SCIENCE CHINA
Life Sciences

• RESEARCH PAPER •

January 2018 Vol.61 No.1: 100–112
10.1007/s11427-016-9107-3

Arabidopsis PRK6 interacts specifically with AtRopGEF8/12 and induces depolarized growth of pollen tubes when overexpressed

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Received April 8, 2017; accepted June 2, 2017; published online August 8, 2017

The pollen receptor kinases (PRK) are critical regulators of pollen tube growth. The *Arabidopsis* genome encodes eight *PRK* genes, of which six are highly expressed in pollen tubes. The potential functions of *AtPRK1* through *AtPRK5*, but not of *AtPRK6*, in pollen growth were analyzed in tobacco. Herein, *AtPRK6* was cloned, and its function was identified. *AtPRK6* was expressed specifically in pollen tubes. A yeast two-hybrid screen of AtPRK6 against 14 *Arabidopsis* Rop guanine nucleotide exchange factors (RopGEFs) showed that AtPRK6 interacted with AtRopGEF8 and AtRopGEF12. These interactions were confirmed in *Arabidopsis* mesophyll protoplasts. The interactions between AtPRK6 and AtRopGEF8/12 were mediated by the C-termini of AtRopGEF8/12 and by the juxtamembrane and kinase domain of AtPRK6, but were not dependent on the kinase activity. In addition, transient overexpression of *AtPRK6::GFP* in *Arabidopsis* protoplasts revealed that AtPRK6 was localized to the plasma membrane. Tobacco pollen tubes overexpressing *AtPRK6* exhibited shorter tubes with enlarged tips. This depolarized tube growth required the kinase domain of AtPRK6 and was not dependent on kinase activity. Taken together, the results show that *AtPRK6*, through its juxtamembrane and kinase domains (KD), interacts with AtRopGEF8/12 and plays crucial roles in polarized growth of pollen tubes.

pollen receptor kinase, pollen tube, polarized growth, RopGEF, *Arabidopsis*

Citation: Yu, Y., Song, J., Tian, X., Zhang, H., Li, L., and Zhu, H. (2018). *Arabidopsis* PRK6 interacts specifically with AtRopGEF8/12 and induces depolarized growth of pollen tubes when overexpressed. Sci China Life Sci 61, 100–112. doi: 10.1007/s11427-016-9107-3

INTRODUCTION

Pollen tubes are polar plant cells with an elongated tubular structure, in which cell expansion occurs exclusively in the cell apex (Zhou et al., 2014). Notable cytoplasmic polarity is one of the features that promotes the proper growth of pollen tubes (Qin and Yang, 2011; Zhang and McCormick, 2008). Due to its polarity, the pollen tube can elongate rapidly to deliver sperm cells to unfertilized ovules (Zhu et al., 2013; Guan et al., 2013; Yang, 2008; Qin and Yang, 2011). This also means that the pollen tube makes an excellent and convenient system to study genes relevant to polarized growth (Qin and Yang, 2011; Guan et al., 2013;Yang, 2008; Lennon and Lord, 2000; Fu, 2015; Zhou et al., 2014).

The receptor like kinase (RLK) protein family is large, containing more than 600 members in *Arabidopsis* and 1,100 in rice (Gish and Clark, 2011; Vaid et al., 2013; Li and Yang, 2016). More than 100 RLKs are expressed in pollen, implying their potential roles for pollen tube growth (Khamsuk, 2011). The largest sub-group of RLKs is the leucine-rich repeat (LRR) RLKs that contain five or six LRR motifs located in the extracellular domain (Gish and Clark, 2011). Within the LRR-RLK group, the pollen receptor kinases (PRKs) coordinate the cellular activity required for pollen tube tip growth and are the most critical regulatory elements for this process (Chang et al., 2013; Kim et al.,

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2002; Muschietti et al., 1998; Zhang et al., 2008; Zhang and McCormick, 2008; Zhao et al., 2013; Li and Yang, 2016). The identification of the first pollen receptor kinase (PRK1) in *Petunia inflate* (Mu et al., 1994), has been followed by the discovery and characterization of other *PRK* genes from different species. *LePRK1* in tomato induced severe depolarized pollen tube growth with blebs forming at the leading edge (Gui et al., 2014), and pollen tubes expressing *LePRK2* exhibited enlarged tips (Zhang et al., 2008).

Using phylogenetic analysis, eight *RLK* genes in *Arabidopsis* were found to be closely related to *LePRK2* (Chang et al., 2013) (http://www. arabidopsis.org). Six of these *RLK*s were found to be highly expressed in pollen tubes and therefore named *AtPRK1* through *AtPRK6* (Chang et al., 2013). The first five *Arabidopsis PRK* genes were successfully cloned, but not the *AtPRK6* gene (Chang et al., 2013). When overexpressed in tobacco pollen tubes, *AtPRK2* led to tubes with severe depolarized growth and swollen tips; while pollen tubes expressing *AtPRK1* showed longer pollen tubes (Chang et al., 2013). These results suggest that PRKs play critical roles in regulating the polarized growth of pollen tubes.

Polarized tip growth of pollen tubes also depends on guanine nucleotide exchange factors (GEFs). GEFs are activators of small G proteins and function by exchanging GDP to GTP to switch on the GTPases (Thomas and Berken, 2010; Zou et al., 2011). The kinase partner protein (KPP) is a plant-specific GEF identified in tomato (Kaothien et al., 2005). Pollen tubes overexpressing LeKPP exhibited depolarized pollen tube growth with abnormal cytoplasmic streaming and F-actin arrangements (Kaothien et al., 2005). The orthologous genes of *LeKPP* in *Arabidopsis* are RopGEFs, which are responsible for the activation of ROPs (small GTPase Rho of plants) (Gu et al., 2006; Berken et al., 2005), the key proteins controlling actin cytoskeleton dynamics in pollen tubes (Gu et al., 2005; Kaothien et al., 2005). Fourteen RopGEF proteins were isolated with a plant-specific ROP nucleotide exchanger domain (PRONE) (Berken et al., 2005), five of which are highly or specifically expressed in pollen tubes (Gu et al., 2006; Li and Liu, 2012; Zhang and McCormick, 2008). When overexpressed in tobacco pollen grains, *AtRopGEF1* induced the most dramatic phenotype: pollen tubes with ballooning tips (Gu et al., 2006). A C-terminus truncation of AtRopGEF12, but not the full length, induced depolarized growth of pollen tubes, suggesting that the RopGEFs C-terminus can inhibit its own activity *in vivo* (Zhang and McCormick, 2007). RopGEFs were defined as the links between the perception of the extracellular cues by the PRKs and the initiation of the ROP-regulated small G protein intracellular cascades that result in polar growth of pollen tubes (Zhang and McCormick, 2007).

