

RESEARCH PAPER

Disruption of a gene for rice sucrose transporter, *OsSUT1*, impairs pollen function but pollen maturation is unaffected

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Abstract

Sucrose transporters (SUTs) are known to play critical roles in the uptake of sucrose from the apoplast in various steps of sugar translocation. Because developing pollen is symplastically isolated from anther tissues, it is hypothesized that SUTs are active in the uptake of apoplastic sucrose into pollen. To investigate this possibility, a comprehensive expression analysis was performed for members of the SUT gene family in the developing pollen of rice (*Oryza sativa* L.) using real-time RT-PCR combined with a laser microdissection technique. Among the five SUT genes, *OsSUT1* and *OsSUT3* were found to be preferentially expressed and had temporal expression patterns that were distinct from each other. Expression of *OsSUT1* in pollen was confirmed by a promoter–GUS fusion assay. The physiological function of *OsSUT1* in pollen was further investigated using retrotransposon insertion mutant lines. While the homozygote of disrupted *OsSUT1* (*SUT1*^{-/-}) could not be obtained, heterozygote plants (*SUT1*^{+/-}) showed normal grain filling. Their progeny segregated into *SUT1*^{+/-} and *SUT1*^{+/+} with the ratio of 1:1, suggesting that the pollen disrupted for *OsSUT1* is dysfunctional. This hypothesis was reinforced *in vivo* by a backcross of *SUT1*^{+/-} plants with wild-type plants and also by *in vitro* pollen germination on the artificial media. However, starch accumulation during pollen development was not affected by disruption of *OsSUT1*, suggesting that the sugar(s) required for starch biosynthesis is supplied by other sugar transporters.

Key words: Gene expression, laser microdissection, pollen germination, pollen maturation, sucrose transporter (SUT), *Oryza sativa* L.

Introduction

Sucrose is the most common sugar molecule translocated in higher plants, and the transmembrane transport of sucrose in higher plants is mediated by sucrose transporter (SUT) proteins. A *SoSUT1* cDNA from spinach was the first reported SUT in higher plants (Riesmeier *et al.*, 1992); since then, more than 60 SUT genes have been cloned. Looking back over the history of SUT research, it was fortuitous that SUTs reported in the early days were localized to vein or phloem and that the proteins were suggested to be a phloem loader, a protein that researchers had long been searching for

as a central player of sucrose loading. However, early reports on the phloem loader SUTs from a number of plant species were followed by a growing body of evidence supporting the idea that SUTs have very diverse roles and that phloem loading is merely one of them. It is now more generally accepted that (plasma membrane-localized) SUTs play critical roles in the uptake of sucrose from the apoplast at various steps in sugar translocation (see Sauer, 2007, for a review).

In addition to phloem loading, SUTs have been well documented to mediate the uptake of sucrose into developing

filial tissues that are symplastically isolated from maternal tissues. In developing seeds, for instance, all nutrients including sucrose are released from maternal tissues into the apoplast surrounding the filial tissues; they are then taken up by outer cell layers of the filial tissues, such as endosperm or the embryo (see Patrick and Offler, 2001, for a review). The uptake of sucrose into developing filial tissues is mediated by SUTs, and the expression and function of these SUTs have been investigated in both grain legumes and cereals. In the developing rice caryopsis, a gene for SUT, *OsSUT1*, is highly expressed in the aleurone (Hirose *et al.*, 2002; Ishimaru *et al.*, 2007), and antisense suppression of *OsSUT1* results in poor grain-filling accompanied by a decreased rate of radiolabelled sucrose uptake into seed tissues (Scofield *et al.*, 2002). Therefore, *OsSUT1* is thought to have a crucial role in the uptake of sucrose by the filial tissues of developing rice caryopsis. In the pea, seed-specific overexpression of SUT was reported to enhance both the sucrose uptake and growth rates of the cotyledons of developing seeds (Rosche *et al.*, 2002; Zhou *et al.*, 2009).

Because pollen grains are symplastically isolated from anther tissues (e.g. filial tissues) of the seeds and accumulate large amounts of starch as in cereal endosperm, SUTs are expected to be active in pollen development. In fact, mRNA or protein of several SUTs has been detected in pollen or pollen tube (Lemoine *et al.*, 1999; Stadler *et al.*, 1999; Hackel *et al.*, 2006; Lauterbach *et al.*, 2007; Sivitz *et al.*, 2008). In tomato, *LeSUT2* has been shown to play a role in pollen tube growth using the antisense suppression technique (Hackel *et al.*, 2006). In the disruption mutants of *Arabidopsis*, *AtSUC1* (At1g71880), pollen having disrupted *AtSUC1* is defective *in vivo*, as shown by segregation distortion; the mutants also have a lower rate of germination *in vitro* (Sivitz *et al.*, 2008).

