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Pollen germination is impaired by disruption of a Shaker K^+ channel OsAKT1.2 in rice

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ABSTRACT

Potassium homeostasis is essential for pollen development and pollen-pistil interactions during the sexual reproduction of flowering plants. Here, we described the role of a Shaker K^+ channel, OsAKT1.2, in rice pollen germination and growth. *OsAKT1.2* is specifically expressed in the tricellular pollen, mature pollen grains and growing pollen tubes. Using CRISPR gene editing, we found that knockout lines did not differ from wildtype in vegetative growth, but showed decreased pollen germination rate both in the germination medium and in vivo. OsAKT1.2-GFP fusion protein was localized in the plasma membrane and enriched at the pollen tube tip. OsAKT1.2 could complement the yeast strain which is deficient in K^+ intake. These findings suggest that OsAKT1.2 is associated with pollen germination and tube elongation in rice.

1. Introduction

The male gametes of flowering plants are immobile and are delivered passively by the pollen tube to the embryo sac which is often deeply buried inside the sporophytic tissues of the pistil (Dresselhaus and Franklin-Tong, 2013; Zhang et al., 2017). After being captured by a compatible stigma, the pollen tube emerges from germination aperture of pollen grain and elongates rapidly through the pistil via cell extension and tip growth, finally it reaches one of the synergid cells of embryo sac, stops growing and ruptures to release two sperm cells to fertilize the egg and central cell (Dresselhaus and Franklin-Tong, 2013; Heslop-Harrison, 1987). The journey of pollen tubes is susceptible to environmental and internal factors (Hedhly et al., 2005; Kakani et al., 2005), sufficient pollen germination rate is therefore one of prerequisites to successful double fertilization.

Considerable progresses have been made in elucidation of molecular mechanism involved in pollen-pistil interactions (Jiang et al., 2005; Lalanne et al., 2004; Miyazaki et al., 2009; Zhang et al., 2008). Many evidences from various sources indicate that K^+, the most abundant cation in plant cells for turgor pressure maintenance and various cellular processes, plays crucial roles in pollen germination and tube growth. Potassium content in mature pollen is about the same or slightly higher than in vegetative tissues (Bashe and Mascarenhas, 1984; Stanley, 1971). Mature pollen from maize and rice, contains about 0.67% (Stanley, 1971) and 0.89% K^+ (Liu et al., 2016), respectively when released from anthers. In vitro germination of barley pollen showed that upon rehydration, concentrated K^+ was observed in the aperture area, which suggests a possible role of K^+ in swelling of pollen by creating osmotic gradient across cell membrane (Rehman et al., 2004). Studies in dicot plants also showed additional 1 ~ 10 mM K^+ was required for optimal pollen germination and tube elongation in growth medium (Bou Daher et al., 2009; Fan et al., 2001).

The first isolation of genes encoding potassium channels from higher plants were Arabidopsis AKT1 (Arabidopsis K$^+$ Transporter 1) (Sentenac et al., 1992) and KAT1 (K$^+$ channel in *Arabidopsis thaliana* 1) (Anderson et al., 1992). Both AtAKT1 and AtKAT1 consist of six membrane-spanning helices and require four α-subunits to assemble a functional K^+ channel, which are similar in structure and amino acid sequence to K^+ channels of the Shaker family found in animals (Gambale and Uozumi, 2006). AtAKT1 functions primarily in K^+ uptake in root cells (Hirsch et al., 1998) and AtKAT1 is required for K^+ uptake in guard cells during light-induced stomatal opening (Kwak et al., 2001). In rice, OsAKT1 is an important inwardly rectifying K^+ channel in roots. Disruption of OsAKT1 leads to inhibition of plant growth (Li et al., 2014), while overexpression of OsAKT1 improves tolerance to drought stress (Ahmad et al., 2016). In contrast to inward K^+ channels, AtGORK (Guard cell Outward Rectifying K$^+$ channel) mediates K^+ release from guard cells (Ache et al., 2000) and AtSKOR

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(Stelar K^+ Outward Rectifier) regulates the transport of K^+ from stelar cells to the xylem in the roots (Liu et al., 2006). Interestingly, AtKC1 (K^+ rectifying Channel 1) encodes a Shaker α-subunit that is silent when expressed alone but interact with subunits of inward channels such as AtKAT1, AtKAT2 or AtAKT2 by forming heteromeric channel (Jeanguenin et al., 2011). In Arabidopsis, pollen germination and tube elongation were impaired when a Shaker K^+ channel SPIK (Shaker Pollen Inward K^+ channel) was lacking (Mouline et al., 2002). Disruption of two K^+ transporters CHX21 and CHX23 localized in endoplasmic reticulum led to failure of entering the micropyle of ovules (Lu et al., 2011). Research in maize and rice suggest K^+ channels and transporters are also involved in the regulation of pollen tube burst (Amien et al., 2010; Liu et al., 2016). Together, these suggest a stable turgor pressure that stretches plasma membrane at the tube tip requires constant K^+ influx from maternal reproductive tissues.

