

# Rice *Importin β1* Gene Affects Pollen Tube Elongation

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**Importin β1 interacts with nuclear transport factors and mediates the import of nuclear proteins. We isolated a pollen-expressed gene, rice *Importin β1* (*Oslmpβ1*), from a T-DNA insertional population that was trapped by a promoterless β-glucuronidase (*GUS*) gene. The *GUS* reporter was expressed in the anthers and ovaries from early through mature developmental stages. Its expression was also observed in all floral organs. However, these patterns changed as the spikelet developed. T-DNA was inserted into the *Oslmpβ1* gene at 339 bp downstream from the translation initiation site. We obtained another T-DNA insertional allele by searching the flanking sequence tag database. In both lines, the wild-type and T-DNA-carrying progeny segregated at a ratio close to 1:1. The latter genotype was heterozygous (*Oslmpβ1/osimpβ1*). Reciprocal crosses between WT and heterozygous plants demonstrated that the mutant alleles could not be transmitted through the male gametophyte. Close examination of the heterozygous anthers revealed that the mutant pollen matured normally. However, *in vitro* assays showed that tube elongation was hampered in the mutant grains. These results indicate that *Oslmpβ1* is specifically required for pollen tube elongation.**

## INTRODUCTION

Fast pollen tube elongation is essential for the fertilization of plant ovules, and pollen grains compete for success among those tubes within the styles. Several genes essential for tube growth have been studied using *Arabidopsis thaliana* mutants. For example, the *poky pollen tube* (*pok*) mutant exhibits very short tubes (Lobstein et al., 2004). The POK protein localizes to the Golgi apparatus, possibly being involved in the retrograde trafficking of vesicles. Furthermore, *seth1* and *seth2* mutations almost entirely block male transmission through reduced pollen germination and tube growth (Lalanne et al., 2004). They encode proteins that are homologous to conserved proteins involved in the first step of the glycosylphosphatidylinositol biosynthesis pathway. This finding suggests that glycosylphosphatidylinositol anchor biosynthesis is essential for pollen tube

wall deposition or metabolism. The gene encoding the exocyst component ortholog, *AtSEC8*, is required for both pollen germination and competitive pollen tube growth (Cole et al., 2005). The significance of the calcium-signaling pathway during pollen germination and tip growth has been investigated with the *no pollen germination 1* (*npg1*) gene, which encodes a protein related to the calmodulin-binding proteins (Golovkin and Reddy, 2003). It has been also known that a signaling regulator, PRK1 plays important roles in pollen tubes development in *Petunia inflata* (Park et al., 2000).

Antisense suppression of *SHY*, which encodes a leucine-rich repeat protein, suppresses tube growth and causes abnormal callose deposits throughout the tube and into the tips (Guyon et al., 2004). Tube growth is also affected by a T-DNA insertional mutant in *AtTAF6*, which encodes a TATA-binding protein-associated factor, indicating that transcriptional regulation of only a specific subset of genes is controlled by this basal transcription factor (Lago et al., 2005). Pollen germination and tube growth also require *COMATOSE*, which encodes a peroximal ABC transporter required for the oxidation of storage lipids during germination (Footitt et al., 2007), as well as the *nicotinate/nicotinamide mononucleotide adenylyltransferase* (*AtNMNAT*) gene, which catalyzes the synthesis of nicotinate adenine dinucleotide from nicotinate mononucleotide (Hashida et al., 2007).

In rice, no mutations defective in their pollen tube elongation have yet been reported. Instead, proteomics analysis has identified a large number of proteins that are specifically present in germinated pollen (Dai et al., 2007). Two-dimensional electrophoresis of approximately 2300 protein spots has revealed 186 that are differentially expressed in mature and germinated pollen. Differentially expressed protein spots have been found, via mass spectrometry, to match 120 diverse protein species. These proteins are involved in various cellular and metabolic processes, with an obvious functional skew toward cell wall metabolism, protein synthesis and degradation, cytoskeleton dynamics, and carbohydrate/energy metabolism.

Nuclear import and export pathways are mediated by nuclear transport receptors - importin proteins - that regulate diverse cellular functions. Most importin proteins contain nuclear localization signals (NLS) that comprise one or two short stretches of basic amino acids (Dingwall and Laskey, 1991). In the cyto-

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sol, proteins that carry NLS bind to importin  $\alpha$  and form a nuclear pore complex. This complex interacts with the nuclear membrane protein importin  $\beta$  via an amino-terminal IBB domain. Then, this trimeric import complex docks to the cytoplasmic face of the nuclear pore complex via importin  $\beta$ , and subsequently is translocated to the nucleoplasmic side. In the nucleus, Ran-GTP binds to importin  $\beta$  and induces the release of import cargoes. This nuclear transport receptor family shares an amino-terminal Ran-GTP-binding domain, which shows limited sequence conservation. Members bind very specifically to the different classes of macromolecules that must be transported across the nuclear envelope. The specificity of these transport processes is ensured by the variety of receptors that recognize different substrates (for a review, see Görlich and Kutay, 1999).