The rice genome encodes five SUT genes (Aoki *et al.*, 2003); however, none of them has been shown to function in pollen grains. In this study, laser microdissection-assisted expression analysis of the rice SUT genes in developing pollen was carried out and functional characterization of one of the SUT genes (*OsSUT1*) was conducted using retrotransposon insertion mutant lines.

Materials and methods

Plant materials

A japonica type wild-type parental rice (*Oryza sativa* L.) cultivar Nipponbare and its three mutant lines containing the retrotransposon *Tos17* insertion within the *OsSUT1* gene were used. Insertion mutants of *Tos17* were selected by BLAST searches against a dataset of *Tos17* flanking sequences in the rice genome (Miyao *et al.*, 2003). Rice plants were grown in the paddy field of the Hokuriku Research Center, Joetsu, Japan (37°06' N, 138°16' E) or in plastic pots filled with soil from the paddy field under outdoor conditions.

Genotyping mutant plants and pollen grains

The genotype of each of the three mutant lines was determined by PCR. Genomic DNA was extracted from mature leaf blades of each plant using the method of Murray and Thompson (1980), and

used as template for the PCR with the primers listed in Table 1. The same PCR primers were also used for genotyping pollen samples.

Preparation of an *OsSUT1* promoter::GUS construct and transformation into rice

A translational fusion of the *OsSUT1* gene promoter with the β -glucuronidase (GUS) reporter gene in a rice transformation vector was constructed as follows. To obtain the *OsSUT1* promoter::GUS construct, a DNA fragment spanning nucleotides -1675 to +19 of the translation start of *OsSUT1* was amplified by PCR, and then ligated into a transformation vector, pZH2B. This vector is a modified version of pPZP202 (Hajdukiewicz *et al.*, 1994), which carries hygromycin resistance. In this construct, the fusion protein consisted of the first seven codons of the *OsSUT1* open reading frame followed by the complete GUS open reading frame. The construct was introduced into *Agrobacterium tumefaciens* strain EHA101 by electroporation. Transformation of this construct into rice tissue was performed by the method of Hiei *et al.* (1994).

GUS staining of transgenic plants

Glumaceous flowers from the *OsSUT1* promoter::GUS transgenic lines were stained for GUS activity at 37 °C in the dark, overnight in X-gluc solution containing 0.5 mM $K_3[Fe(CN_6)]$, 0.5 mM $K_4[Fe(CN_6)].3H_2O$, 0.3% (v/v) Triton X-100, 20% (v/v) methanol, and 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide cyclohexylamine salt in 100 mM sodium phosphate buffer (pH 7.0). GUS staining was examined with a stereomicroscope (SZX10, Olympus). Some of the GUS-stained samples were fixed with FAA fixative, embedded in paraffin, and examined with a microscope (BX50, Olympus).

Laser microdissection (LM) and real-time RT-PCR

Anther sampling was performed in the morning before flowering of the day commenced. Anthers were collected from glumaceous flowers and fixed in acetone at 4 °C for 3 h. The fixative was

Table 1. PCR primers used in this study

Purpose	Oligo name	Sequence
NC7083 genotyping	7083-L	TATTGCTGGCATGGTGGTAA
	7083-R	TACAATAGCAGCATGCCACC
NF8036 genotyping	8036-L	TCCCAGTATGATCCAGAAGCC
	8036-R	TCCTTAAGTTGAAGTCCCG
NF2752 genotyping	2752-L	CCTACTGGGATGCCTTCTGT
	2752-R	TGGAAGTCCTTGAGTGACCA
<i>Tos17</i> left border, genotyping	<i>Tos17</i> -L	ATTGTTAGGTTGCAAGTTAGTTAAGA
<i>OsSUT1</i> RT-PCR	<i>OsSUT1</i> -L	TCATCCCTCAGGTGGTCATCG
	<i>OsSUT1</i> -R	CTTGGAGATCTTGGGCAGCAG
<i>OsSUT2</i> RT-PCR	<i>OsSUT2</i> -L	GTCATACCACAGGTTATTGTGTC
	<i>OsSUT2</i> -R	GAATTGCAAAGAAATGGCCG
<i>OsSUT3</i> RT-PCR	<i>OsSUT3</i> -L	TCCTCTTCGACACCGACTG
	<i>OsSUT3</i> -R	CAGCAGATCGAGTTAAGGAG
<i>OsSUT4</i> RT-PCR	<i>OsSUT4</i> -L	CGTTGTTCCGCGAGATAGTAGT
	<i>OsSUT4</i> -R	GTGTTCTGCTCAGCCAAATCC
<i>OsSUT5</i> RT-PCR	<i>OsSUT5</i> -L	TCGGCATGGTGTCCATGAG
	<i>OsSUT5</i> -R	CAATGGCAAGACCTTGGCC
RUBIQ1 RT-PCR	RUBIQ1-L	GGAGCTGCTGCTGTTCTTGG
	RUBIQ1-R	CACAATGAAACGGGACACGA