Here we report a Shaker K^+ channel OsAKT1.2 which is a homolog of OsAKT1 in rice (Golldack et al., 2003). Unlike *OsAKT1*, *OsAKT1.2* transcript was not detected in vegetative organs such as root and leaf, but was highly expressed in tricellular pollen and germinated pollen. OsAKT1.2 is localized at the tip of the pollen tube and may act as an inwardly rectifying K^+ channel. Disruption of *OsAKT1.2* in rice results in decreased pollen germination rate.

2. Materials and methods

2.1. Plant materials and growth conditions

Rice plants were cultivated under natural condition in summer growing season in Beijing. The pYLCRISPR plasmid was used as the backbone to construct *OsAKT1.2* genome editing vectors. We used CRISPR-P 2.0 online tool to design the base pairing sequence of the sgRNA targeting the exon, following the instructions described by Liu et al. (Liu et al., 2017). To ensure specificity, the off-target sites of sgRNAs must below score 0.3 or have at least 4 mismatches. The plasmid construct was sequenced and then introduced to *Agrobacterium tumefaciens* strain EHA105. Rice callus induced from the *japonica* cultivar Zhonghua11 (*Oryza sativa* ssp. *Japonica*) was infected with transformed EHA105 as described by Toki et al. (Toki et al., 2006). The T1 plant lines identified as homozygous knockout were used for research.

2.2. Gene expression analysis

Total RNA was extracted from various tissues by using the RNeasy plant mini kit (QIAGEN). Isolated RNA was reverse transcribed to cDNA by using SuperScript III reverse transcriptase (Invitrogen). 2 × Plus Master Mix (CWBIO) was used for semi-quantitative RT-PCR. Real-time fluorescence quantitative RT-PCR was performed using SYBR Green Real-Time PCR Master Mix (Invitrogen). The primer sequences and amplification sizes are listed in Table S1.

2.3. In vitro pollen analysis

Alexander staining: The tips of rice glumes were cut off and florets were soaked in Alexander staining solution (Alexander, 1969). After overnight staining, the anthers were crushed by the tip of a tweezer to release pollen grains for microscopic observation.

4′,6-diamidino-2-phenylindole (DAPI) staining: pollen were fixed in Carnoy fixative (ethanol vs acetic acid = 3:1) for 2 h at room temperature and stained in 1 μg/mL DAPI solution (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 50% glycerol, pH 7.3) for 30 min in the dark. Stained pollen were observed under UV light.

The semi-solid germination medium (Liu et al., 2016) was dissolved by heating in a microwave oven and pipetted onto a glass slide. The blooming florets were cut off from panicle and pollen were shaken onto the medium that had solidified for 5 min. Images of pollen germination were obtained under a microscope.

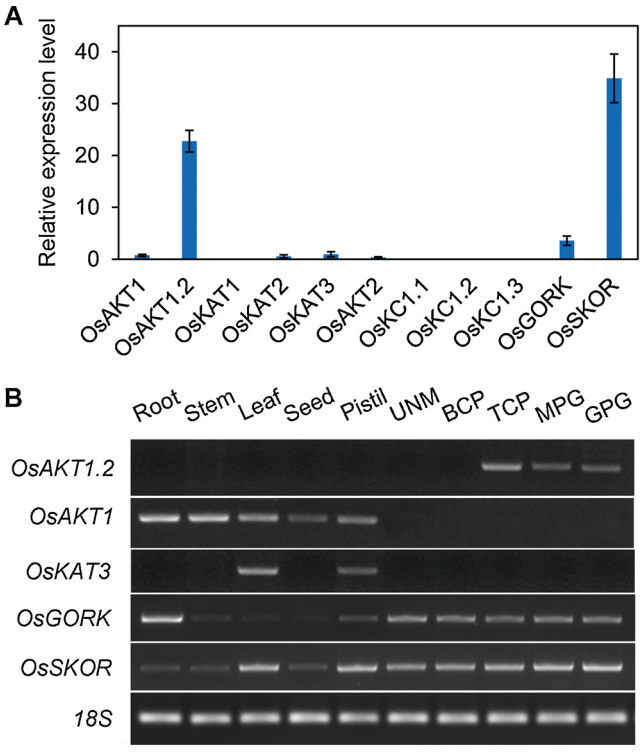


Fig. 1. *OsAKT1.2* is specifically expressed in pollen. (A) Real-time quantitative RT-PCR detection of the expression of eleven K^+ channel genes in rice pollen. (B) The tissue expression patterns of *OsAKT1.2*, *OsAKT1*, *OsKAT3*, *OsGORK* and *OsSKOR* were detected by semi-quantitative RT-PCR. Total RNA was isolated from root, stem, leaf, seed, pistil, uninucleate microspore (UNM), bicellular pollen (BCP), tricellular pollen (TCP), mature pollen grain (MPG) and germinated pollen grain (GPG).