LePRKs were revealed to interact with KPP through the intracellular domain *in vitro* (Kaothien et al., 2005). AtPRK2 induced depolarization depending on its interaction with different RopGEFs (Zhang and McCormick, 2007; Zhao et al., 2013; Chang et al., 2013). AtPRK2 interacted with AtRopGEF12 both *in vitro* and *in vivo* (Zhao et al., 2013; Zhang and McCormick, 2007). The interaction between AtRopGEF1 and AtPRK2 is also essential for the polarized growth of pollen tubes (Chang et al., 2013). Together these numerous studies show that PRKs regulate pollen tube polar growth through initiating the ROP signaling pathway through physical interaction with different RopGEFs.

Very recently, *Arabidopsis* PRKs were reported to guide pollen tubes during double fertilization (Takeuchi and Higashiyama, 2016; Li and Yang, 2016; Zhou and Yang, 2016). The pollen tubes in *atprk6* mutants lost the ability to react to the attractant peptide AtLURE1, which is secreted from the synergid cell. This suggested that AtPRK6 is essential for specific ovule targeting (Takeuchi and Higashiyama, 2016). However, more remains to be understood about how *AtPRK6* regulates polarized growth of pollen tubes. Here, we provide evidence that overexpression of *AtPRK6* directly led to abnormal polarized pollen tube growth. Pollen tubes overexpressing *AtPRK6* were shorter with enlarged or swollen tips. In addition, AtPRK6 was shown to interact with AtRopGEF8/12. The AtPRK6 and AtRopGEF8/12 interactions were confirmed by bimolecular fluorescence complementation (BiFC) and shown to occur at the plasma membrane. Further detailed analysis found that both the juxtamembrane domain (JMD) and the kinase domain (KD) of AtPRK6 and the C-terminal domain (CT) of the two RopGEF proteins contributed to the interactions. Taken together, our results indicated that *AtPRK6* is involved in tip growth of pollen tubes, and that this regulation relies on the KD of AtPRK6 while being independent of its kinase activity.

RESULTS

Expression patterns of *AtPRK6*

Since an earlier attempt at cloning the *AtPRK6* gene was not a success (Chang et al., 2013), a fresh attempt was made. Using the first strand cDNA synthetized from the total RNA of flower tissues, *AtPRK6* was successfully cloned.

Semi-quantitative RT-PCR assays detected transcripts of *AtPRK6* in flowers, but not in the vegetative seedling, root and leaf tissues (Figure 1A). To investigate the detailed expression patterns of *AtPRK6* in different tissues during plant development, transgenic plants with the *GUS* (β-glucuronidase) reporter gene driven by the native promoter of *AtPRK6* were developed. Results showed that no GUS accumulation was detected in young seedlings (15-day-old) (Figure 1B(a)). In 6- to 8-week-old plants, GUS accumulation was mainly detected in flowers at stage 7 (as defined by Alvarez-Buylla et al., 2015), but not in flowers younger than

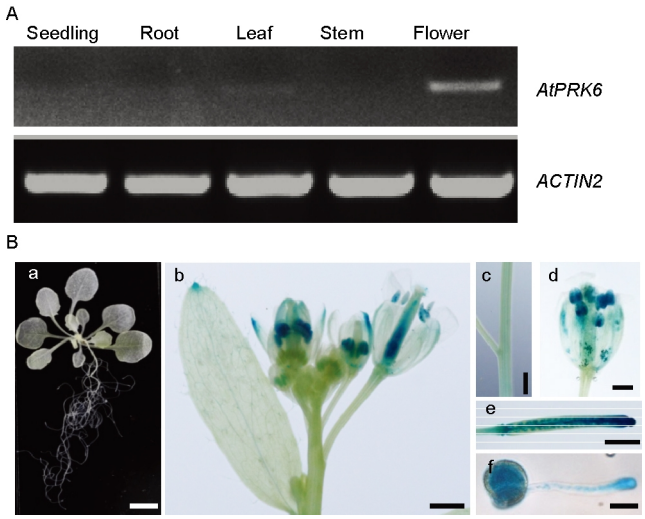

Figure 1 Expression pattern of *AtPRK6* in *Arabidopsis*. A, *AtPRK6* mRNA levels were detected for seedlings and different tissues by RT-PCR. Actin was used as a control. B, Histological GUS assays for *AtPRK6*$_{Pro}$*-GUS* transgenic plants. a, 15-day-old seedling (Bar=1 cm); b, inflorescence (Bar=1 cm); c, stem (Bar=0.2 cm); d, close-up image of a single flower (Bar=0.1 cm); e, young silique (Bar=0.5 cm); f, germinated pollen grain with growing pollen tube (Bar=10 μm).

