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RESEARCH PAPER

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

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Abstract

There is little known about the function of rice hexokinases (*HXK*s) *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 Pyrophosphorylase4* 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.*, 2016).

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.*, 2006*a*, 2006*b*, 2009; Claeyssen and Rivoal, 2007). In rice, there are 10 HXK isoforms (Cho *et al.*, 2006*a*), 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 O_2-deficient germination since the *hxk7* 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 30/20 °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 *hxk5-1* 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 *hxk5-1* mutant were determined by genomic DNA PCR using the *Tos17*-specific T1 primer (5′-CCAGTCCATTGGATC TTGTATCTTGTATATAC-3′) and the *OsHXK5* gene-specific F1 (5′-TATATGGGGTCCTAAAGAAGCTA-3′) and R1 (5′-AAAAAGATGGTAAGATTTCAAGG-3′) primers.

The other three mutant alleles, *hxk5-2*, *hxk5-3*, and *hxk5-4*, 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′-GCGGGGCATCTCGGACGCCA-3′) was cloned into an entry vector, pOs-sgRNA, and then cloned into a destination vector, pH-Ubi-cas9-7, 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 *hxk5-2*, *hxk5-3*, and *hxk5-4* were determined by Sanger sequencing of the target PAM site. PCR amplicons were generated using gene-specific F2 (5′-GGTTAAAACGCCCAAGTCAGTG-3′) and R2 (5′-AAGGTCAGTCGGTCCTAATCAGAG-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. The *Ubiquitin5* gene (*OsUbi5*; LOC_Os01g22490) was used as an internal PCR control.

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_6 media 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-$MgCl_2$ buffer (0.2 M Tris, 4 mM $MgCl_2$, 0.5% Triton X-100, pH 7.5) in a glass Petri dish on ice (Pfosser *et al.*, 1995). Nuclei were stained in 50 μg l^{-1} propidium iodide solution with 50 μg l^{-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_3BO_3, 1 mM $CaCl_2$, 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 a 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 overnight to clear the tissue, and then stained for 5 h with 0.2% aniline blue in 0.1 M K_2HPO_4. The stained ovaries were mounted on glass slides and fluorescence was observed using a 365-nm excitation filter and a 397-nm barrier filter on a BX61 microscope (Olympus).

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 OsHXK5

To introduce a catalytically inactive mutant form of OsHXK5 into the *hxk5* 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 *hxk5-1* 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 $OsHXK5^{G113D}$.

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*, 2006*a*). To identify HXKs that are expressed during late stages of pollen development and germination, we examined six Affymetrix rice microarray 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_Os05g45590) 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 microarray 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 *HXK*s 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 late 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 *hxk5-1* 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 *hxk5-1* mutant plants. This showed no homozygous *Tos17* insertion mutant line (*hxk5-1/hxk5-1*); all appeared to be either wild-type (NP) or heterozygote (*HXK5/hxk5-1*; 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,