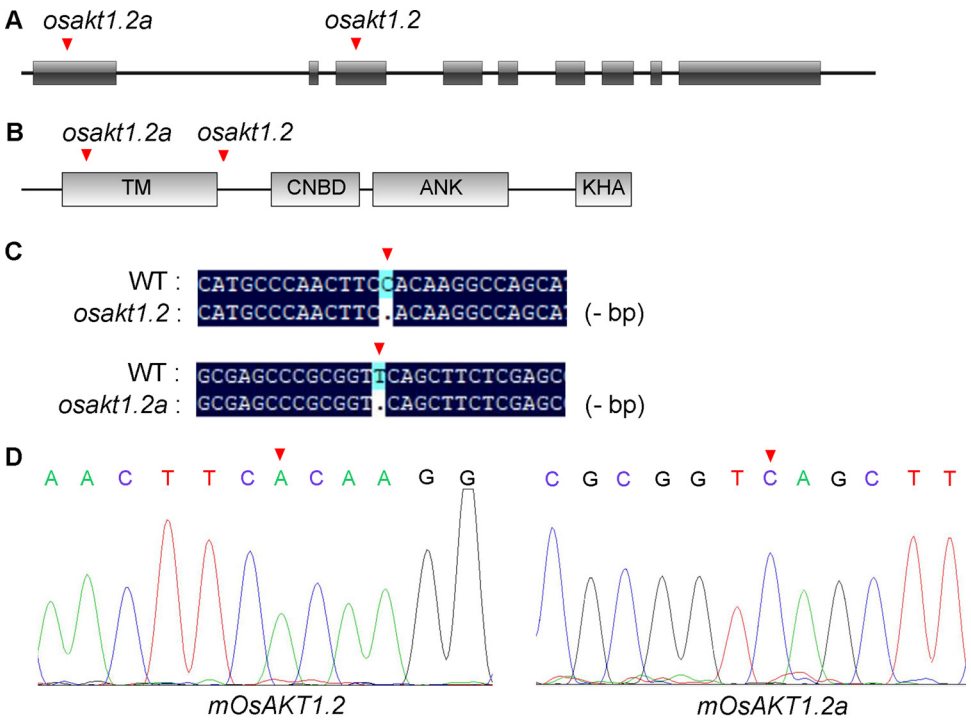
2.4. Aniline blue staining for observation of pollen germination in vivo

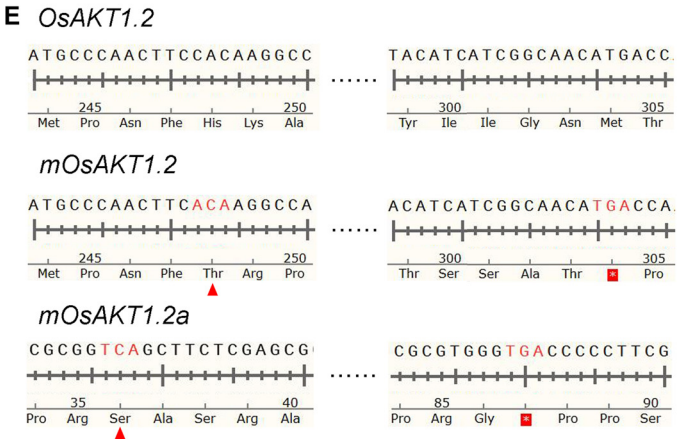
Blooming florets were marked at noon and cut off after 6 h of pollination. Florets were fixed in 1 mL Carnoy fixative for more than 2 h at room temperature and washed with deionized water for 5 times. Subsequently, florets were softened in 1 M NaOH at 55 °C for 30 min and washed with deionized water for 3 times. Florets were stained with 0.1% (w/v) aniline blue solution in the dark overnight. The pistil was taken out onto glass slide and covered with coverslip gently. The pollen tube was observed under a fluorescent microscope.

2.5. Subcellular localization

The *OsAKT1.2*coding sequence (without a stop codon) was constructed into pBI221-GFP vector to generate a fusion of OsAKT1.2 with GFP at the C-terminus. OsAKT1.2-GFP and the plasma membrane localization marker PIP2-mCherry were co-transformed into rice protoplasts from leaf sheaths according to PEG3350 (polyethylene glycol)-mediated transformation method as described by Chen et al. (Chen et al., 2006). The fluorescence signals of transformed protoplast cells were observed with a confocal microscopy (Leica TCS SP5). GFP was excited by 488 nm laser and mCherry was excited by 543 nm laser.

The *OsAKT1.2*coding sequence (without a stop codon) was constructed into pUN1392-GFP vector to generate a fusion of OsAKT1.2 with GFP at the C-terminus. The constructs above were bombarded into 50 mg lily pollen that had been hydrated and degreased by gene gun method (Finer et al., 1992). The pollen was incubated in 3 mL germination solution at 26 °C for 8 h at 65 rpm. The fluorescence in the pollen tube was observed under GFP channel.