infiltrated into anther samples under vacuum for 5 min at 4 °C. The fixed tissues were then embedded in paraffin (Paraplast-XTra, Sigma), sectioned to 10 µm using a rotary microtome (RM2145, Leica) and mounted on PEN membrane frame slides (Arcturus). The sections were deparaffinized by xylene and air-dried just prior to LM. Pollen grains were collected from the sections by LM (Veritas, Arcturus) and total RNA was extracted using a PicoPure RNA isolation kit (Arcturus) following the manufacturer's instructions. RNA quality was checked by microcapillary electrophoresis (BAS2000, Agilent Technologies). Approximately 100 pg reaction⁻¹ of total RNA was used for real-time quantitative RT-PCR with a One Step SYBR PrimeScript RT-PCR Kit II (Takara Bio Inc.) and Smart Cycler II (Cepheid). The results obtained for the different RNAs were normalized to the transcript level of a rice polyubiquitin gene (*RUIBQ1*; Wang *et al.*, 2000; accession number U37687), which shows highly constitutive expression in various rice tissues.

Total number and maturity of the pollen grains

Glumaceous flowers one or two days prior to anthesis were collected, immediately fixed in 50% (v/v) ethanol, and stored at room temperature until use. Anthers were excised from the glumaceous flowers, stained with an I₂/KI solution, and the total number of the pollen grains was counted. Only pollen grains densely stained by the I₂/KI solution were counted as mature pollen.

In vitro pollen germination and collection of the germinated pollen

Pollen grains were germinated on 1% agar medium containing 20% (w/v) sucrose and 20 mg l⁻¹ K₂B₄O₇ following the method of Kariya (1989). Glumaceous flowers just after anthesis were sampled and gently shaken above the germination medium in Petri dishes to collect pollen grains. After incubation for 20 min at 20 °C, pollen grains were stained by an I₂/KI solution and the numbers of germinated pollen grains were counted under a microscope (BX50, Olympus). In the preliminary experiment it was confirmed that the sucrose concentration of the medium and the time of incubation are optimal for Nipponbare, the cultivar used in this study, which is different from that used by Kariya (1989). Some of the germinated pollen grains were picked up with pipette tips under a stereomicroscope (SZX10, Olympus), and their DNA was extracted to determine genotype by PCR using a PicoPure DNA isolation kit (Arcturus) following the manufacturer's instructions.

Results

Expression of *SUT* genes in the developing pollen

To examine which of the five members of the rice *SUT* gene family is expressed in the developing pollen grains, real-time RT-PCR analysis combined with laser microdissection (LM) was employed. A typical image of LM and electrophoresis of a resultant RNA sample are shown in Supplementary Fig. S1 at *JXB* online. LM was performed using anthers chemically fixed with pure acetone; these preparations yielded higher quality RNAs than from the more commonly used ethanol/acetic acid fixative, although preservation of specimen structural integrity was somewhat inferior (data not shown). Because anthesis of rice glumaceous flowers occurs in an orderly manner within an inflorescence, the days to the anthesis (DTA) can be estimated for each spikelet according to their position in the panicle. Thus, the developmental stages of the pollen

were classified into five classes according to the estimated DTA of each glumaceous flower, as follows: stage I (5–7 DTA), stage II (4–5 DTA), stage III (2–3 DTA), stage IV (1 DTA), and stage V (0 DTA). At stage I, starch was not detectable in the pollen by I₂/KI staining, and the cellular remnant of the tapetum was evident in the anther (Fig. 1A). At stage II, starch began to accumulate in the pollen (Fig. 1A). By real-time RT-PCR, only two *SUT* genes, *OsSUT1* and *OsSUT3*, were detectable in the pollen; mRNA for the other three *SUT* genes was very low, if at all (Fig. 1B). The temporal expression patterns of the two *SUT* genes were different; the mRNA level of *OsSUT1* was not detectable in stage I, increased suddenly in stages II and III, and then decreased through stages IV and V; whereas expression of *OsSUT3* was already detectable at stage I; it then increased and maintained the highest level during stages II to V.