The first plant *Importin β* genes were identified from rice by cloning two (*Oslmpβ1* and *Oslmpβ2*) cDNA clones (Matsuki et al., 1998). Transcripts of both genes were detected in roots, shoots, and calli. *In vitro* binding assays have indicated that rice importin  $\beta1$  assembles a complex with importin  $\alpha1$  and an NLS protein, and also binds to the nuclear envelope (Jiang et al., 1998). Moreover, Ran-GTP, but not Ran-GDP, interacts with rice importin  $\beta1$  and dissociates the heterodimer formed between rice importins  $\alpha1$  and  $\beta1$ . In HeLa cells, the rice importin  $\beta1$  mediates nuclear envelope-docking of the NLS proteins and their subsequent translocation into the nucleus (Jiang et al., 1998).

Although many importin  $\beta$ -like proteins exist in the *Arabidopsis* genome, only a few have been characterized. The nuclear export receptor XPO1, which specifically binds to leucine-rich nuclear export signal (NES), has been functionally analyzed (Haasen et al., 1999). The *Arabidopsis* protein interacts with *Arabidopsis* Ran and with NESs of plant and human proteins. Export activity within a plant cell has been demonstrated *in vivo* using an assay system that employs green fluorescent protein (GFP) fusion proteins. This finding demonstrates the high conservation of that nuclear export pathway among animals, yeast, and plants (Haasen et al., 1999). Among the 17 members of the importin  $\beta$  family, HASTY was the first shown to have an essential role in plants. A loss-of-function mutation of *HASTY* affects many different processes in *Arabidopsis* growth and accelerates its developmental program (Bollman et al., 2003; Telfer and Poethig, 1998). In contrast, mutation of another *Arabidopsis* exportin, PAUSED, causes delayed leaf initiation and transition to flowering (Hunter et al., 2003). Furthermore, a *hasty/paused* double mutant has a more severe phenotype than either single mutant (Hunter et al., 2003), suggesting that these two exportins act on different pathways. *Sensitive to ABA and Drought 2* (*SAD2*) mutations in another member of the importin  $\beta$  family cause ABA hypersensitivity in seed germination and seedling growth (Verslues et al., 2006). This suggests a role for nuclear transport of importin  $\beta$  proteins in ABA signal transduction.

In this study, we examined the role of *Importin β1* in rice development, using loss-of-function mutants to investigate their defects in pollen tube elongation and fertilization rates.

## MATERIALS AND METHODS

### Plant growth

Seeds from wild-type japonica rice (*Oryza sativa* cv. Dongjin) and heterozygous (*Oslmpβ1/oslmpβ1*) plants were germinated on a Murashige and Skoog (MS) medium containing 0.44% MS basal salts, 3% sucrose, 0.2% phytigel, and 0.55 mM myo-inositol (Sigma, USA). The seedlings were grown for 1 week at 27°C under continuous light, then transplanted to soil in the greenhouse and raised to maturity.

### Histochemical GUS assay and microscopic analyses

Histochemical GUS staining was performed as described by Kim et al. (2009). For light-microscopic analysis, the tissues were fixed in a solution containing 50% ethanol, 5% acetic acid, and 3.7% formaldehyde, and then embedded in Technovit 8100 Resin (Kulzer and Co., Germany). These samples were sectioned to 3- to 10- $\mu$ m thickness with a microtome (Leica, Germany), and observed under a microscope (Nikon, Japan) using bright- and dark-field illumination.

### Isolation of T-DNA flanking sequence by TAIL-PCR and identification of a fusion transcript between the *Oslmpβ1* and *GUS* genes

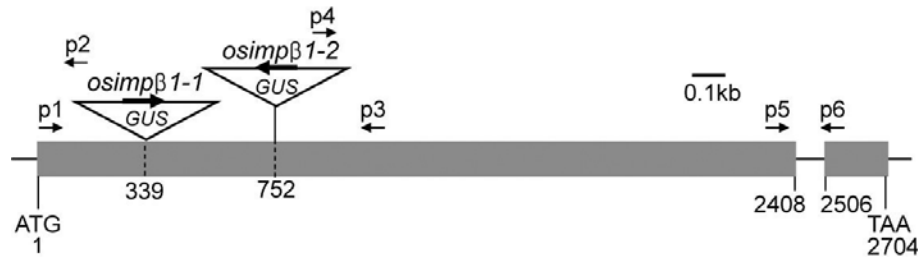
PCRs were performed in 50  $\mu$ l of a mixture containing 2 ng of cDNA, 10 $\times$  Ex Taq buffer, 0.2 mM dNTP, 0.5 unit of Ex Taq polymerase (Takara, Japan), and 1  $\mu$ M of the primers. Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) was conducted as described previously (Jung et al., 2005; Liu et al., 1995). The template was cDNA prepared from the panicles of *Oslmpβ1/oslmpβ1-1* plants collected at the mature stage. The specific primers were gus1 (5'-GCCGTAATGAGTGAC CGCATCG-3'), gus2 (5'-ATCTGCATCGGCGAACTGATCG-3'), and gus3 (5'-CACGGGTTGGGGTTTCTACAGG-3'). Arbitrary primers included ad1 [5'-NTCGA(G/C)T(G/C)G(A/T)GTT-3'], ad2 [5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3'], and ad3 [5'-(A/T)GTGNAG(A/T)ANCANAGA-3']. The third PCR product was subjected directly to sequencing using the gus3 primer. The fusion transcript between *Oslmpβ1* and *GUS* was amplified by the p1 primer (5'-TCCTGCTATCAGCTCAATCT-3', 17 bp downstream from the translation initiation site of the *Oslmpβ1* gene; Fig. 1) and by the p2 primer (5'-CATCACTTCTGATTATTG ACC-3', 316 bp downstream from the translation initiation site of the *GUS* gene; Fig. 1).