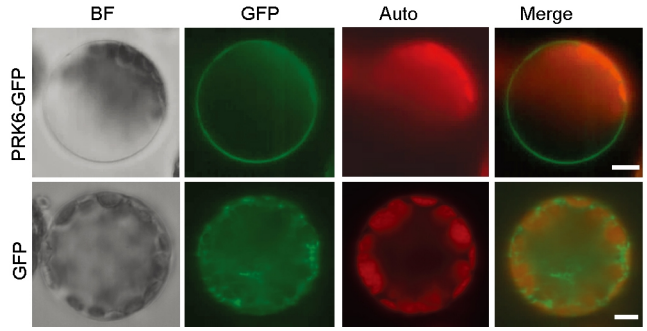

Figure 2 Subcellular localization of AtPRK6 in *Arabidopsis* mesophyll cells. A *PRK6::GFP* fusion plasmid (upper) or GFP alone (control, lower) were transiently expressed in mesophyll cells. BF, Bright field image; GFP, fluorescence image; Auto, chloroplast fluorescence; Merge, merged image. Bar=10 μm.

stage 7 or in stems (Figure 1B(b) and (c)). Further observations revealed that the accumulation was predominantly detected in stamen, pollen grains and young siliques (Figure 1B(d) and (e)), indicating a pollen-related expression pattern. To further analyze the expression of *AtPRK6* during pollen tube growth, pollen grains of *AtPRK6*$_{Pro}$*-GUS* plants were cultured on pollen germination medium (see methods) for 4 h. GUS activity assays indicated that *AtPRK6* was expressed highly in pollen tubes. These results suggested that the expression of *AtPRK6* is mainly associated with pollen grains and pollen tubes.

AtPRK6 was localized to the plasma membrane

To determine the subcellular localization of AtPRK6, a *35S-PRK6::GFP* fusion plasmid was constructed (referred to as PRK6-GFP in Figure 2), and transiently transformed into *Arabidopsis* protoplast cells. Fluorescence signals produced by AtPRK6-GFP fusion proteins (Figure 2, upper panels) were observed on the plasma membrane, while protoplasts expressing *GFP* control plasmids showed universal signals across the cells (Figure 2, lower panels), indicating plasma membrane localization of AtPRK6.

AtPRK6 interacts with AtRopGEF8/12 at the plasma membrane

In order to identify any potential relationships between AtPRK6 and the 14 RopGEF proteins, a yeast-two-hybrid screen was carried out. The cytosolic domain (CD) of AtPRK6 (PRK6-CD, 290–659 aa) was used as a bait against the 14 RopGEFs (referred to as GEFs in Figures 3 and 4) as preys. Yeast cells expressing AtPRK6 and AtRopGEF8 or expressing AtPRK6 and AtRopGEF12 showed healthy growth on selection medium (SD/-Trp/-Leu/-His) (Figure 3), suggesting that AtPRK6 interacted with AtRopGEF8/12 through the CD.

The interactions between AtPRK6 and AtRopGEF8/12 were confirmed by filter-lift assays and BiFC (Figure 4). In the filter-lift assays, the β-gal (β-galactosidase) activity was detected in the cells harboring AtPRK6-CD as bait and AtRopGEF8/12 as prey, but not in the negative control cells (expressing AtPRK6-CD and AtRopGEF13 or activation domain alone (AD) (Figure 4A)). In BiFC experiments, the interactions between AtPRK6 and AtRopGEF8/12 were confirmed (Figure 4B). While protoplasts expressing cCFP-AtPRK6 and nVenus failed to yield any fluorescence signals (Figure 4B, upper panels), GFP fluorescence signals were consistently detected at the plasma membrane in protoplasts co-expressing cCFP-AtPRK6 and either nVenus-AtRopGEF8 or nVenus-AtRopGEF12 (Figure 4B, middle and lower panels). All of these results indicated that AtPRK6 interacts with AtRopGEF8/12 through the CD and that their interactions occur at the plasma membrane.

The interacting domains of AtPRK6 and AtRopGEF8/12

To reveal which domains are responsible for the interactions between AtPRK6 and AtRopGEF8/12, the CD of AtPRK6 was divided into two sub-domains: the JMD (290–397 aa) and the KD (398–659 aa) (Figure 5A). Results showed that deletion of either domain abolished AtPRK6 interaction with the two RopGEFs, which indicated that both the JMD and KD contributed to the interaction (Figure 5B, upper).

Since the KD of AtPRK6 is essential for interaction, the dependence of the interaction on its kinase activities was tested in Y2H assays. As previously reported, mutagenizing the ATP binding lysine of a kinase protein leads to the loss of kinase activity in both mammalian and plants (Chou et al.,

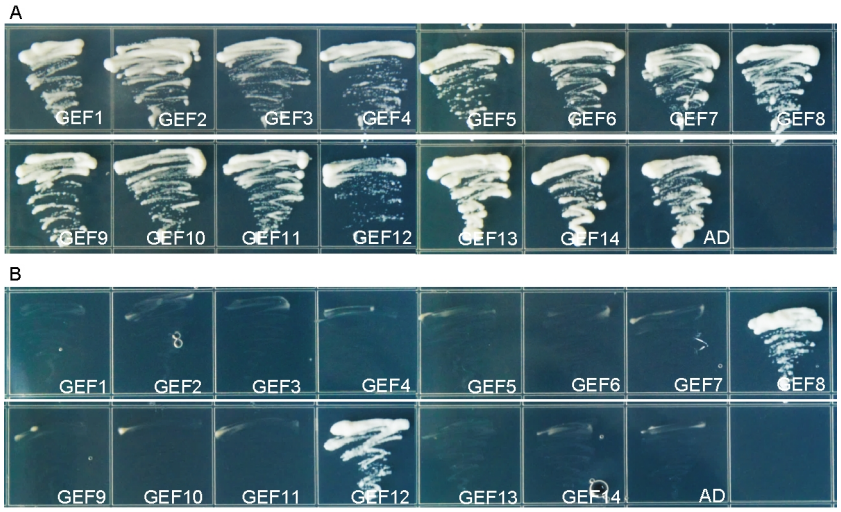

Figure 3 Yeast two-hybrid pairing of AtPRK6 with 14 RopGEFs. A and B, Yeast cells harboring both the CD of AtPRK6 and full-length GEF were grown on selective medium (SD/-Trp/-Leu) (A) and test medium (SD/-Trp/-Leu/-His containing 1.5 mmol L^{-1} 3-AT) (B). Cells with both AtPRK6-CD-BD and empty AD were used as negative control.