The expression of *OsSUT1* was then examined by histochemical staining using transgenic plants harbouring a *OsSUT1* promoter::GUS construct. No staining was detected in the glumaceous flowers at stage I or earlier (Fig. 2A, B, C); clear staining was then observed in both the anther wall and pollen at stage II and later (Fig. 2D, E, F). These results are consistent with those from the RT-PCR analysis.

Disruption of *OsSUT1* impairs pollen function

To investigate the functions of *OsSUT1* and *OsSUT3* in pollen, gene disruption mutants of the two genes were searched for from a population of rice mutants generated by insertion of a retrotransposon Tos17. Three mutant lines of *OsSUT1* were identified, but none were identified for *OsSUT3*. It was therefore decided to study *OsSUT1* function in pollen further using three mutant lines, NC7083, NF8036, and NF2752, in which insertions of Tos17 occurred in exons 3, 4, and 10, respectively (Fig. 3). Populations of these three mutant lines segregated into two genotypes, homozygous wild-type (*SUT1*+/+) and heterozygous for the Tos17 insertion (*SUT1*+/-); however, no homozygous plants for the insertion (*SUT1*-/-) were identified (data not shown). Three hypotheses were developed to explain the absence of *SUT1*-/- plants: (i) both male and female gametes with disrupted *OsSUT1* are functional, but the *SUT1*-/- zygote cannot develop to maturity, (ii) a male gamete is not formed, or is dysfunctional, and (iii) a female gamete is not formed or dysfunctional. To determine which of these three hypotheses is valid, the progeny of *SUT1*+/- plants were grown and investigated further. The germination frequency of the seeds from *SUT1*+/- plants ranged between 90% and 97% in four independent observations, which was not different from that of the seeds from *SUT1*+/+ plants for all three mutant lines. However, homozygous plants did not develop once again, and the segregation ratio of *SUT1*+/+ and *SUT1*+/- were 1:1 in all the three mutant lines (Table 2). The *SUT1*+/- plants grew normally and no differences were detected between *SUT1*+/+ in the major

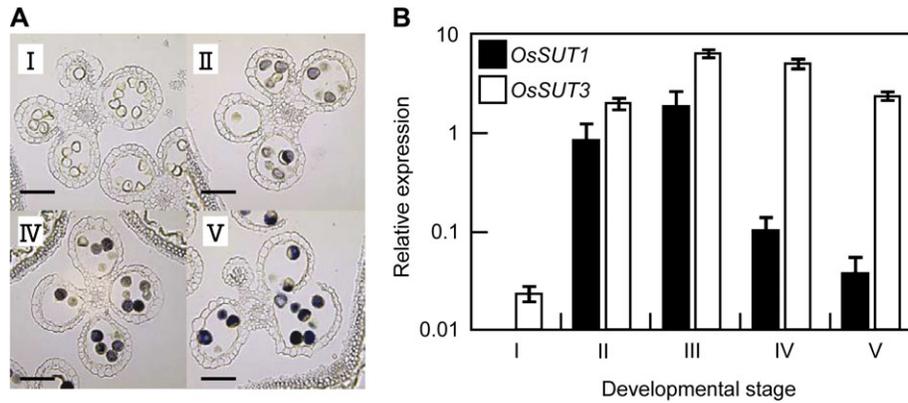


Fig. 1. (A) The iodine-stained cross-sections of the anthers at different developmental stages used in the real-time RT-PCR. Bars=0.1 mm. (B) Transcript levels of *OsSUT1* and *OsSUT3* in the pollen at different developmental stages. The transcript levels the SUT genes determined by real-time RT-PCR were standardized against those of a constitutive ubiquitin gene *RUBI1Q1* and expressed on a logarithmic scale. Values are the means of three independent replications (bars indicate SE). See Materials and methods for the details of the developmental stage.