### Genotyping the mutant plants

PCRs for genotyping were performed in 50  $\mu$ l of a mixture containing 20 ng of plant DNA, 10 $\times$  Ex Taq buffer, 0.2 mM dNTP, 0.5 unit of Ex Taq polymerase (Takara), and 1  $\mu$ M of the primers. The protocol included 35 cycles of 94°C for 60 s, 60°C for 90 s, and 72°C for 120 s. Primers for genotyping were 5'-TCCTGCTATCAGCTCAATCT-3' (17 bp downstream of the translation initiation site of *Oslmpβ1*; p1 in Fig. 1), 5'-TAGCAAT GAGCCCAAGGCAT-3' (1050 bp downstream from the translation initiation site of *Oslmpβ1*; p3 in Fig. 1), 5'-CATCACTT CCTGATTATTGACC-3' (316 bp downstream from the translation initiation site of *GUS*; p2 in Fig. 1), and 5'-ATGGCAGTGA ATTAACATAGC-3' (2924 bp downstream from the translation initiation site of the *GUS* gene; p4 in Fig. 1).

### Quantitative real-time RT-PCR

Total RNAs were isolated using Tri Reagent (MRCI Inc., USA). For the first-strand cDNA synthesis, 1  $\mu$ g of total RNA was reverse-transcribed in a total volume of 20  $\mu$ l that contained 10 ng of oligo(dT)<sub>12-18</sub> primer, 2.5 mM deoxynucleotide triphosphate (dNTP), and 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Promega, USA) in a 5 $\times$  reaction buffer. Quantitative real-time PCR was performed with a Roche Lightcycler, using SYBR premix ExTaq (Takara). PCR reactions were carried out in a final volume of 20  $\mu$ l of reaction solution containing 10  $\mu$ l PCR mixture, 1  $\mu$ l cDNA, and 0.5  $\mu$ M of each primer, under the following conditions: 1 min at 95°C, then 45 cycles at 94°C for 15 s, 60°C for 13 s, and 72°C for 12 s. At the end of the reaction, the specificity of the primer set was confirmed by a melting curve. The *Ubiquitin 1* (Os03g0221500, LOC\_Os03g12140) mRNA level was used to normalize the expression ratio for each



**Fig. 1.** Schematic diagram of *OsImpβ1* gene and T-DNA insertion positions in *osimpβ1* mutants. Black boxes represent exons; intervening lines, introns. Positions of T-DNA insertion are indicated with triangles. Numbers indicate nucleotide positions from the start ATG codon. Primer sets of p1 and p3, and of p1 and p2 were used for genotyping progenies of *OsImpβ1/osimpβ1-1* selfed, *OsImpβ1/osimpβ1-1* (female) × WT (male), and WT (female) × *OsImpβ1/osimpβ1-1* (male) lines. Primer sets of p1 and p3, and of p3 and p4 were used for genotyping progenies of *OsImpβ1/osimpβ1-2* selfed line.

gene. Primers for the *Ubiquitin 1* gene were 5'-CACGGTCAA CAACATCCAG-3' and 5'-TGAAGACCCTGACTGGGAAG-3'. The primers for *OsImpβ1* were 5'-GGCATACTCCAGGGTAT AAA-3' (2311 bp downstream from the translation initiation site of the gene; p5 in Fig. 1) and 5'-ACTCCCTCAGGAATCAA CA-3' (2608 bp downstream from the translation initiation site of the gene; p6 in Fig. 1). Changes in gene expression were calculated according to the delta-delta  $C_t$  method.

#### *In vitro* pollen germination

Pollen grains from dehisced anthers (both wild-type and mutant) were placed on a slide glass at 35°C for 2 h in a pollen germination medium consisting of 1 mM CaCl<sub>2</sub>, 1 mM KCl, 0.8 mM MgSO<sub>4</sub>, 1.6 mM H<sub>3</sub>BO<sub>3</sub>, 30 μM CaSO<sub>4</sub>, 0.03% casein, 0.3% 2-(N-morpholino) ethanesulfonic acid, 10% sucrose, and 12.5% polyethylene glycol. The humidity was maintained above 90%. Germinated pollen grains were then stained in a GUS assay solution at 37°C for 3 h. The grains were observed with a microscope (Nikon), using bright-field illumination.

#### 4', 6-daimidino-2-phenylindole (DAPI) staining

Pollen grains were fixed in an ethanol:acetic acid (3:1) solution for 1 h at room temperature, then dehydrated through an ethanol series (75, 55, and 35%). They were transferred to Tris-HCl buffer (pH 7.5) with 200 ng ml<sup>-1</sup> DAPI, and incubated for 1 h at 60°C. The stained pollen was monitored under UV light with a Zeiss Airplane 2 microscope.

#### Ultrastructural analyses by transmission electron microscopy

Anthers sampled from *OsImpβ1-1/osimpβ1-1* plants at the mature stages were fixed for 4 h in cacodylate buffer (pH 7.2) that contained 2% paraformaldehyde (Sigma) and 2% glutaraldehyde (Sigma). They were then rinsed with the same buffer and post-fixed for 1 h in cacodylate buffer containing 1% osmium tetroxide (Pelco International, USA). After dehydration, the specimens were embedded in London Resin White (London Resin Co., UK). Ultra-thin sections (40 to 60 nm thick) collected on uncoated nickel grids (300 mesh) were stained with 4% uranyl acetate and examined at 60 to 80 kV with a JEOL 1200 transmission electron microscope (JEOL Ltd., Japan).