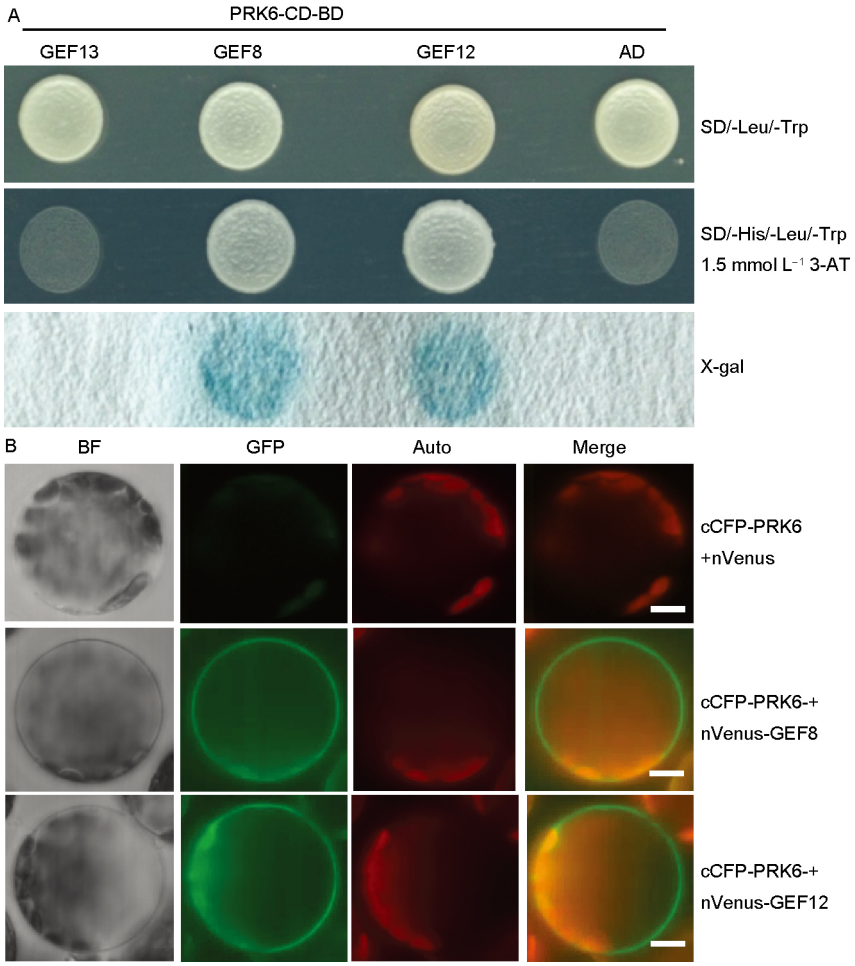

Figure 4 Interactions between AtPRK6 and AtRopGEF8/12. A, The β-galactosidase activity assays were carried out using the filter-lift method. Yeast cells co-expressing AtPRK6-CD and either AtRopGEF8 or 12 were grown on selective medium (SD/-Trp/-Leu/-His) supplied with 1.5 mmol L^{-1} 3-AT. Cells expressing AtPRK6-CD with AtRopGEF13 or AD were used as negative controls. B, BiFC analysis of interaction between AtPRK6 and AtRopGEF8/12 in *Arabidopsis* mesophyll protoplasts. The full length of AtPRK6 and AtRopGEF8/12 were fused with cCFP and nVenus, respectively. Protoplast cells co-expressing cCFP-AtPRK6 and nVenus were used as negative control (upper). Cells co-expressing cCFP-AtPRK6 and nVenus-AtRopGEF8 (middle) or nVenus-AtRopGEF12 (lower) displayed green fluorescence on the plasma membrane (Bar=10 μm).

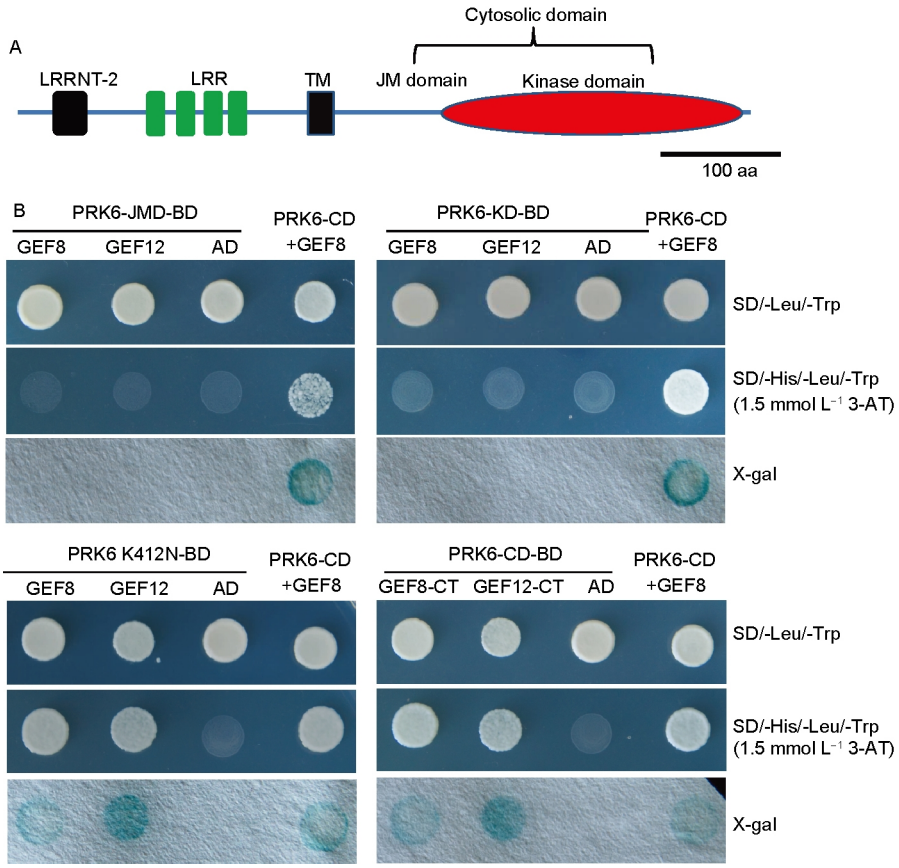

Figure 5 The juxtamembrane domain and kinase domain of AtPRK6 and the C-terminus of AtRopGEF8/12 are essential for their interactions. A, Schematic of the predicted domains of the AtPRK6 protein. The CD was divided into the JMD (290–397 aa) and the KD (398–659 aa). B, The interactions between AtPRK6-JMD and AtRopGEF8/12 (upper left), AtPRK6-KD and AtRopGEF8/12 (upper right), AtPRK6-K412N (kinase-inactive form) and AtRopGEF8/12 (bottom left) and AtPRK6-CD and AtRopGEF8-CT (440–523 aa)/AtRopGEF12-CT (444–515 aa) (bottom right) were tested in the yeast two-hybrid system.