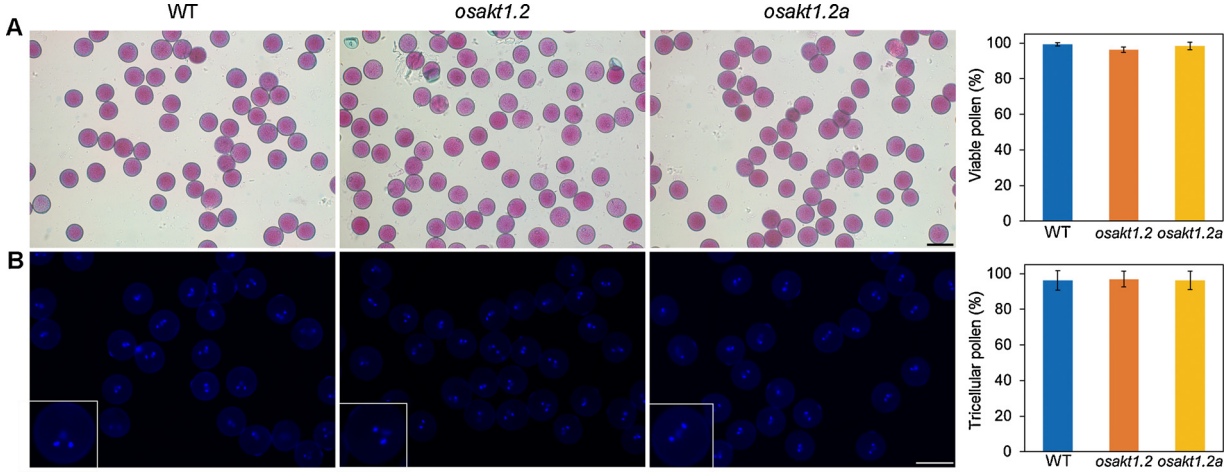


Fig. 2. CRISPR/Cas9-mediated gene editing in stable transgenic rice plants. (A) *OsAKT1.2* gene structure and CRISPR target sites. (B) OsAKT1.2 contains a transmembrane domain (TM), a putative cyclic nucleotide binding domain (CNBD), an ankyrin domain (ANK), and a domain rich in hydrophobic and acidic residues (KHA). mOsAKT1.2 and mOsAKT1.2a lacked CNBD, ANK and KHA domain. (C) Sequencing revealed a 1-bp deletion in the third exon in *osakt1.2* and a 1-bp deletion in the first exon in *osakt1.2a*. (D) Sequence peaks of *mOsAKT1.2* and *mOsAKT1.2a*. (E) *mOsAKT1.2* and *mOsAKT1.2a* created a transcoding mutation and premature termination. The red arrows denote the mutation sites.

Fig. 3. The observation of mature pollen grains. (A) Alexander staining of wildtype (WT), *osakt1.2* and *osakt1.2a* pollen grains and the percentage of viable pollen grains. (B) DAPI staining of WT, *osakt1.2* and *osakt1.2a* pollen grains and the percentage of tricellular pollen grains. Scale bar, 50 μm.