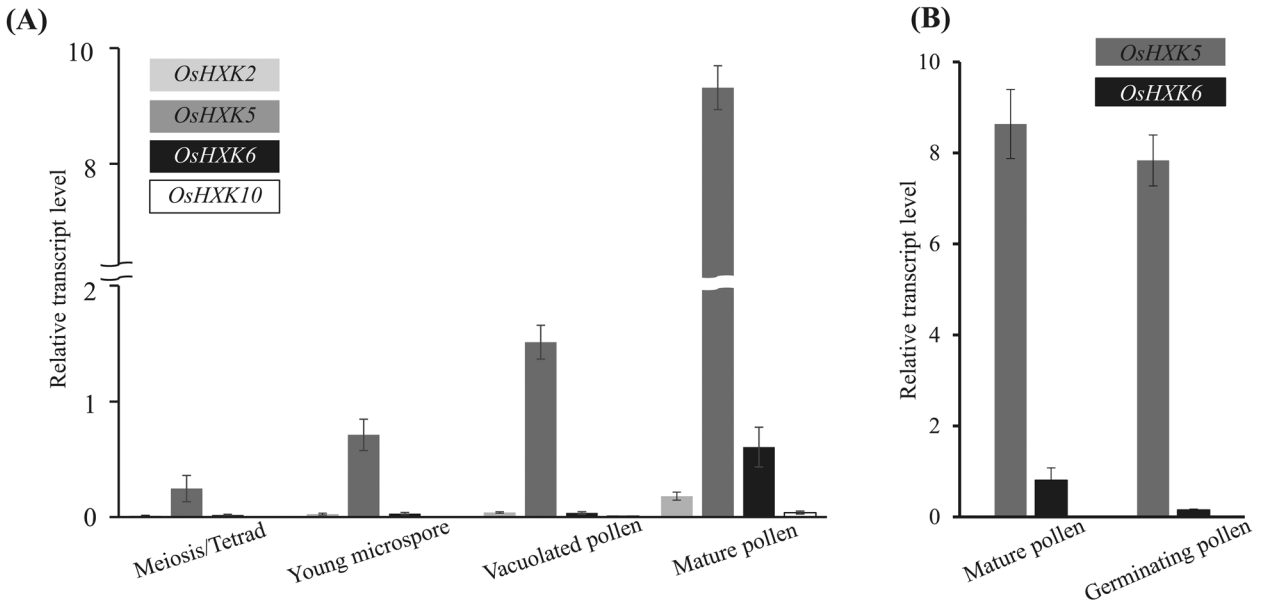

Fig. 1. Expression of rice hexokinase *HXK* genes in anthers at different stages of pollen development. (A) Quantitative RT-PCR analysis of *HXK*s in anthers from meiosis to the mature pollen stage. (B) Quantitative RT-PCR analysis of *HXK5* and *HXK6* in mature pollen and germinating pollen. Data are means (±SE) of three replicates.