1987; Li et al., 2006). Similar mutation in tomato PRK genes was shown to be inactive (Muschietti et al., 1998; Kim et al., 2002). Here, a kinase inactive *AtPRK6* was generated by mutagenizing the ATP binding residue K412 to an N (referred to as PRK6 K412N in Figure 5B). The K412N mutant did not affect the interactions between AtPRK6 and AtRopGEF8/12 (Figure 5B, lower left), suggesting that the kinase active site, ATP binding, and kinase activity are not critical to the interaction in AH109.

To identify which sites in AtRopGEF8/12 mediate the interaction, we co-expressed the CT domain of either AtRopGEF8 or AtRopGEF12 with AtPRK6 in the yeast AH109 strain. The results revealed that AtRopGEF8/12 interacted with AtPRK6 through their CT domains (Figure 5B, lower right).

These results provided strong evidence that both the JMD and KD of AtPRK6 and the CT domains of AtRopGEF8/12 contributed to the interaction between AtPRK6 and AtRopGEF8/12 and that the interactions are not dependent on the kinase activity of AtPRK6.

Overexpression of *AtPRK6* in tobacco pollen grains leads to shorter pollen tubes with enlarged tips

To further analyze the potential function of *AtPRK6* during pollen tube growth, the full-length cDNA sequence of *AtPRK6* gene was fused with *GFP* under the control of the *LAT52* promoter (*LYCOPERSICON ANTHERSPECIFIC 52*, a gene specifically expressed in pollen) (Twell et al., 1991). The $LAT52_{Pro}$-*AtPRK6::GFP* construct was then delivered into tobacco pollen grains via particle bombardment. After cultured for 4 h on pollen growth medium, the pollen tubes overexpressing *AtPRK6::GFP* exhibited a depolarized growth, i.e., the tubes were significantly shorter with enlarged (balloon-like) tips (Figure 6A and B). Statistical results showed that more than 80% of the pollen tubes overexpressing *AtPRK6* lost their polarity (Figure 6C), and more than 90% of these pollen tubes were shorter than 0.4 mm (Figure 6D). Overall, *AtPRK6*-overexpressing tubes were 60% shorter than tubes overexpressing only GFP on average (Figure 6E), and the average width of the tips was twice as wide as that of control (Figure 6F). To further dissect the depolarized phenotype caused by *AtPRK6* overexpression, the subcellular morphologies of growing pollen tubes were observed. Large vacuoles were visible in the tips of the *AtPRK6* overexpressing pollen tubes (Figure 6B, lower, see indented area; Figure S1 in Supporting Information), but no similar vacuoles were viewed in the control tube tips (Figure