#### Transient expression of GFP and RFP fusion constructs in protoplasts

Protoplasts were prepared from rice mesophyll cells. They were harvested and incubated in an enzyme solution (1.5% cellulase RS, 0.5% macerozyme, 0.1% pectolyase, 0.6 M mannitol, 10 mM MES, 1 mM CaCl<sub>2</sub>, and 0.1% bovine serum albumin) for 4

to 5 h at 26°C with gentle agitation (50 to 75 rpm). The protoplasts were isolated from the undigested material with a nylon mesh (20 μm) and centrifuged at 40 to 60 × *g* for 2 min. They were then re-suspended in 5 ml of KMC solution (117 mM KCl, 82 mM MgCl<sub>2</sub>, and 85 mM CaCl<sub>2</sub>) and pelleted again by centrifugation for 90 s at 40 to 50 × *g*. These pelleted protoplasts were re-suspended in an EP3 solution (70 mM KCl, 5 mM MgCl<sub>2</sub>, 0.4 M mannitol, and 0.1% MES; pH 5.6). For our transfection experiment, 1 × 10<sup>6</sup> protoplasts were electroporated with 20 μg each of the fusion constructs, using the Gene Pulser Xcell (BioRAD, USA) at 300 V and 450 μF. Following transformation, expression of the fusion constructs was monitored at various times with a Zeiss LSM510 confocal laser scanning microscope. The primers for constructing the *OsImpβ1-GFP* fusion vectors were 5'-CCCGGGGGTACAGCCATGAATATC AC-3' (the ATG start codon of *OsImpβ1* is underlined) and 5'-TCGAGCTTAAGAAACCAGTGCTTGTT-3' (the TAA stop codon is underlined).

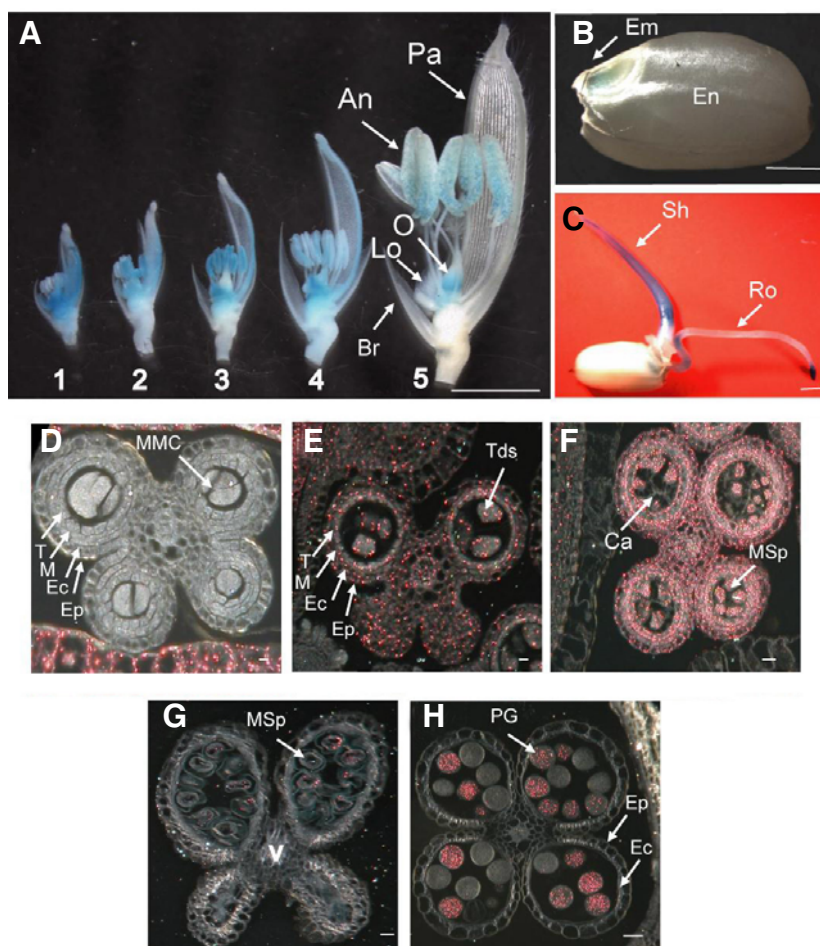
## RESULTS

### Isolation of a promoter trap line using GUS activity in a pollen-expressed gene

We have previously generated insertional mutant populations by T-DNA (Jeon et al., 2000; 2002; Jung et al., 2003; Ryu et al., 2004). Within that T-DNA, a promoterless *GUS* gene is placed immediately next to the T-DNA border so that insertion of the T-DNA within a gene can generate a fusion between an endogenous gene and the *GUS* reporter. *GUS* analysis of the developing spikelets has allowed us to identify several lines with *GUS* activity in their pollen (Han et al., 2006).

Here, we report the characterization of Line 0-19302. In the spikelets, *GUS* expression was observed in the anthers and ovary from the early through mature developmental stages (Fig. 2A). The activity was also observed in the palea/lemma and lodicules during earlier stages, but disappeared later (Fig. 2A). The assay indicated that the gene was also expressed in the embryo of mature seeds (Fig. 2B) and shoots of 4 day old seedling (Fig. 2C). Finally, *GUS* was strongly detected in the root tips of the seedling (Fig. 2C).