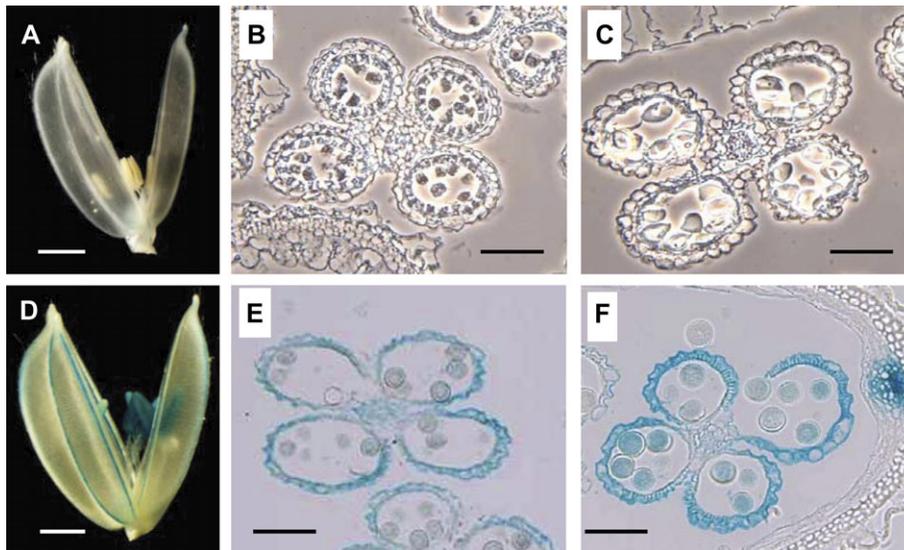


Fig. 2. Analysis of *OsSUT1* expression in the glumaceous flowers of transgenic plants harbouring a *OsSUT1* promoter::GUS construct. (A, D) Whole glumaceous flowers at stage I (A) and stage III (D). Bars indicate 1 mm. (B, C, E, F) Cross-sections of anthers of various developmental stages; (B) earlier than stage I (around 10 DTA), (C) stage I, (E) stage II, (F) stage IV. Bars indicate 0.1 mm. (B) and (C) are phase-contrast images to show the anatomy of the anthers better.

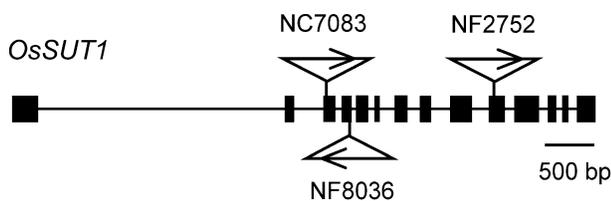


Fig. 3. A diagram showing the exon/intron structure of *OsSUT1* gene and the position of the insertion of retrotransposon *Tos17* in the three mutant lines used in this study. The arrowheads indicate the direction of *Tos17* at the respective insertion sites.

agronomic traits, including days to heading, plant height, or panicle number per plant (data not shown). In addition, both the total number of spikelets and the percentage of filled grains per panicle did not differ between the two

genotypes (Table 3). If the first hypothesis were true, the segregation ratio of *SUT1*^{+/+}:*SUT1*^{+/-} should be 1:2, with one-quarter of the caryopses (*SUT1*^{-/-} zygotes) being infertile or immature. If the third hypothesis were true, the segregation ratio should be 1:1, and half of the caryopses with *SUT1*⁻ female gametes would be infertile. Thus, the second hypothesis appears to be valid; in this case, the segregation ratio should be 1:1, with the number of filled grains unaffected. To confirm this, *SUT1*^{+/-} plants were backcrossed to wild-type plants. A total of 74 F₁ seeds were obtained using *SUT1*^{+/-} zygotes of the three insertion lines as pollen parents, but none of the F₁ plants inherited the *OsSUT1* mutant allele, indicating that pollen function is, in fact, impaired by disruption of the *OsSUT1* gene (Table 4).

Table 2. Segregation of the progeny of the three *SUT1*+/- parent lines

Line	Total	Genotype			<i>P</i> ^a
		<i>SUT1</i> +/+	<i>SUT1</i> +/-	<i>SUT1</i> -/-	
NC7083	94	46	48	0	0.837
NF2752	109	52	57	0	0.632
NF8036	104	56	48	0	0.433

^a Probability calculated by χ^2 -test when 1:1 segregation of *SUT1*+/+ and *SUT1*+/- is hypothesized.

Table 3. The number of spikelets and the filled grain percentage per panicle of *SUT1*+/+ or *SUT1*+/- plants. Values are the means of 5–7 individual plants of each genotype. 'ns' shows not significantly different between the two genotypes (*t* test).