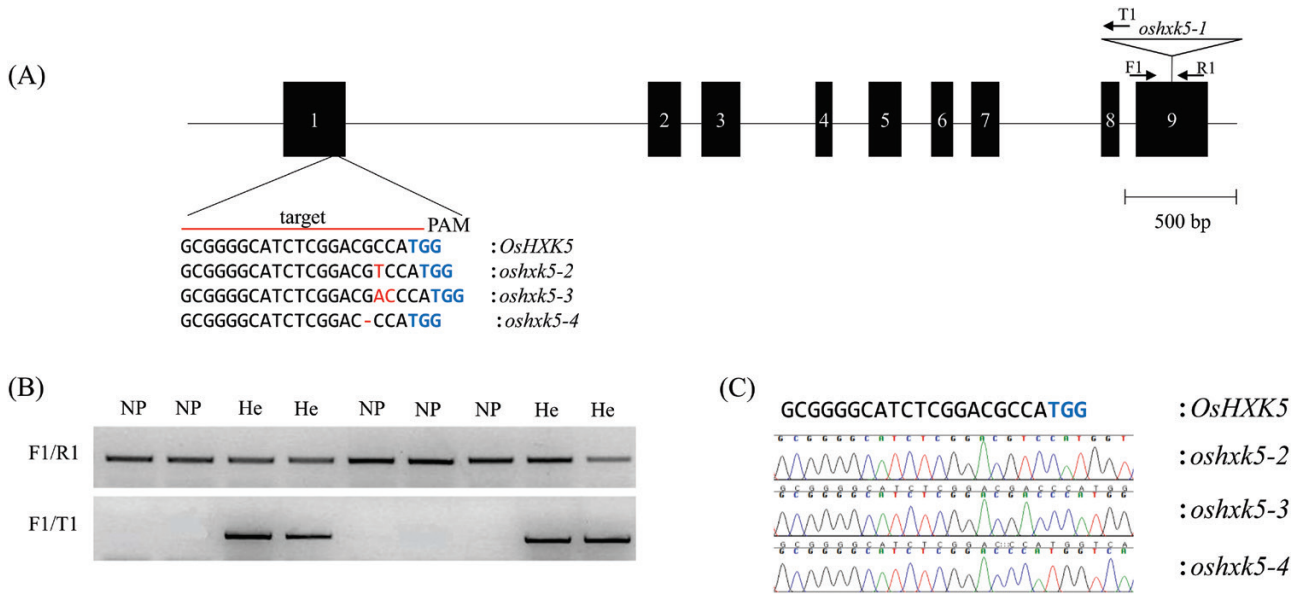

Fig. 2. Molecular characterization of the rice *HXK5* mutants *hxk5-1*, *hxk5-2*, *hxk5-3*, and *hxk5-4*. (A) Schematic diagram of *Tos17* in *hxk5-1* and the CRISPR/Cas9-generated indel mutant alleles. The nine exons of *OsHXK5* are indicated by the numbered boxes. Primers for genotyping are indicated with arrows. *Tos17* is inserted into the ninth exon of *OsHXK5*. The mutants carrying one and two nucleotide insertions, and one nucleotide deletion are designated as *hxk5-2*, *hxk5-3*, and *hxk5-4*, respectively, and are highlighted in red. (B) PCR analysis of genomic DNA from the progeny of self-fertilized *HXK5/hxk5-1* plants. An F1/R1 primer set was used for the wild-type *HXK5* allele (top) and an F1/T1 primer set was used for the *hxk5-1* allele (bottom). NP, mutant background cv. Nipponbare genotype; He, heterozygote. (C) Sequence alignment of the CRISPR/Cas9 target sites in the *OsHXK5* mutant alleles. The target site protospacer adjacent motif is highlighted in blue.

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 F_1 plants yielded the heterozygous genotype; all were wild-type. However, when the heterozygous line was used as a female donor, the genotypes of 44 F_1 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 hxk5-1 *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 *hxk5-1* heterozygote. Unsurprisingly, approximately half of the regenerated plants were wild-type and the other half were *hxk5-1* homozygous mutants. The regenerated *hxk5-1* 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 *hxk5-1* homozygous plants showed the same diploid levels as the wild-type plants, representing the 2C DNA content (Supplementary Fig. S2A). However, the homozygous *hxk5-1* 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 *hxk5-1* 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* hxk5-1 *heterozygous mutant plants*

Parent plant	Percentage of observed/expected genotypes of progeny (actual numbers recorded)		
HXK5/hxk5-1	Wild-type	*HXK5/hxk5-1*	*hxk5-1/hxk5-1*
	51.1/25 (47/92)	48.9/50 (45/92)	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* hxk5-1 *heterozygous plants*

Genetic cross		Percentage of observed/expected genotypes of progeny (actual numbers recorded)	
Paternal	**Maternal**	**Wild-type**	***HXK5/hxk5-1***
HXK5/hxk5-1	Wild-type	100/50 (45/45)	0/50 (0/45)
Wild type	*HXK5/hxk5-1*	52/50 (23/44)	48/50 (21/44)

Genotypes of F_1 plants were determined by PCR using gene- and *Tos17*-specific primers.

Production and characterization of additional hxk5 *homozygous mutant alleles*

To further confirm the male-sterile phenotype of *hxk5-1*, we utilized the CRISPR/Cas9 system as an alternative to anther culture for producing homozygous mutant lines. A region in the first exon was selected as the guide RNA target. Of 16 independent transgenic lines obtained, three were homozygous mutants at the target site lines, namely *hxk5-2*, *hxk5-3*, and *hxk5-4*. The *hxk5-2*, *hxk5-3*, and *hxk5-4* alleles (background cv. DJ) respectively had one nucleotide (T) insertion, two nucleotide insertions (AC), and one nucleotide deletion (G) (Fig. 2A, C). The fertility of these plants ranged from ~5–10%, with mean values of 6.4%, 6.2%, and 7.2% for *hxk5-2*, *hxk5-3*, and *hxk5-4*, respectively (Fig. 4A, B). The fertility levels were comparable to that of *hxk5-1*.

Pollen analysis of the hxk5 *homozygous mutant*

To further understand male sterility in the *hxk5* mutant pollen, we examined iodine staining in *hxk5-1* and *hxk5-2* homozygous plants. We found that all of the mutant pollen grains