To observe these expression patterns at the cellular level, we cross-sectioned the anthers at five developmental stages. At the microspore mother cell stage, *GUS* expression was not detected in the anthers (Fig. 2D). *GUS* expression was found in all cell types of anthers at the meiotic (Fig. 2E) and tetrad stages (Fig. 2F), but was restricted to only the pollen at the late-vacuolated (Fig. 2G) and mature-pollen stages (Fig. 2H). About half the pollen grains were stained by *GUS* assay, indicating



**Fig. 2.** Expression profiles of the *Oslmpβ1* gene using GUS assay. (A) Temporal and spatial expression patterns of the *Oslmpβ1*-GUS gene fusion product in *Oslmpβ1/osimpβ1-1* spikelets at various developmental stages: Samples 1, pollen mother cell stage; 2, meiosis stage; 3, tetrad stage; 4, late vacuolated stage; 5, mature pollen stage. (B) Expression patterns in a mature seed. (C) Expression patterns in a seedling. Bar = 2 mm. Cross sections of anthers at pollen mother cell stage (D), meiosis stage (E), tetrad stage (F), late vacuolated stage (G), and mature pollen stage (H). Photographs were taken with dark-field microscope (D-H), where GUS activity appeared as red coloring. An, anther; Br, bract; Ca, callose; EC, endothelial cell layer; Em, embryo; En, endosperm; Ep, epidermal cell layer; Lo, lodicles; M, middle layer; MMC, microspore mother cells; MSp, microspores; O, ovary; Pa, palea; PG, pollen grain; Ro, root; Sh, shoot; T, tapetal cell layer; Tds, tetrads; V, vascular region. Bars = 40 μm.

segregation of the GUS reporter gene in male gametophyte (Figs. 2G and 2H). The cross-section analysis showed that GUS expression in the palea/lemma was the highest at the microspore mother cell stage, and the expression disappeared at mature-pollen stage, indicating that GUS patterns in developing pollens were antiparallel with those in developing palea/lemma (Figs. 2D-2H).

#### T-DNA insertion occurs in a gene encoding Importin β1

We determined the T-DNA flanking sequence for Line 0-19302 via thermal asymmetric interlaced PCR (Jung et al., 2005). T-DNA was inserted into the rice *Importin β1* (*Oslmpβ1*) gene at 339 bp downstream from the translation initiation site (Fig. 1A). The *Oslmpβ1* protein was previously reported to mediate nuclear envelope docking of nuclear localization signal (NLS) containing proteins and their subsequent translocation into the nucleus (Jiang et al., 1998; Matsuki et al., 1998). In order to obtain another allele, we searched our flanking sequence tag (FST) database (An et al., 2003; Jeong et al., 2006). This resulted in the identification of Line 2C-10311, which carries the T-DNA insertion at 752 bp downstream from the *Oslmpβ1* translation initiation site (Fig. 1).

#### Analyses of *Oslmpβ1* expression

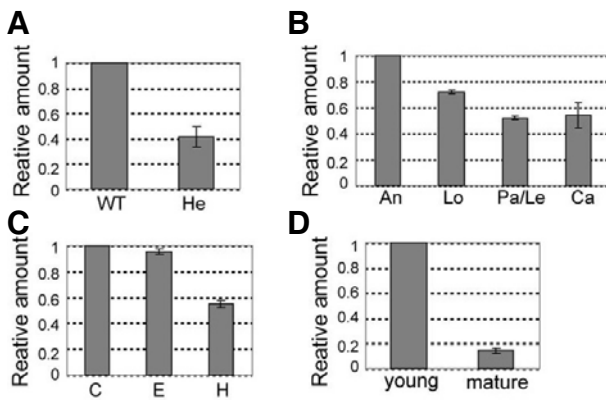
We measured transcripts of *Oslmpβ1* by using quantitative real-time RT-PCR with gene-specific primers with total RNAs prepared from various organs (P5 and P6 in Fig. 1). As expected, the transcript level in heterozygous anthers (*Oslmpβ1/*

*osimpβ1-1*) was about half of the amount measured from the wild-type anthers (Fig. 3A). Quantitative real-time RT-PCR analyses in mature spikelets showed that transcription was the highest in the anthers but only moderate in the other floral organs (Fig. 3B). Furthermore, amount of transcript was higher in the root tip and root elongation zone than in the root hair zone (Fig. 3C). In the palea/lemma, transcription was greater at the young stage compared with the mature stage (Fig. 3D). Overall, these results are in good agreement with our GUS data.

#### T-DNA insertions in *Oslmpβ1* cause aberrant transmission through male gametophytes

All of the progeny from the T-DNA tagging lines were normal. Therefore, we determined the T-DNA segregation in T2 progeny of Line 0-19302 by analyzing GUS expression and hygromycin resistance (Table 1). As a result, the segregation ratio of wild-type and T-DNA-carrying progeny was 143:150, that is, close to 1:1. We observed a similar pattern (94:90) from Line 2C-10311. These results indicate that transmission of T-DNA was hampered in the gametophyte. We genotyped the progeny from the selfed population of the primary transgenic plants and observed that all the T-DNA-carrying progeny were heterozygous (Table 1). Therefore, we could predict that either the male or female gametophytes would be defective in the insertional lines.

