5610755)

**Fig. 4.** Reduced fertility in rice hxk5 mutants. (A) Representative images of mature hxk5 mutant panicles. NP and DJ, Nipponbare and Dongjin background wild-type (WT) genotypes. (B) Mean fertility of the hxk5 mutants. Ubi::OsHXK5<sup>G113D</sup> represents the mean of six independent transgenic lines with a catalytically inactive mutant form of OsHXK5 in the hxk5 null mutant. Other data are means (±SE) from at least three different plants.

![Fig. 5.](https://academic.oup.com/jxb/article/71/1/116/5610755)

**Fig. 5.** Pollen grain staining and germination assays in rice hxk5-1 and hxk5-2 homozygotes. (A) Pollen grains of the wild-type Nipponbare (NP), hxk5-1, and hxk5-2 stained with an I<sub>2</sub>/KI solution. Pollen grains stained with Hoechst 33342 are shown in the insets, where red arrowheads indicate generative nuclei and white arrowheads indicate vegetative nuclei. (B) In vitro germination ratio of mature pollen grains prepared from the wild-types NP and Dongjin (DJ), hxk5-1, and hxk5-2. (C) Mean length of elongated pollen tubes measured 30 min after germination from the wild-types and the mutant lines. Data in (B, C) are means (±SE) of three replicates and significant differences compared to the wild-types were determined using Student’s t-test: *P<0.01. (D) Representative images of in vitro germinated pollen grains at 10 min after germination from the wild-types and the mutant lines. Scale bars are 40 μm. (E) In vivo pollen germination assays on pistil stigmas after pollination. Spikelets were collected 10 min after full pollination, and the pistils from the NP wild-type and the mutant lines were stained with aniline blue solution.
efficiency of 91.7% and 90.6% for cvs NP and DJ, respectively, whereas hxk5-1 homozygote grains germinated with an efficiency of only 36.6% (Fig. 5B). The mean length of the pollen tube was 191.4 µm at 30 min after germination in the wild-type (cv. NP), but was only 108.6 µm in the hxk5-1 homozygotes (Fig. 5C). Similarly, hxk5-2 homozygote pollen grains germinated with an efficiency of 32.6% (Fig. 5B). The mean length of pollen-tube growth was 201.4 µm at 30 min after germination in the wild-type (cv. DJ), but only 107.5 µm in the hxk5-2 homozygotes (Fig. 5C). In iodine staining analysis, germinated mutant pollen grains were stained much more intensely than the wild-types (Supplementary Fig. S3), indicating a defect in starch utilization in the mutant pollen.

In vivo pollen germination assays conducted 10 min after pollination on the stigma and followed by aniline blue staining showed efficient pollen germination and tube growth for the wild-type grains (cv. NP) as a representative. In contrast, only limited numbers of hxk5-1 and hxk5-2 grains germinated on the stigma (Fig. 5E).

Taken together, these results demonstrated that pollen germination and tube growth were significantly reduced in the hxk5 mutants compared to the wild-types. Thus, the pollen tubes of the mutant could not reach the ovary when competing with wild-type pollen in the heterozygous mutant. However, a few hxk5 mutant pollen grains were able to fertilize when not competing with wild-type pollen in the homozygous mutant, and thus were able to produce some seeds.

Biochemical analysis in anthers of hxk5 mutants

To determine the contribution of HXK5 to the total hexokinase activity in rice anthers carrying mature pollen grains, we examined hexokinase activity in the anthers of the wild-types (cvs. NP and DJ) and the hxk5-1, hxk5-2, hxk5-3, and hxk5-4 mutants at the mature stage. We found that the total hexokinase activity in the hxk5 mutants was reduced to ~70% of that in the wild-types (Fig. 6A). We also measured starch and soluble sugars in the mature anthers and found that the those of the hxk5 mutants contained more glucose, fructose, and sucrose compared to the wild-types, but less starch (Fig. 6B): the anthers of the homozygous mutants had 75% of the starch found in the wild-type, which was consistent with the relative weak starch staining of their pollen grains (Fig. 5A). Given that starch biosynthesis during the final phase of pollen maturation is critical for normal pollen development and starch is the main reserve energy source for pollen germination (Min et al., 2013; Zhang et al., 2013; Khan et al., 2015; Lee et al., 2016; Wu et al., 2016), our results suggested that the reduced starch content contributed to male sterility in the hxk5 mutants.

Production of transgenic rice expressing a catalytically inactive form of HXK5 in hxk5-1

It has been reported that a catalytically inactive form of OsHXK5, OsHXK5-G113D, complements the sugar-insensitive phenotype of the Arabidopsis glucose insensitive2-1 mutant (Cho et al., 2009). To examine whether the male-sterile phenotype of rice hxk5 mutants was due to a lack of glucose sensing, OsHXK5-G113D fused with a Myc tag was introduced into hxk5-1 under the control of the maize Ubiquitin1 promoter, which has been shown to drive strong gene expression in mature pollen (Cornejo et al., 1993). Among the 21 transgenic plants, six lines (designated OsHXK5-G113D) were selected by protein gel-blot analysis of leaves and of anthers carrying mature pollen using an anti-Myc antibody (Supplementary Fig. S4). The mean fertility rate of the T0 plants was 7.4% (Fig. 4A, B), which suggested that catalytically inactive OsHXK5 could not rescue the hxk5 male-sterile phenotype.