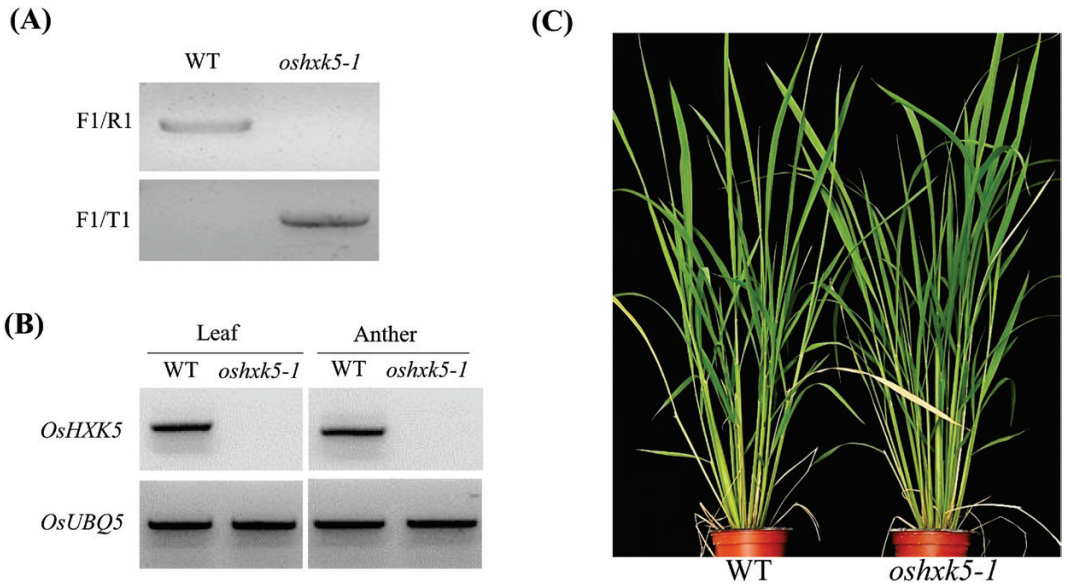

Fig. 3. Molecular and phenotypic analysis of rice *hxk5-1* mutants produced via anther culture. (A) Genotype analysis of the *hxk5-1* homozygous mutant. An F1/R1 primer set was used for the wild-type (WT) *HXK5* allele (top), and an F1/T1 primer set was used for the *hxk5-1* allele (bottom). (B) Expression analysis of *HXK5* in leaf tissues and anthers harboring mature WT and *hxk5-1* pollen grains. *OsUBQ5* was used as the internal PCR control. (C) Mature WT and *hxk5-1* 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

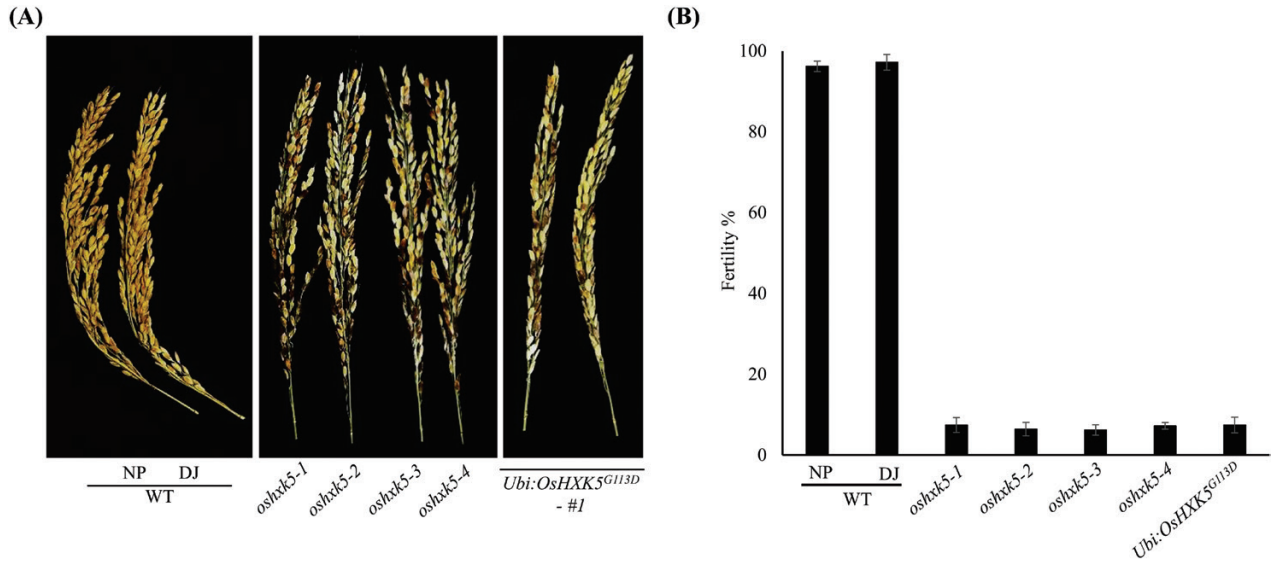

Fig. 4. Reduced fertility in rice *hxk5* mutants. (A) Representative images of mature *hxk5* mutant panicles. NP and DJ, Nipponbare and Dongjin background wild-type (WT) genotypes. (B) Mean fertility of the *hxk5* mutants. *Ubi::OsHXK5*^{G113D} represents the mean of six independent transgenic lines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