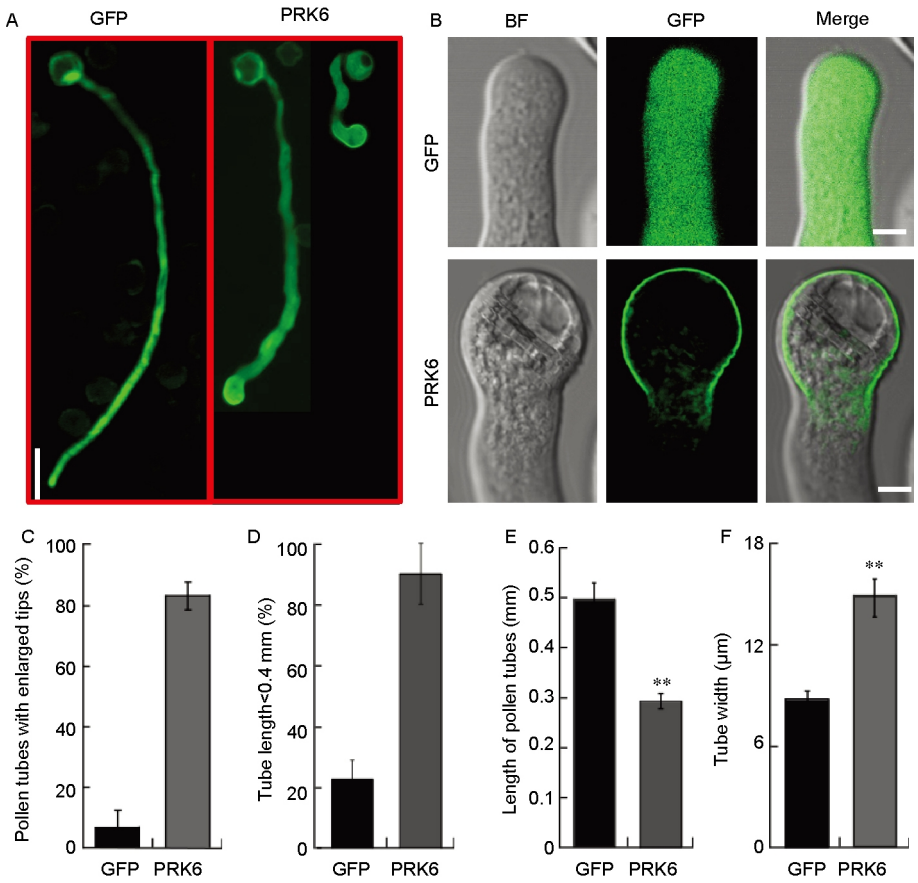

Figure 6 AtPRK6 induced tip enlargement of pollen tubes. A, Overexpression of AtPRK6 in tobacco pollen tubes. Full length AtPRK6 was fused with the N-terminal of GFP driven by the pollen-specific promoter *LAT52* and transiently expressed in tobacco pollen tubes. Pollen tubes expressing *LAT52*$_{Pro}$*-GFP* were used as control (Bar=50 μm). B, Close-up pictures of the tip area, enlarged in cells overexpressing AtPRK6. Pollen tubes expressing GFP were control. (Bar=5 μm). C, Percentage of shorter pollen tubes with enlarged tips. Data were collected from 60 to 80 pollen tubes from three independent experiments. Data are presented as mean±SD. D, Percentage of pollen tubes shorter than 0.4 mm in AtPRK6 or GFP expressed pollen tubes. Data were collected from 60 to 80 pollen tubes from three independent experiments. Data are presented as means±SD. E and F, Average length (E) and tip width (F) of pollen tubes expressing AtPRK6 or GFP. Data were collected from 60 to 80 pollen tubes from three independent experiments. Data are presented as means±SD (P<0.01, t-test).

6B, upper and Figure S1 in Supporting Information). The accumulation of large vacuoles at the tip may contribute to the observed tip enlargement.

The kinase domain, but not kinase activity, of AtPRK6 is essential for its function

As shown above, the kinase domain of AtPRK6 is essential for its interaction with AtRopGEF8/12, while the interaction does not depend on the kinase activity of AtPRK6. To investigate whether the kinase domain and/or the kinase activity affects AtPRK6's function in pollen tubes, we examined the overexpression of two PRK6 mutants (referred to as RPK6 K412N and RPK6 ΔKD in Figure 7) in tobacco pollen tubes. Similar to wild-type AtPRK6 (Figure 7B), over-expression of AtPRK6 K412N induced depolarized pollen tube growth with enlarged tips (Figure 7C). On the other hand, over-expression of AtRPK6 ΔKD lost the ability to induce the depolarized growth of pollen tubes (Figure 7D). These results suggest that the kinase domain is essential for the function of AtPRK6, and its function does not depend on its activity.

DISCUSSION

Overexpression of AtPRK6 induced depolarized pollen tube growth

PRKs are a sub-group of RLKs, which are membrane-localized proteins with serine/threonine kinase activities, and function in polar growth of pollen tubes (Lee et al., 1996; Tang et al., 2002; Zhang et al., 2008; Gui et al., 2014; Chang et al., 2013; Zhao et al., 2013; Muschietti et al., 1998). Previous studies demonstrated that *LePRK1* and *LePRK2*, two LRR-RLK (leucine rich repeat receptor like kinases), are specifically expressed in pollen grains of *Solanum lycopersicum* (tomato) (Muschietti et al., 1998), and pollen tubes overexpressing *LePRK2* had significantly decreased pollen tube lengths and increased pollen tube tip widths (Salem et al., 2011). In *Arabidopsis*, there are eight *PRK* genes, six of which (*AtPRK1* through *AtPRK6*) were reported to be highly expressed in pollen grains (Chang et al., 2013). Here, we successfully cloned the *AtPRK6* gene, which was unanalyzed due to an unsuccessful cloning attempt in the study described

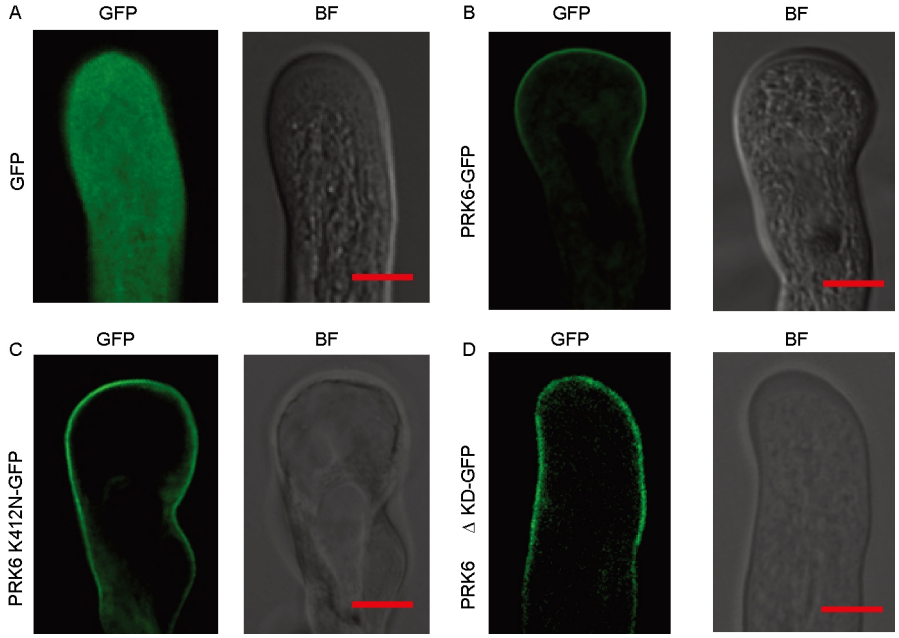

Figure 7 The kinase domain of AtPRK6 contributed to tip enlargement of pollen tubes. A, Pollen tubes expressing GFP were used as control. B–D, Tobacco pollen tubes overexpressing the full-length AtPRK6 (B), the kinase activity site mutagenized AtPRK6 (PRK6 K412N) (C) and kinase domain-truncated AtPRK6 (PRK6 ΔKD) (D) were imaged. Bars=5 μm.

above, and further investigated its potential function in pollen tube growth. During our preparation of this current article, a study reporting that AtPRK6, together with several PRK family members, functions in pollen tube reorientation during reproduction in *Arabidopsis* was published (Takeuchi and Higashiyama, 2016). In a semi-*in-vivo* assay, only the *prk6* pollen tubes lost responsiveness to the peptide AtLURE1, while the other tested *prk* mutants still showed a reaction to AtLURE1. Furthermore, severe defects in growth and attraction *in vivo* were observed in *prk3*/*prk6*/*prk8* and *prk1*/*prk3*/*prk6* triple mutants. These data suggested that AtPRK6 is essential for targeting pollen tube growth toward ovules through the sensing of the species-specific AtLURE1 attractant in cooperation with other PRKs in *Arabidopsis* (Takeuchi and Higashiyama, 2016).