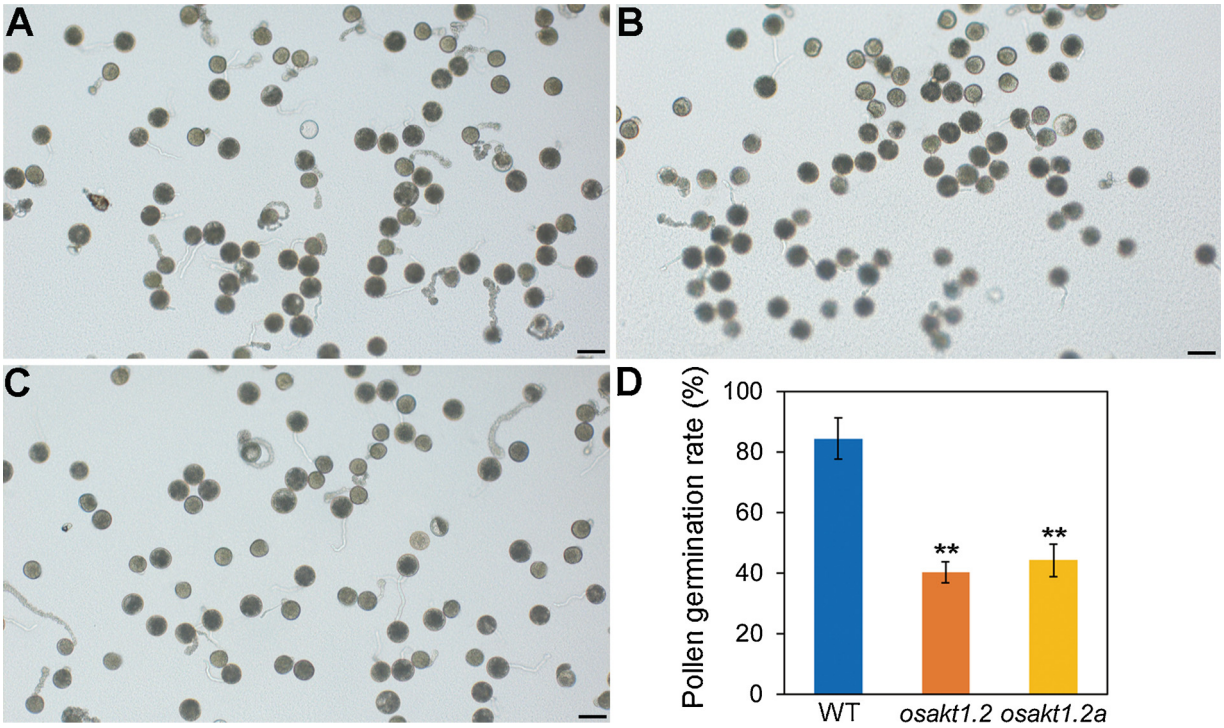


Fig. 4. Pollen germination in vitro. (A-C) The pollen germination in vitro of wildtype (WT) (A), *osakt1.2* (B) and *osakt1.2a* (C). Scale bar, 50 μm. (D) The pollen germination rate of WT, *osakt1.2* and *osakt1.2a*. *Asterisks* denote statistically significant values relative to WT (Student's t-test, **, $P < 0.01$).

2.6. K^+ channel complementary assay for K^+ uptake-deficient yeast

The coding sequences of full-length *OsAKT1.2, mOsAKT1.2, OsGORK* and *OsSKOR* were constructed into pYES3/CT vector respectively. The constructs were transformed into the competent cells of K^+ uptake-deficient yeast strain R5421 (Gaber et al., 1988; Ko and Gaber, 1991) through the lithium acetate method. The competent cells were spread onto SD/-Trp +100 mM KCl and cultivated until colonies grew up to 2 mm in diameter. Colonies were incubated in liquid medium of SD/-Trp +100 mM KCl overnight. The bacterial pellet was collected by centrifugation and resuspended with different volumes of ddH_2O to make sure OD600 was 0.5. Stock solution and diluted solution were dropped onto arginine and phosphoric medium containing different K^+ concentration and incubated at 30 ˚C for 6 days in the dark (Rodrigueznavarro and Ramos, 1984).

2.7. Phylogenetic analysis

Protein sequences of OsAKT1.2 and its homologs from these indicated species were obtained from the database of the national center for biotechnology information. The phylogenetic tree was generated with MEGA7 software using the maximum-likelihood method and 1000 bootstrap replicates.

3. Results and discussion

3.1. OsAKT1.2 is specifically expressed in pollen

To investigate the role of K^+ in the reproduction of rice, we focus on Shaker K^+ channels that may involve in male gametophyte development and pollen tube growth. A BLAST search using the protein sequence of Shaker K^+ channel AtAKT1 in Arabidopsis showed that eleven Shaker K^+ channels exist in rice genome (Fig. S1, Table S2). Expression pattern of these K^+ channel genes were analyzed using real-time quantitative RT-PCR and revealed that three genes *OsAKT1.2*, *OsGORK* and *OsSKOR* showed relatively high-level expression in pollen (Fig. 1A). Subsequently, the tissue expression patterns of five K^+ channel genes were detected by semi-quantitative RT-PCR. The expression patterns of *OsAKT1* and *OsAKT1.2* are not overlapping. *OsAKT1.2* was highly expressed in tricellular pollen (TCP), mature pollen grain (MPG) and germinated pollen grain (GPG), but was not detected in pistil, root, stem, leaf and seed. *OsAKT1* was expressed in all vegetative organs, pistil and seed, but not in male gametophyte, while *OsKAT3* was detected only in leaf and pistil. Unlike *OsAKT1.2*, there were constitutive expressions of *OsGORK* and *OsSKOR* in vegetative and reproductive organs (Fig. 1B). Therefore, our work focused on the gene *OsAKT1.2*.

3.2. The pollen germination rate of functionally deficient mutant osakt1.2 was significantly decreased

OsAKT1.2 consists of nine exons and eight introns and has an open reading frame encoding 902 amino acid (aa) residues with a predicated molecular weight of 97.7 kDa and an isoelectric point of 8.3 (Fig. 2A). In order to investigate whether *OsAKT1.2* plays a role in pollen development, we generated two independent CRISPR/Cas9 knockout mutant lines *osakt1.2* and *osakt1.2a*. Sequencing of PCR products from T1 generation plants revealed a 1-bp deletion in the third exon in *osakt1.2* and a 1-bp deletion in the first exon in *osakt1.2a*, both deletion will result in frameshift mutations and premature terminations. The truncated mOsAKT1.2 and mOsAKT1.2a were predicated to contain only 303 amino acids and 86 amino acids, respectively, both missing the C-terminal domain (including cyclic nucleotide-binding domain, ankyrin domain and KHA domain which involve in the formation of channel tetramers) (Daram et al., 1997) (Fig. 2B–E and Fig. S2).