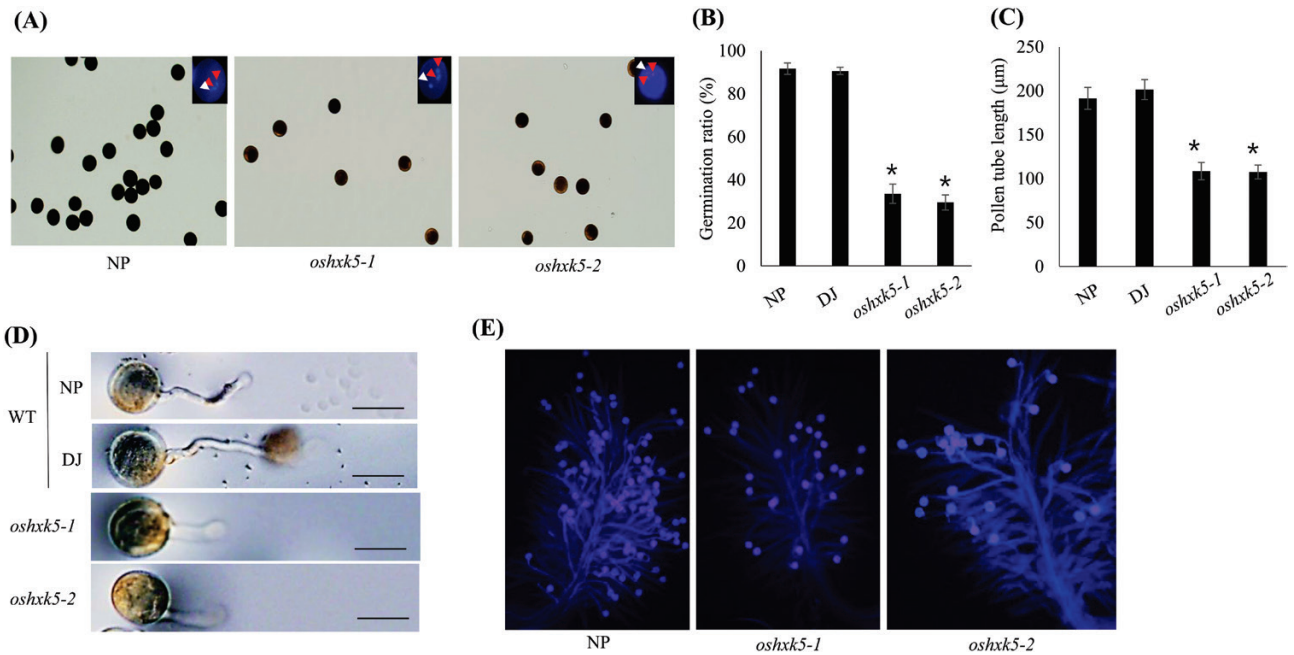

Fig. 5. Pollen grain staining and germination assays in rice *hxk5-1* and *hxk5-2* homozygotes. (A) Pollen grains of the wild-type Nipponbare (NP), *hxk5-1*, and *hxk5-2* stained with an I_2/KI solution. Pollen grains stained with Hoechst 33342 are shown in the insets, where red arrowheads indicate generative 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, D). 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 T_0 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