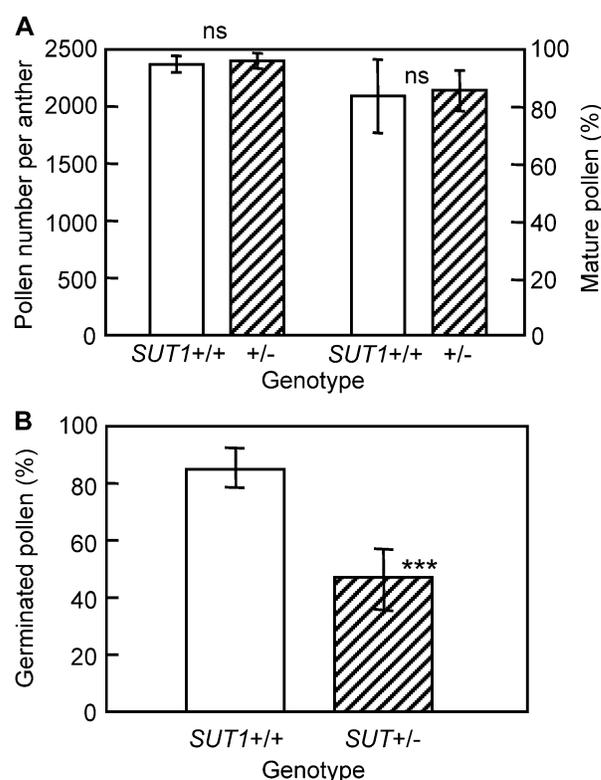
Line	Genotype	No. of spikelet per panicle	Filled grain percentage (%)
NC7083	<i>SUT1</i> +/+	50.0±3.4	92.6±3.0
	<i>SUT1</i> +/-	50.8±2.9 ns	92.4±2.7 ns
NF2752	<i>SUT1</i> +/+	52.4±3.5	93.0±1.2
	<i>SUT1</i> +/-	51.4±3.0 ns	95.2±1.1 ns
NF8036	<i>SUT1</i> +/+	48.0±2.8	92.3±2.6
	<i>SUT1</i> +/-	47.0±6.7 ns	94.0±0.7 ns

Pollen germination is impaired by disruption of OsSUT1, but pollen maturation is unaffected.

To investigate further how *OsSUT1* disruption affects the development and/or function of pollen, the total number of the pollen in an anther, the rate of matured pollen, and the pollen germination rate were examined using a disruption line NF2752. The total number of pollen grains in an anther and the percentage of matured pollen, as assessed by iodine staining, were not different, irrespective of the genotype (Fig. 4A). In another observation using all three disruption lines, the rate of matured pollen did not differ irrespective of the genotypes in each disruption line (see Supplementary Table S1 at *JXB* online). This suggests that a male gamete with disrupted *OsSUT1* is able to develop into a mature pollen grain. This also implies that disruption of *OsSUT1* does not affect pollen development, but rather some other pollen function(s) during the process of fertilization, for example, pollen germination or pollen tube elongation. Pollen germination in the mutants was assessed by an *in vitro* pollen germination assay carried out on agar medium. The percentage of germinated pollen was found to be significantly lower in *SUT1*+/- plants than in wild-type *SUT1*+/+ plants (Fig. 4B), suggesting that disruption of *OsSUT1* does in fact impair pollen germination. Germinated pollen grains of *SUT1*+/- plants were then individually genotyped by PCR revealing that 35 out of 40 (88%) of the germinated pollen grains had a wild-type (*SUT1*+) genotype (Fig. 5; Table 5).

Table 4. Genotypes of F₁ progeny of wild-type Nipponbare backcrossed with the three *SUT1*+/- lines

Female plant	Pollinator	No. of F ₁	F ₁ genotype	
			<i>SUT1</i> +/+	<i>SUT1</i> +/-
Nipponbare (<i>SUT1</i> +/+)	NC7083 (<i>SUT1</i> +/-)	27	27	0
	NF2752 (<i>SUT1</i> +/-)	28	28	0
	NF8036 (<i>SUT1</i> +/-)	19	19	0
Total		74	74	0

**Fig. 4.** Total number, maturity, and germination rate of the pollen of *SUT1*+/+ or *SUT1*+/- plants of the insertion mutant line NF2752. (A) The number of pollen (left) and the percentage of matured pollen (right) in an anther. Measurements of seven glumaceous flowers per plant were averaged and processed as the value of each plant, and the means of five individual plants for each genotype were plotted. Vertical bars indicate SD, and 'ns' shows not significantly different between the two genotypes (*t* test). (B) The *in vitro* pollen germination rate of *SUT1*+/+ or *SUT1*+/- plants. Five to seven observations per plant were averaged and processed as the value of each plant, and the means of 16 individual plants for each genotype were plotted. Vertical bars indicate SD, and the asterisks (***) show significant difference by *t* test (*P* < 0.001).