The development of floral organs and flowering of *osakt1.2* were normal (Fig. S3A, S3B). Comparison with wildtype plants did not reveal any significant difference in seed setting and plant height (Fig. S3C–E). Pollen grains of wild type, *osakt1.2* and *osakt1.2a* showed no obvious difference in Alexander staining (Fig. 3A). *osakt1.2* and *osakt1.2a* pollen grains contained one loosely stained vegetative nucleus and two condensed sperm nuclei like wildtype (Fig. 3B). Because *OsAKT1.2* is

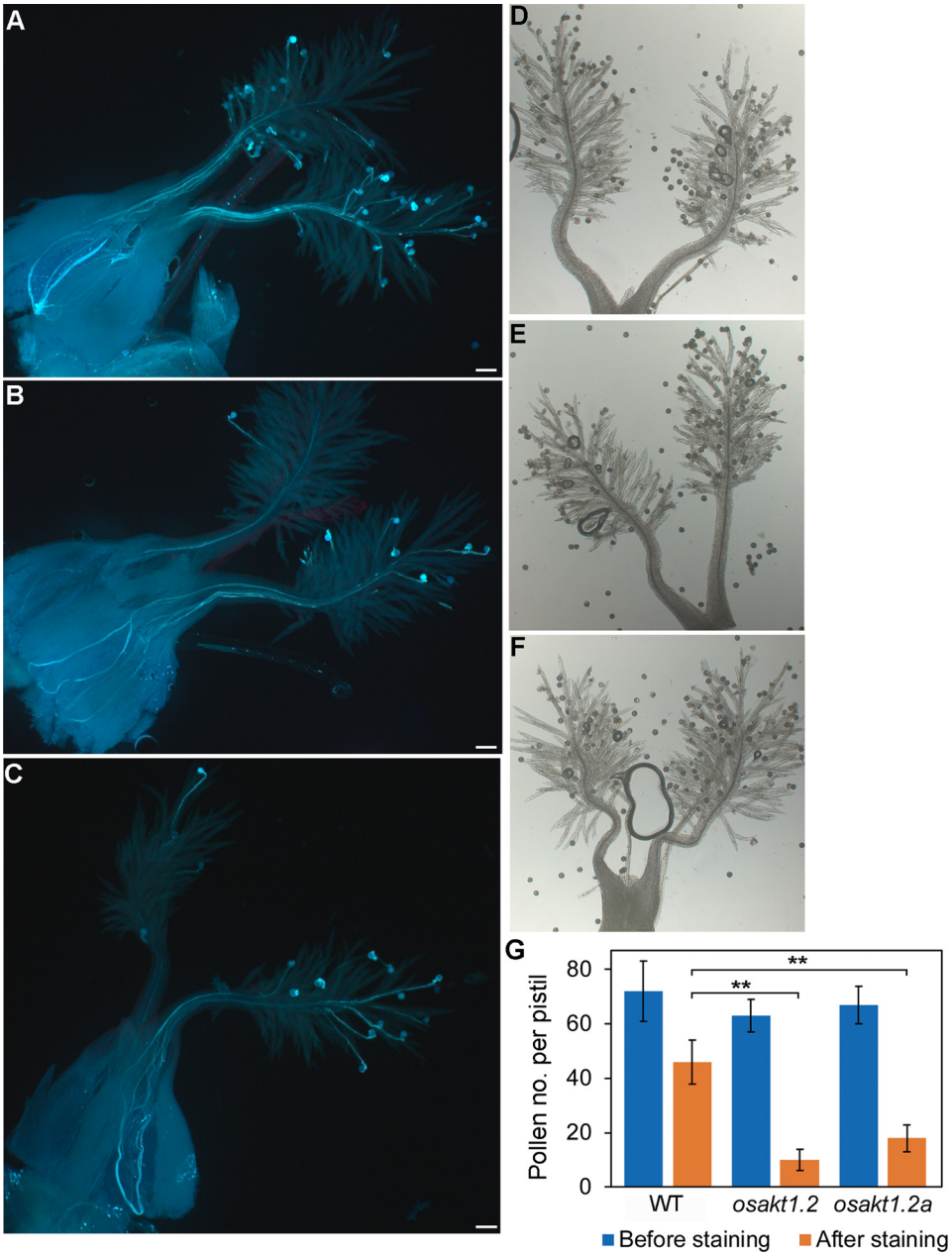


Fig. 5. Pollen germination in vivo. (A-C) The observation of pollen germination in vivo of wildtype (WT) (A), *osakt1.2* (B) and *osakt1.2a* (C) after staining with aniline blue. Scale bar, 100 μm. (D-F) The pollen on stigma of WT (D), *osakt1.2* (E) and *osakt1.2a* (F) before staining. (G) Statistics of pollen number per pistil before or after aniline blue staining. (Student's t-test, **, $P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