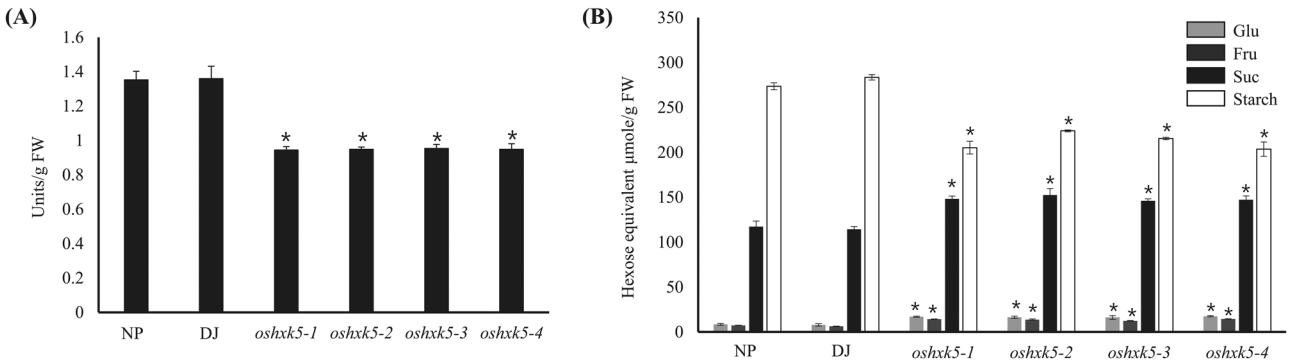

Fig. 6. Biochemical analysis of rice *hxk5* mutant anthers harboring mature pollen grains. (A) Hexokinase activity of mature anthers from the wild-types Nipponbare (NP) and Dongjin (DJ) and homozygous *hxk5* lines. Units are nmol min^{-1} at pH 7.5 and at room temperature. (B) Starch and soluble sugar 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: **P*<0.01.

catalyses the first irreversible step of sugar metabolism through the phosphorylation of hexoses into hexose-6-phosphate. This serial reaction therefore potentially constitutes an important regulatory step in starch biosynthesis in mature pollen grains (Dai *et al*, 1999; Winter and Huber, 2000). HXK activity in carbohydrate catabolism is also critical for supplying the building blocks and energy during pollen germination and tube growth (Dai *et al.*, 2007).

Little is known about how HXKs regulate growth and development in rice. In this study, among the 10 identified rice HXK genes (Cho *et al.*, 2006*a*), we found that *HXK5* is highly expressed in the late stages of pollen development when starch begins to accumulate (Raghavan, 1988;Yamagata *et al.*, 2010) (Fig. 1, Supplementary Fig. S1). The isolated *Tos17* insertion mutant line *hxk5-1* did not bear any homozygotes from self-pollinated heterozygotes (Fig. 2,Table 1). Reciprocal-cross experiments between *hxk5-1* and wild-type plants revealed a male-sterile phenotype in the mutant pollen (Table 2). Pollen analysis of the *hxk5* mutant alleles showed reduced hexokinase activity, partly impaired starch biosynthesis, and a severe sterile phenotype (Figs 3–6). A pollen germination assay further supported the presence of a defect in *hxk5-1* pollen germination and tube growth (Fig. 5).

It was notable that, while the heterozygous plants did not produce homozygous progeny, anther-derived homozygous *hxk5-1* plants and *hxk5-2*, *hxk5-3*, and *hxk5-4* plants that were generated using the CRISPR/Cas9 system produced some homozygous seeds (Fig. 4, Supplementary Fig. S2B). This suggests that the *hxk5* mutant pollen grains could not compete with wild-type grains. However, since the grains contained a considerable amount of starch (nearly 75% of the wild-type level), the mutant retained some fertilization capability despite its relatively slow germination and tube growth (Figs 5, 6). Other HXKs may have contributed to the residual hexokinase activity that could direct starch biosynthesis. Although pollen separated from anthers was not directly examined due to the technical challenges involved, we believe that pollen represents the most metabolically active component in mature anthers and hence it contributes to a large portion of the hexokinase activity and starch content in the anthers. Therefore, it was not surprising that *hxk5* mutant pollen could fertilize in self-pollinated homozygous lines.

It is not currently known whether the male sterility of *HXK5*-deficient pollen is primarily due to insufficient starch biosynthesis or to a defect in pollen germination and tube growth. In general, HXK functions in both the starch biosynthesis and utilization pathways (Supplementary Fig. S5). Thus, an insufficient starch content may affect subsequent processes after pollen maturation. The results from our present study suggest that OsHXK5 is a major HXK isoform that functions during pollen germination and tube growth. A deficiency in HXK5 may affect the building blocks and energy production required for that process (Supplementary Fig. S5). We hypothesize that HXK5 functioning is critical for pollen maturation, germination, and tube growth in rice.

In a previous study, Xu *et al.* (2008) found that suppression of *OsHXK10* expression using RNAi caused a reduction in transcripts in the anther walls of the transgenic plants. 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 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 *hxk6-1* 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 *hxk5*/*hxk6* 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 *hxk5* 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 *hxk5-1* 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^{G113D}-Myc*.

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

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