To explore additional biological functions of AtPRK6, we overexpressed AtPRK6 in tobacco pollen grains, and our results clearly showed that AtPRK6 overexpression induced abnormal, depolarized pollen tube growth. The pollen tubes were shorter and had swollen tips (Figure 6A and B). Different pollen tubes expressing *AtPRK6* showed tip enlargement to different extents (Figure 6C–F), indicating that *AtPRK6* may truly have a specific function in polarized tube growth. Together with previous studies (Chang et al., 2013), evidences now support that all six pollen-expressed *AtPRK* genes function in polarized pollen tube growth. However, to fully understand the function of AtPRK6 in tip growth of pollen tubes, more genetic data will be needed in the future.

The pollen tube is a special cell that grows in a tubular fashion to deliver sperm cells to the egg (Zhou et al., 2014). This process is essential for seed formation in flowering plants. Pollen tubes possess two basic features: rapid growth over a long distance in one direction and capability to sense directional cues to redirect growth (Gu et al., 2005). The pollen tube grows only at one tip and is precisely guided towards ovules through sensing of the AtLURE1 attractant peptide (Takeuchi and Higashiyama, 2016). Our evidence that AtPRK6 is involved in tip growth of pollen tubes confirms the role of *AtPRK6* in polarized growth.

Using the inactive mutant (PRK6 K412N), our results provided strong evidence that the function of *AtPRK6* relies on the kinase domain, but not on its kinase activity (Figure 7). In addition, our RT-PCR results and transgenic plant analysis demonstrated a pollen tube-specific expression pattern of *AtPRK6* (Figure 1B), which is consistent with the previous results (Chang et al., 2013; Takeuchi and Higashiyama, 2016).

We also showed by two independent methods that AtPRK6 was localized to the plasma membrane (Figure 2; Figure 6B, lower). Careful observation revealed a stronger signal accumulation to the membrane in the tip area of the cell (Figure 6B, lower), which was consistent with our GUS activity results (Figure 1B(f)) and the previous report (Takeuchi and Higashiyama, 2016). These data support the idea that AtPRK6 is a tip-located receptor that is recruited to the cell membrane at the pollen tube apex when pollen tubes are growing (Takeuchi and Higashiyama, 2016).

Single *atprk* mutants exhibited no defect in pollen grain germination (Chang et al., 2013; Takeuchi and Higashiyama, 2016), which we also observed in *atprk6* mutants (Figure S2 in Supporting Information). Meanwhile, severe defects in

pollen tube germination or growth were observed in double or triple *atprk* mutants (Chang et al., 2013; Takeuchi and Higashiyama, 2016), suggesting that *AtPRK6* most likely function redundantly with other *AtPRK* genes. In addition, PRK proteins perceive multiple guidance signals from the female or male tissues for the precise guidance to the ovule, much like other RLK proteins have been reported to (Takeuchi and Higashiyama, 2016; Wang et al., 2016). Studies in tomato indicated that *LePRK2* mediates pollen tube growth through binding to different peptides at different stages (Muschietti et al., 1998; Tang et al., 2002; Johnson and Preuss, 2003; Tang et al., 2004; Zhang et al., 2008). In *petunia*, PRK2 mediates reproductive success by binding the peptide SHY (Muschietti et al., 1998; Gu et al., 2004). In *Arabidopsis*, *PRK6* guides pollen tube growth by perceiving the attractant peptide AtLURE1 (Takeuchi and Higashiyama, 2016). As reported, AtRopGEF8 can activate the pollen expressing small GTPase protein AtROP11 (Li and Liu, 2012). It was hypothesized that AtROP11 may be involved in signaling sensing during pollen tube guidance to ovules (Gu et al., 2004). Our results showed that AtPRK6 interacts with AtRopGEF8. Therefore, it is possible that AtPRK6 is also dependent on the perception of other extracellular signals, and thus a *atprk6* mutation is not critical and does not show a phenotype, but AtPRK6 overexpression disrupts the signaling and leads to a depolarized growth phenotype in tobacco pollen tubes.

AtPRK6 interacted with AtRopGEF8/12 through JM and KD

The mechanism by which PRKs regulate the polar growth of pollen tubes was proposed to be through RopGEF-activation of ROPs signaling pathways (Chang et al., 2013). ROPs are small GTPases that control the rate of tip growth via tip-localized calcium fluxes (Li et al., 1999), the dynamics of tip F-actin for maintainence of polar growth (Gu et al., 2005), and vesicle trafficking via RICs (Lee et al., 2008). A yeast two-hybrid screen showed that AtPRK6 interacts with AtRopGEF8/12 (Figure 3). *Arabidopsis* PRK2 was previously shown to interact with AtRopGEF1 and AtRopGEF12 (Chang et al., 2013; Zhao et al., 2013). These results together indicate that AtRopGEF12 interacts with not only AtPRK2 but also AtPRK6, suggesting that RopGEF proteins can work with multiple AtPRK proteins to regulate pollen tube growth. Since both PRK and RopGEF members exhibited functional redundancy, the regulation of polar pollen tube growth may involve multiple signals downstream of the PRKs and RopGEFs.

AtPRK6 also interacts with AtRopGEF8, 9, 12, & 13, which are all highly or prominently expressed in pollen tubes (Takeuchi and Higashiyama, 2016; Zhang and McCormick, 2007). However, the current results in yeast were not totally consistent with the published results, i.e., only AtRopGEF8/12 were confirmed to interact with AtPRK6 (Figures 3 and 4), while the interactions between AtPRK6 and AtRopGEF9/13 were not detected (Figure 3). In this study, only the CD of AtPRK6 (PRK6-CD) was used as bait in the yeast two-hybrid system to screen the RopGEFs (Figures 3 and 4A), while Takeuchi and Higashiyama used the full-length CDS sequence of *AtPRK6* in *N. benthamiana* BiFC assays (Takeuchi and Higashiyama, 2016).