highly expressed in germinated pollen, we tested whether *OsAKT1.2* disruption could impair pollen germination in vitro. While the glumes opened naturally, the pollen of wildtype, *osakt1.2* and *osakt1.2a* were shaken off onto semisolid germination medium, the pollen germination was observed after 15 min and the germination rate was calculated. It was observed that germination rate of *osakt1.2* (40.3%) and *osakt1.2a* (44.3%) were significantly less than that of wildtype (84.5%) (Fig. 4).

To investigate in vivo tube growth behavior in CRISPR mutants, the pistils of self-pollination were stained with aniline blue. Although pollen tubes elongation and ovule targeting were observed in *osakt1.2* and *osakt1.2a* pistils, the average amount of pollen on the stigma of *osakt1.2* (10) and *osakt1.2a* (18) after staining was greatly reduced in comparison to wildtype (46) (Fig. 5A–C, G). We hypothesized that a portion of the mutant pollen failed to adhere to the stigma due to impaired germination. Because during the dyeing process, several rinses were carried out to wash away the part of pollen that didn't germinate. To verify the hypothesis, we counted the number of pollen grains per pistil before staining and found no significant difference among wildtype (72), *osakt1.2* (63) and *osakt1.2a* (67) (Fig. 5D–G). The above experiments indicated that the pollen germination rate of *osakt1.2* and *osakt1.2a* were significantly decreased compared with wildtype both in vitro and in vivo.

Unexpectedly, disruption of *OsAKT1.2* did not completely inhibit pollen germination. One possible reason is that by the time of anthesis, most mature pollen grains have already accumulated enough K^+ to initiate germination, however, in the absence of OsAKT1.2, maintenance of K^+ concentration is affected due to decreased K^+ influx from female reproductive tissues. In vivo, some *osakt1.2* pollen tubes could reach the ovule, so the seed setting rate of *osakt1.2* is not significantly different from that of wildtype. Similarly, the Arabidopsis *SPIK* showed

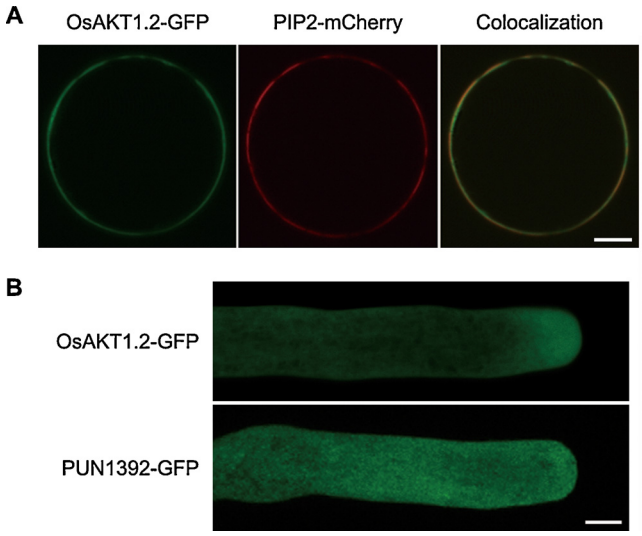


Fig. 6. OsAKT1.2 is localized at the tip of the pollen tube. (A) Plasma membrane localization of OsAKT1.2 in rice protoplast cells. Scale bar, 10 μm. (B) Enrichment of OsAKT1.2 at the tip of lily pollen tube. Scale bar, 10 μm.

preferential expression in pollen. *spik-1* mutation only mildly affected the overall germination rate, but reduced the tube length by an average factor of 40%–50% (Mouline et al., 2002). Despite of impaired tube growth, seed setting rate of *spik-1* plants was not significantly different from that of wild-type plants.

3.3. *OsAKT1.2 is localized at the tip of pollen tube*

To determine the subcellular localization of OsAKT1.2, we transiently transformed rice protoplasts with constructs *ubi*::OsAKT1.2-GFP and PIP2-mCherry (plasma membrane localization marker). When co-expressed in rice protoplast, the green (OsAKT1.2-GFP) and red fluorescence (PIP2-mCherry) showed remarkable overlap (Fig. 6A), thus indicating localization of OsAKT1.2 in the plasma membrane. Furthermore, we clarified in which compartment of the pollen tube was OsAKT1.2 localized by transient expression in lily pollen tubes. The fluorescent signals of OsAKT1.2-GFP concentrated at the tip of pollen tube, while the free GFP signals were evenly distributed throughout the pollen tube (Fig. 6B). These results indicate that OsAKT1.2 is a plasma membrane protein and enriched at the tip of the growing pollen tube.