Discussion

In common with many major food crops, rice stores starch in pollen to provide the building blocks and energy necessary for its germination and tube growth, and hence it is commonly used as an indicator for viable pollen. However, only a few enzymes likely to be functional in the starch biosynthesis pathway during pollen development have been defined in rice (Mu et al., 2009; Lee et al., 2016). Sucrose is transported from the photosynthetic source organs to the non-photosynthetic sink anthers that carry the pollen, where it is hydrolysed by invertase into glucose and fructose (Koch, 2004). HXK then

![Fig. 6](https://academic.oup.com/jxb/article-fig/71/1/116/5610755) Biochemical analysis of rice hxk5 mutant anthers harboring mature pollen grains. (A) Hexokinase activity of mature anthers from the wild-types Nipponbare (NP) and Dongjin (DJ) and homozygous hxk5 lines. Units are nmol min⁻¹ at pH 7.5 and at room temperature. (B) Starch and soluble sugar contents of mature anthers from the wild-types and homozygous hxk5 lines. All data are means (±SE) from at least three different plants. Significant differences compared to the wild-types were determined using Student’s t-test: *<0.01.
This reduction disrupted dehiscence due to inhibited cell-wall thickening in the anthers, and reduced pollen germination and fertility. In contrast, the pollen appeared to be morphologically normal with levels of cell wall polysaccharides and starch similar to those found in the wild-type. Therefore, it is likely that the function of HXK5 that we examined is distinct from that of HXK10 in rice anthers. Mutation of OsSUT1 affects pollen germination rather than maturation, and reinforces the necessity of sucrose in pollen germination (Hirose et al., 2010). Similarly, a mutation in OsSPS1 results in starch being accumulated normally during pollen maturation, but pollen germination is reduced by 50% (Hirose et al., 2014). Therefore, it is likely that the function of OsHXK5, which affects both pollen maturation, germination, and tube growth, is distinct from that of OsSUT1 and OsSPS1, mutations of which only disrupt germination and tube growth.

The functions of the other OsHXKs need to be characterized. In particular, OsHXK6, which is constitutively expressed at low levels throughout anther development, is a close homolog to OsHXK5 (Fig. 1). Huang et al. (2015) found that OsHXK6 physically associates with Restorer-of-Fertility 6 (RF6). RF6 encodes a pentatricopeptide-repeat family protein, which controls pollen development by promoting the processing of aberrant cytoplasmic male sterility (CMS)-associated transcripts and rescues the Honglian CMS phenotype. Our preliminary analysis of a rice hlxk6-1 T-DNA mutant allele showed normal Mendelian progeny segregation ratios in the self-pollinated heterozygous plants (data not shown), suggesting that OsHXK6 may not be critical for normal pollen development in our wild-type cv. DJ background. It would be of value to examine a double-mutant of hlxk5/hlxk6 to understand any functional redundancy.

OsHXK5, in addition to OsHXK6, is primarily present in mitochondria, but has the potential to locate to the nucleus using a nuclear-localization signal sequence that functions in sugar signaling and sensing (Cho et al., 2009). We tried to complement the low fertility of the hlxk5 mutant by introducing a catalytically inactive OsHXK5-G113D, but none of the resulting OsHXK5-G113D transgenic rice plants showed normal fertility (Fig. 4). It is likely that the kinase activity of HXK5 is critical for pollen development, germination, and tube growth in rice. It has recently been reported that glucose sensing via AtHXK1 is involved in the glucose-mediated inhibition of pollen tube growth in Arabidopsis, and that this is independent of the catalytic function of AtHXK1 (Rottmann et al., 2018). We have previously reported that OsHXK5 and OsHXK6 have glucose sensing functions similar to AtHXK1 (Cho et al., 2009). It would therefore be interesting to determine whether these HXKs have a similar role in sugar signaling and sensing in the glucose-mediated inhibition of pollen tube growth in rice.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Digital expression profiles of OsHXK genes using Affymetrix microarray data prepared from developing pollen of *japonica* rice and downloaded from NCBI GEO.

Fig. S2. Characterization of hlxk5-1 homozygous rice plants derived from anther culture.
Fig. S3. Iodine staining of pollen grains of wild-type and hxk5-1 and hxk5-2 mutants at 30 min after germination.

Fig. S4. Protein gel–blot analysis of transgenic rice plants expressing Ubi::OsHXK5<sub>3</sub> (Myc).

Fig. S5. A model of carbohydrate metabolism in rice pollen grains.

Acknowledgements

This work was supported by grants from the Next Generation BioGreen 21 Program of the Rural Development Administration of Korea (PJ013172012019) and from the Mid-Career Researcher Program of the National Research Foundation of Korea (NRF-2017R1A2B4009687). The authors declare no conflicts of interest.

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