To determine whether this defect was due to a problem in the development and production of male or female gametophytes, we performed reciprocal crosses between wild-type and *Oslmpβ1/*



**Fig. 3.** Quantitative real-time RT-PCR analyses of *Oslmpβ1* expression. (A) Quantitative real-time RT-PCR analysis of *Oslmpβ1* in wild-type and hetero (*Oslmpβ1/osimpβ1-1*) anthers at mature pollen stage. (B) Spatial expression levels of *Oslmpβ1* in mature floral organs (C) Temporal expression patterns of *Oslmpβ1* in wild-type roots at 10 days after germination. (D) Temporal expression patterns of *Oslmpβ1* in wild-type palea/lemma at young and mature stages. An, anther; C, root cap; Ca, carpel; E, elongation zone; H, root hair zone; Le, lemma; Lo, lodicule; Pa, palea. The *Oslmpβ1* expression levels were normalized with respect to the internal control *Ubiquitin 1* (*Os03g0221500*, *LOC\_Os03g12140*) gene. The gene expression levels were calculated by the delta  $C_t$  Method.

*osimpβ1-1* heterozygous plants (Line 0-19302). When the latter were used as the female, both WT and heterozygous progeny were obtained (Table 1). However, when *Oslmpβ1/osimpβ1-1* plants were used as the pollen donor, only wild-type progeny were produced (Table 1). These experiments indicate that the *osimpβ1* mutation caused a defect in the male gametophyte.

#### ***Oslmpβ1* is not essential for pollen maturation**

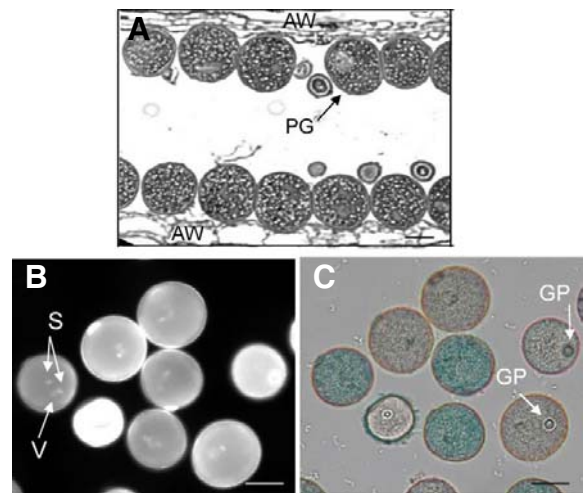
Because mutations in *Oslmpβ1* prevented the transmission of genetic materials through the male gametophyte, we examined ultrastructure of *Oslmpβ1/osimpβ1-1* anthers at the mature pollen stage. Longitudinal sections, analyzed by transmission electron microscopy (TEM), revealed that all the pollen grains were morphologically normal (Fig. 4A), thereby indicating that the *osimpβ1-1* pollen reached the mature stage without any developmental defect. Using DAPI staining to determine whether the grains successfully achieved the trinuclear stage, we noted that all contained three nuclei, with the two more brightly stained small organelles being the sperm nuclei, and the larger and diffusely stained one being the vegetative cell nucleus (Fig. 4B). GUS staining enabled us to distinguish *osimpβ1-1* pollen grains from the wild-type (Fig. 4C). We also tested pollen viability via fluorescein diacetate staining and observed that both types of pollen were equally viable (data not shown).

#### **Pollen tube elongation is delayed in *osimpβ1-1***

Because the *osimpβ1-1* pollen grains matured normally, we analyzed pollen germination and elongation under *in vitro* conditions. Here, 98% (501/509) of the wild-type grains germinated and elongated normally (Fig. 5A), compared with a rate of 46% (347/755) for the *Oslmpβ1/osimpβ1-1* grains (Fig. 5B). GUS staining of these germinated pollens showed that those unelongated grains were mostly GUS-positive while those that lengthened normally were GUS-negative (Fig. 5C). Therefore, we may conclude that mutations of the *Oslmpβ1* gene affect

**Table 1.** Genetic analyses of male or female gene transfer caused by mutations of *Oslmpβ1*

Cross (female x male)	No. plants WT	No. plants <i>Oslmpβ1/osimpβ1</i>	Observed ratio	Expected ratio	$\chi^2$
<i>Oslmpβ1/osimpβ1-1</i> selfed Line 0-19302	143	150	1 : 1.05	1 : 1	0.17
<i>Oslmpβ1/osimpβ1-2</i> selfed Line 2C-10311	94	90	1 : 0.96	1 : 1	0.09
<i>Oslmpβ1/osimpβ1-1</i> x WT	26	29	1 : 1.12	1 : 1	0.16
WT x <i>Oslmpβ1/osimpβ1-1</i>	52	0	N/A	N/A	N/A