3.4. *OsAKT1.2 has the function of K^+ uptake in yeast*

To determine the K^+ channel activity of OsAKT1.2, the constructs pYES/CT-OsAKT1.2, pYES/CT-mOsAKT1.2 (truncated OsAKT1.2), pYES/CT-OsGORK and pYES/CT-OsSKOR were transformed into K^+ uptake deficient yeast strain R5421 respectively. Transformed strains were dotted on arginine phosphoric medium with different external K^+ concentrations. The yeast strain transformed with OsAKT1.2 could grow on a medium containing a low concentration of K^+ (1 mM and 0.5 mM) (Fig. 7), whereas yeast strain transformed with mOsAKT1.2, OsGORK and OsSKOR could not grow under low concentration of K^+ (1 mM and 0.5 mM), unless the medium containing a relatively high concentration of K^+ (5 mM) (Fig. 7). This suggest that OsAKT1.2 could complement K^+ uptake deficient yeast strain and OsAKT1.2 is a Shaker K^+ channel with the function of K^+ uptake. Phylogenetic analysis using K^+ channel genes from rice, Arabidopsis and maize showed that OsAKT1.2 was in the same branch with OsAKT1 and AtAKT1 (Fig. S1), and it had been reported that OsAKT1 and AtAKT1 were inwardly rectifying K^+ channels (Ahmad et al., 2016; Dennison et al., 2001; Li et al., 2014; Sentenac et al., 1992). The other two highly expressed K^+ channels OsGORK and OsSKOR were in the same branch with AtGORK and AtSKOR (Fig. S1), and consistent with complementary assay, AtGORK and AtSKOR were outwardly rectifying K^+ channels (Ache et al., 2000; Johansson et al., 2006; Liu et al., 2006). The above researches indicated that OsAKT1.2 has the function of K^+ uptake and could be an inwardly rectifying K^+ channel.

4. Conclusion

K^+ is an essential cation for pollen germination and tube growth. It is widely accepted that K^+ together with other osmoticums controlled turgor in pollen (Weisenseel and Jaffe, 1976; Messerli and Robinson, 2003; Dutta and Robinson, 2004). The balance between turgor pressure and cell wall loosening is a critical factor driving pollen germination and tube growth (Zonia et al., 2006). Here, we reported that plasma membrane localized K^+ channel OsAKT1.2 is required for pollen germination and tube growth. Interestingly, the *OsAKT1.2* displays an expression pattern complementary to that of *OsAKT1*. *OsAKT1*.2 was expressed in male gametophyte, whereas *OsAKT1* showed universal expression across all tested samples except male gametophyte. Among 11 Shaker K^+ channels in rice, only *OsAKT1.2* is exclusively expressed in late stages of pollen development, which implies an important role of *OsAKT1.2* for pollen germination and pollen tube growth. The unchanged seed setting rate is probably a result of functional redundancy of transporters that expressed in pollen. It is known that cross membrane K^+ influx was carried out not only through K^+ channels but also by K^+ transporters (Gierth and Maser, 2007; Véry and Sentenac, 2003; Wang and Wu, 2013). It's possible that potassium transporters in pollen such as rice homologues of CHX21 and CHX23 might perceive the shortage of K^+ within the pollen tube, and compensate for the role of OsAKT1.2. The exact mechanism of coordination between K^+ channels and K^+ transporters still awaits elucidation.

Author contributions

T.W. and L.L. planned and designed the research, L.L. and F.Y. wrote the manuscript. F.Y. performed the research and analyzed data.

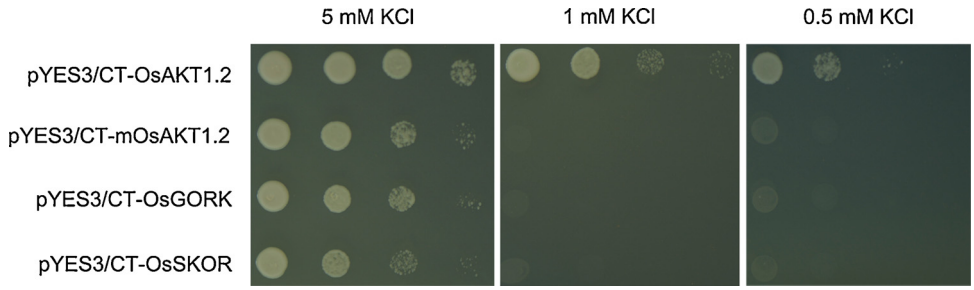


Fig. 7. Complementation assay of OsAKT1.2 in a K^+ uptake deficient yeast strain. OsAKT1.2 could complement K^+ uptake deficient yeast strain, while mOsAKT1.2, OsGORK and OsSKOR could not.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2020.153140.

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