Discussion

In this study it was revealed that, in developing rice pollen, *OsSUT1* and *OsSUT3* are the most abundantly expressed among the five *SUT* genes in the rice genome. Compared

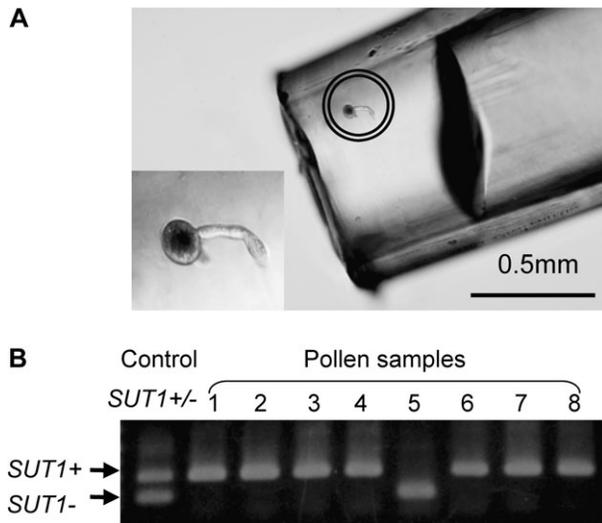


Fig. 5. Identification of the genotypes of individual pollen grain. (A) A pollen grain germinated on the agar medium was picked up with a fine pipette tip (marked in the circle). A magnified image of the pollen is lower left. (B) A typical result of genotyping by PCR, showing that seven out of eight pollen grains have wild type *OsSUT1* (*SUT1+*) gene. A PCR product with the DNA template from the leaves of *SUT1+/-* plant was used as a control.

Table 5. Genotypes of the pollens germinated on the agar media

	Genotype		<i>P</i> ^a
	<i>SUT1+</i>	<i>SUT1-</i>	
Number ^b	35	5	<0.001
Ratio	0.88	0.13	

^a Binominal probability of obtaining the results observed or less number of *SUT1-* genotype when equal ability of pollen germination for each genotype is hypothesized.

^b Three glumaceous flowers from each of five individual plants of NF2752 were used.

with the other four SUT genes, the expression and function of *OsSUT1* was the best characterized. *OsSUT1* was shown to be expressed in various tissues of rice plants; for example, it has been repeatedly shown to localize to the phloem of the leaf blades, leaf sheaths, and internodes (Matsukura et al., 2000; Scofield et al., 2007a, b); it is also expressed in both filial and maternal tissues in the developing caryopsis (Furbank et al., 2001; Hirose et al., 2002). By contrast, tissue-specific expression of *OsSUT3* has remained unclear, although *OsSUT3* mRNA expression has been detected in various organs at low and constant levels (Aoki et al., 2003; Scofield et al., 2007b). Thus, the relatively higher expression of *OsSUT3* and *OsSUT1* represents a novel and distinctive feature of developing pollen. To date, there are two plant species in which multiple SUT genes have been shown to be expressed in pollen: in *Arabidopsis* (*AtSUC1*, Stadler et al., 1999; *AtSUC3*, Meyer et al., 2004) and in *Plantago major* (*PmSUC1*, Lauterbach et al., 2007; *PmSUC3*, Barth et al., 2003). In both cases, the two SUTs belong to different classes in the phylogenetic tree, which is speculated to reflect

the complex roles of SUTs in pollen (Sauer, 2007). However in rice pollen, *OsSUT1* and *OsSUT3* belong to the same phylogenetic group (Aoki et al., 2003).

Altogether, our analysis of the gene disruption mutant revealed *OsSUT1* to be essential for function(s) in pollen to fertilize the ovule normally probably through its function(s) in pollen germination and/or pollen tube growth, but not for starch accumulation during pollen maturation, although some of the data were obtained using a single disruption line. While physiological function of *OsSUT1* was previously examined through the antisense suppression technique, no evidence for the function of the gene in pollen was reported (Ishimaru et al., 2001; Scofield et al., 2002). A possible explanation for this is that antisense suppression was insufficient to bring about detectable distortion of the segregation in the progeny or an increase in the number of infertile caryopsis while it was sufficient to cause poor grain-filling and retarded early growth of the seedlings. The role of SUTs in pollen germination and/or pollen tube growth has previously been reported for tomato and *Arabidopsis* (Hackel et al., 2006; Sivitz et al., 2008). However, *OsSUT1* mutant phenotypes are distinct from those reported. In *Arabidopsis*, mutant plants with homozygous *AtSUC1* gene disruption can be recovered, although the segregation is distorted in the progeny of the heterozygous plants (Sivitz et al., 2008). In rice, however, homozygous *SUT1-/-* plants have never been recovered among more than 500 progeny of *SUT1+/-* plants examined during the course of this study. This suggests that a lack of *AtSUC1* can be partially compensated for by other SUTs, including *AtSUC3*; while in rice, *OsSUT1* is essential to pollen for normal fertilization. By contrast, a fraction of the pollen grains germinated *in vitro* had the *SUT1-* genotype, indicating that *OsSUT1* is not essential for pollen germination, at least on the agar medium. Presumably, *SUT1-* pollen fails to fertilize due to pollen tube elongation that is slower than in *SUT1+* pollen, even if it can germinate on the stigma. Indeed, an attempt was made to look at the elongation rate of the pollen tube on the agar medium, but reproducible assessment was difficult which is probably related to extraordinarily shorter longevity of rice pollen (a few minutes after dehiscence of the anther) than other plant species. However, from our analysis presented here, together with data from studies of *AtSUC1* and *LeSUT2*, it is suggested that pollen germination represents a third mode of action for SUTs, in addition to phloem loading and seed maturation. Recently, Slewinski et al. (2009) characterized a disruption mutant of *ZmSUT1*, a maize SUT gene closely related to *OsSUT1*, and revealed that *ZmSUT1* functions in phloem loading. Notably, they recovered mutation homozygous plants of *ZmSUT1* used for their study, showing that unlike *OsSUT1*, disruption of *ZmSUT1* does not result in the complete dysfunction of pollen. However, it is not clear whether any segregation distortion was observed among the genotypes, which is potentially brought about by impaired pollen function. It is interesting that the two closely related SUT genes have different physiological functions; *ZmSUT1* acts as a phloem loader but is not