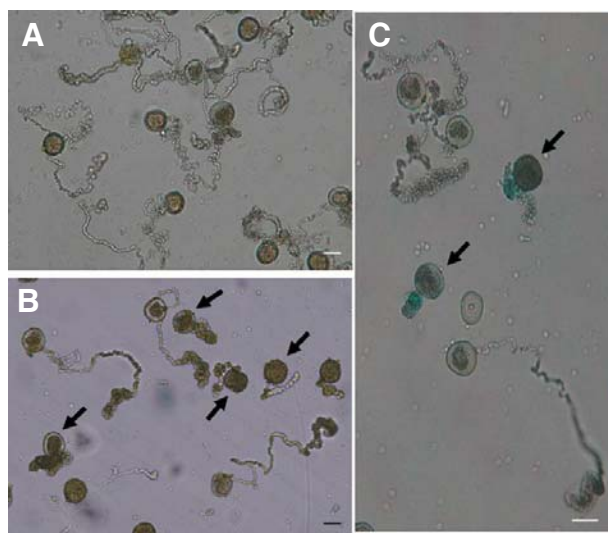


**Fig. 4.** Transmission electron microscopy analysis of *Oslmpβ1/osimpβ1-1* anther. Longitudinal sections of *Oslmpβ1/osimpβ1-1* anther at mature stage (A). Pollen grains from the *Oslmpβ1/osimpβ1-1* anther at mature stage were stained with DAPI (B) and GUS solutions (C). Stained pollen grains were monitored with a fluorescence microscope (B) and a bright-field microscope (C). AW, anther wall; GP, germination pore; PG, pollen grain; S, sperm nuclei; V, vegetative nucleus. Bars = 50  $\mu$ m.

pollen tube elongation.

#### ***Oslmpβ1* is located at the nucleus**

Jiang et al. (1998) have previously reported that the Cy3-labeled rice Importin  $\beta 1$  is accumulated predominantly in the nuclear envelope of tobacco BY-2 cells. To confirm that observation, we made a fusion molecule by linking the carboxy-terminal end of the full-length *Oslmpβ1* cDNA to the *green fluorescent protein* (*GFP*) gene. This construct was placed under the control of the *35S* promoter and the *nos* terminator (Fig. 6A). As a positive control, the cDNA of a previously characterized nuclear protein, *OsmADS3*, was fused to the *red fluorescent protein* (*RFP*) gene and located between the same regulatory elements (Kang et al., 1998). When the chimeric molecules were co-introduced into protoplasts prepared from rice mesophyll cells, both green and red fluorescence signals were detected in the nucleus (Figs. 6B-6D).



**Fig. 5.** *In vitro* pollen germination assays. Mature pollen grains from wild-type (A) and *OsImpβ1/osimpβ1-1* (B) anthers were germinated for 2 h in the pollen germination medium. Germinated pollens from the *OsImpβ1/osimpβ1-1* anther were stained in GUS solution for 3 h before microscope observations (C). Thick arrows indicate the *osimpβ1-1* pollen. Bars = 50  $\mu$ m.

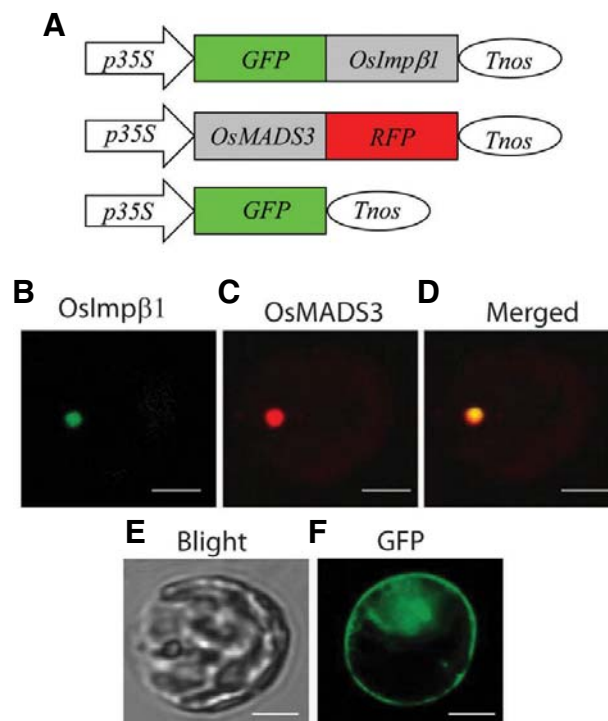
## DISCUSSION

### T-DNA tagging using the GUS reporter gene is an efficient way to identify gametophyte-defective mutants

Screening of gametophytic mutants is difficult because it generally requires a large number of plants to be assayed under a microscope (Howden et al., 1998; Lalanne et al., 2004; Procissi et al., 2001). Therefore, we have previously utilized a method of enriching such mutants by screening the lines that express the GUS reporter gene in male gametophytes. In doing so, we have identified mutants defective in that reproductive organ. For example, this strategy has led to the discoveries of mutations in the *Undeveloped Tapetum 1 (Udt1)* gene, a major regulator of early tapetum development (Jung et al., 2005), and in the *Wax-deficient anther (Wda1)* gene that is involved in very-long-chain fatty acid biosynthesis (Jung et al., 2006). This protocol has also enabled us to identify another mutant in the *RICE IMMATURE POLLEN 1 (RIP1)* gene, a regulator of late pollen development (Han et al., 2006). Here, we identified *osimpβ1* mutants defective in their pollen tube elongation using the GUS trap system.

### The *OsImpβ1* gene is ubiquitously expressed in proliferating cells

Our T-DNA vector used for the gene trap has promoterless *GUS* gene near the right border of T-DNA. Therefore, it could generate a translational fusion when it is inserted within a gene in a correct orientation. The resulting GUS expression pattern should represent endogenous activity of the tagged gene. It is different from transgenic expression from exogenously introduced promoter-GUS constructs, which may show position effect. GUS analysis of our T-DNA tagged line showed that the *OsImpβ1* gene was expressed in all floral organs, from early developmental stages to maturity. Expression was higher in young floral buds, with transcript levels decreasing thereafter. Upon maturation, expression could no longer be detected in



**Fig. 6.** Subcellular localization of *OsImpβ1*. (A) Schematic diagrams of fusion constructs of GFP and *OsImpβ1* cDNA (upper) and of *OsMADS3* cDNA and *RFP* (middle). Fusion constructs were placed between the CaMV 35S promoter (*p35S*) and *nos* terminator (*Tnos*). Protoplasts isolated from mesophyll cells were co-transfected with *p35S-GFP-OsImpβ1-Tnos* and *p35S-OsMADS3-RFP-Tnos* vectors, and green fluorescent signal (B) or red fluorescent signal (C) was examined 30 h after transfection. (D) Merged photo of (B) and (C). (E) Bright field microscopy of the protoplast observed in (B)-(D). (F) Control GFP signal in a mesophyll protoplast obtained by transiently expressing *p35S-GFP-Tnos*. Bars = 10  $\mu$ m.

any organs except pollen grains and ovaries. In vegetative organs, the gene was expressed in seedling roots and shoots, but declined in its induction in mature tissues. Therefore, it was apparent that *OsImpβ1* is expressed and functional in actively proliferating cells.

### The *osimpβ1* is a male-gametophytic mutant

Our *osimpβ1* mutant lines exhibited segregation distortion. The *osimpβ1-1* homozygous progeny of Line 0-19302 were not present during four consequent generations produced by selfing *OsImpβ1/osimpβ1-1* plants. Homozygous progeny also were lacking in another allele, Line 2C-10311. Reciprocal crosses between heterozygous plants and the wild type indicated a problem in transmitting the *OsImpβ1* gene through the male gametophyte. Such a male-specific defect can result from damage during microsporogenesis or gametogenesis. However, our microscopic examinations revealed no characteristics that would distinguish the mutant pollen from the wild-type, nor did any affect sperm cell development and division.

Even if there are two more genes closely linked to *OsImpβ1* in the phylogenetic tree (Supplementary Fig. S1), expression pattern of the genes suggest that *OsImpβ1* plays predominant roles in reproductive stages. Although *OsImpβ1* (LOC\_Os05g28510) and LOC\_Os03g18350 showed similar expression pat-

terms, *Oslmpβ1* expression was higher than LOC\_Os03g18350 especially during floral development. The third gene LOC\_Os12g38110 was highly expressed in germinating and developing seeds, but weakly during floral development.

### ***Oslmpβ1* is necessary for pollen tube growth**

Pollen tube growth requires changes in gene expression, and the proteins needed for this must be transported into the nucleus. Within 1 h of pollination, a large number of proteins are synthesized in the germinating pollen (Taylor and Hepler, 1997). Many of these contain a nuclear localization signal and are probably transported by importin protein family members. It is likely that the failure to transfer these specific proteins affects tube elongation.

Under *in vitro* conditions, the *osimpβ1* pollen germinated normally but its tube elongation was hampered. Therefore, the mutant grains were unable to compete with wild-type gametophytes. This resulted in a male-specific transmission defect. Because this mutant phenotype was not observed during pollen maturation and germination, and not at all in the female gametophytes, it is evident that *Oslmpβ1* is absolutely required for pollen tube elongation. It is possible that the rice Importin β1 protein is necessary for nuclear-targeting of a group of proteins that are needed specifically for tube elongation. For example, the pollen tube interacts intimately with the nutrient-rich extracellular matrix of the stylar tract, and adhesion factors implicated in this tube growth have been identified (Lord and Russell, 2002). Studies of nucleocytoplasmic receptors in yeast and mammals have indicated that some receptors regulate the transport of a large number of functionally diverse proteins, whereas others have a more limited range of cargo molecules (for reviews, see Görlich, 1997; Nigg, 1997). Possible targets are the MIKC\* MADS-proteins, which bind motifs enriched in the proximal region of late pollen-specific *Arabidopsis* promoters (Verelst et al., 2007). A considerable number of putative direct-target genes for the AtMIKC\* transcription factor complexes are either known or have proposed functions in pollen tube growth. We investigated the nuclear localization of *Oslmpβ1* by transiently expressing GFP-*Oslmpβ1* in mesophyll cells. *Oslmpβ1*-GFP was observed mostly in and around the nucleus, similar to subcellular localization of the GFP-tagged SAD2 encoding *Arabidopsis* Importin β. However, our result is different from those from tobacco BY-2 cells, where *Impβ1* was localized to the nuclear envelope (Jiang et al., 1998). Therefore, we do not rule out the possibility that the N-terminal fusion interfered with a proper localization of *Oslmpβ1*.

Alternatively, *Oslmpβ1* may not have target specificity but is required when a cell is in large demand for protein import to the nucleus. Pollen tubes grow at extremely high rates *in vivo* and, consequently, have a very great need for energy (Lord and Russell, 2002). It will be interesting to determine whether *Oslmpβ1* controls general translocation of nuclear proteins by testing various GFP-nuclear proteins in the *osimpβ1* mutants. Because we did not obtain any homozygous progeny, however, we were unable to examine the effect of this *osimpβ1* mutation on seedling growth. In particular, it will be interesting to determine whether the *Oslmpβ1* gene is required for root tip growth.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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