essential to pollen function while *OsSUT1* is essential to pollen function and plays an important role in grain-filling but not in phloem loading (Ishimaru *et al.*, 2001; Scofield *et al.*, 2002).

It is natural to hypothesize that sucrose taken up by *OsSUT1* represents an energy source and/or osmoticum for germinating pollen. However, the temporal patterns of the transcript levels of *OsSUT1* seem inconsistent with the presumed roles; *OsSUT1* mRNA accumulates more specifically during the starch accumulation period and decreases thereafter during pollen maturation. It has been reported that, in pollen, temporal differences can exist between the transcript levels and protein levels of a given gene. In *Arabidopsis*, the transcript level of *AtSUC1* is highest at the tricellular stage, followed by a decrease in mature pollen (Bock *et al.*, 2006). However, *AtSUC1* protein could be detected by immunofluorescence microscopy only after the onset of germination (Stadler *et al.*, 1999). A similar phenomenon was observed for a monosaccharide transporter *AtSTP9* (Schneidereit *et al.*, 2003). Although localization of *OsSUT1* protein is yet to be examined, it can be speculated that the transcription and the translation of *OsSUT1* are under similar regulation. It should be noted that these possible roles of sucrose may also be fulfilled by monosaccharides. In fact, preferential expression of monosaccharide transporters (i.e. MSTs) has been reported repeatedly; in *Arabidopsis*, as many as five MST genes are expressed in the pollen grain and/or pollen tube (see Büttner, 2007, for a review). Moreover, *Arabidopsis* pollen does germinate on agar media containing glucose but lacking sucrose (Sivitz *et al.*, 2008). However, in their analysis of an insertion mutant of one of the MST genes (*PMT1*) in *Petunia hybrida*, Garrido *et al.* (2006) found no defect in pollen function. Further study is clearly needed to characterize the physiological function of MSTs in relation to the function(s) of SUTs in pollen.

It was surprising that disruption of *OsSUT1* did not affect starch accumulation in pollen because *OsSUT1* is highly expressed in the starch-accumulating pollen and also because pollen grains are symplastically isolated from the surrounding tissue like developing endosperm, where *OsSUT1* function is crucial (Ishimaru *et al.*, 2001; Scofield *et al.*, 2002). Because *OsSUT3* is expressed in pollen along with *OsSUT1* (Fig. 1), it may be possible that *OsSUT3* plays a role to supply the sucrose for starch accumulation. Here again, it is also possible that MSTs import hexoses as substrates for starch synthesis. Castro and Clément (2007) suggested that hexoses are predominantly imported and utilized for pollen maturation based on both the sugar levels and the activities of sugar metabolizing enzymes separately measured in different fractions of lily anther, i.e. anther wall, locular fluid, and pollen. In addition, expression of the genes for MSTs has been repeatedly documented as mentioned above. However, no evidence has been presented for the function of the MSTs in developing pollen. Consequently, it remains an important and open question of which protein(s) loads the sugar for starch synthesis into pollen.

In conclusion, by conducting the first comprehensive expression analysis of members of the rice SUT gene family, it was determined that both *OsSUT1* and *OsSUT3* are predominantly expressed in developing rice pollen. *OsSUT1* was also suggested to be essential for normal pollen germination, but not for pollen maturation or starch accumulation; whereas the function of *OsSUT3* remains undetermined.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. The percentage of matured pollen in the three gene disruption lines.

Supplementary Fig. S1. Typical images showing the procedure of LM to obtain the developing pollen and qualitative assessment of the resultant RNA.

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