In our study, details of the interactions between AtPRK6 and AtRopGEF8/12 were investigated. It was demonstrated that the CD of AtPRK6 interacted with the CT domain of AtRopGEF12 (Figure 5B), which is consistent with the previous results from the *N. benthamiana* BiFC assay and co-immunoprecipitation assay (Takeuchi and Higashiyama, 2016). Furthermore, our data identified both the JMD and KD of AtPRK6 are essential for the interaction, and provided detailed information about the interaction between AtPRK6 and AtRopGEF8 (Figure 5B). AtRopGEF8 is specifically expressed in pollen grains and can activate AtROP11 (Li and Liu, 2012). AtRopGEF8 expression also induces depolarized growth of pollen tubes (Gu et al., 2006). Our research similarly showed that the overexpression of *AtRopGEF8* induced depolarization of pollen tube growth (Figure S3 in Supporting Information).

Another interesting finding in this report is that the potential kinase-dead mutation AtPRK6 K412N retained the ability to interact with AtRopGEF8/12, while the kinase domain-truncated AtPRK6 did lose the ability to interact with AtRopGEF8/12 (Figure 5B), suggesting that the kinase activity of AtPRK6 is not essential for the interaction in the yeast two-hybrid system. In addition, our data showed that AtPRK6 function required the kinase domain, but was independent of its kinase activity (Figure 7). Since the downstream effectors of AtPRK6-AtRopGEF8/12 were not isolated, it remains to be discovered whether the kinase activity is essential for formation of any three-protein complex.

The working model of AtPRK6 regulation of polarized growth of pollen tubes

A mechanism of how PRKs regulate the polar growth of pollen tubes was proposed previously (Chang et al., 2013; Zhang and McCormick, 2007). Our current article supported this model while adding detailed information, especially for AtPRK6. The extracellular LRR domains of AtPRK6 sense the guiding signal of AtLURE1, and as signal transduction cascades inside the cell, the JMD and KD of AtPRK6 interact with AtRopGEF8/12 to activate the downstream ROPs signaling pathways, which further regulate the polarized growth of pollen tubes (Figure 8). *Arabidopsis* genome encodes eleven *ROP* genes falling into four groups. The group I (*AtROP8*), group II (*AtROP9*, *AtROP10* and *AtROP11*) and group IV (*AtROP1*, *AtROP3* and *AtROP5*) members were identified to express in pollen tubes (Gu et al., 2003; Gu et

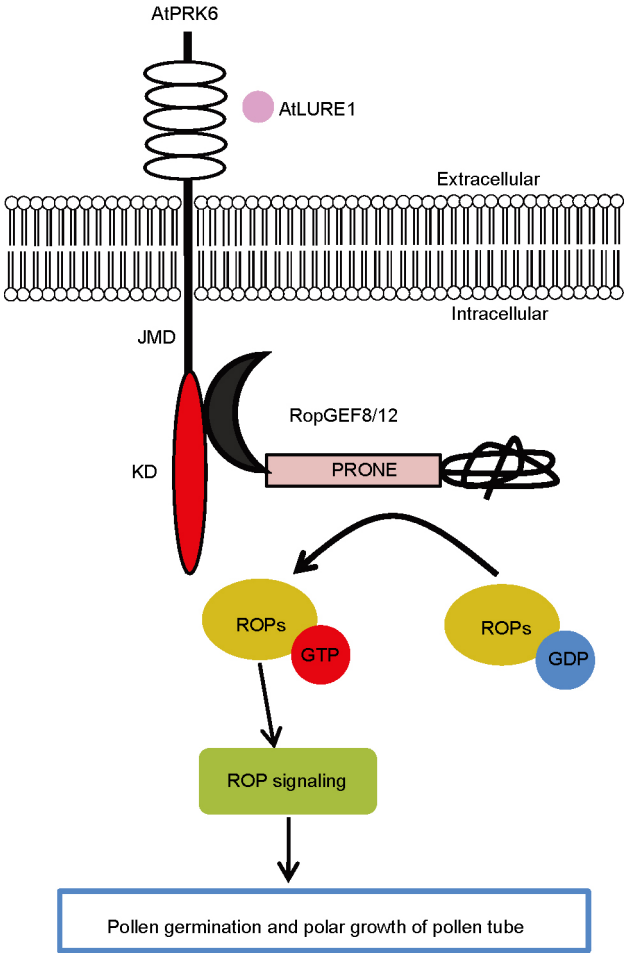

Figure 8 A working model of *AtPRK6*-induced, depolarized pollen tube growth. Plasma membrane-localized AtPRK6 has both an extracellular LRR domain and a CD (which is divided into two subdomains: juxtamembrane and kinase). The extracellular domain is responsible for the perception of AtLURE1, to guide the pollen tube growth towards unfertilized ovules. Meanwhile, AtPRK6 (through its JMD and KD) interacts with AtRopGEF8/12, which may activate ROPs by exchanging their GDP for GTP, to further regulate polar pollen tube growth through downstream ROP signaling pathways. This model is suggested by Chang et al. (Chang et al., 2013) and Zhang and McCormick (Zhang and McCormick, 2008).

al., 2004). *AtROP1*, *AtROP3* and *AtROP5* were reported to act redundantly to promote pollen tube elongation (Gu et al., 2004; Gu et al., 2003). The specific functions of *AtROP8–AtROP11* remain unknown. ROP proteins localize at the tip and control polarized tip growth in pollen tubes (Li et al., 1999; Gu et al., 2003; Gu et al., 2004). ROPs were reported to regulate pollen tube growth by controlling two counteracting pathways: the RIC3 and RIC4 pathways. RIC3 pathway activates Ca^{2+} signaling that leads to F-actin disassembly, whereas, RIC4 pathway promotes F-actin assembly (Chang et al., 2013; Gu et al., 2005). Thus these two pathways work together to regulate the influence of F-actin dynamics and F-actin/Ca^{2+} dependent exocytosis on polarized pollen tube growth (Chang et al., 2013; Yang and Fu, 2007; Yang and Fu, 2007). The connections between ROPs, RopGEFs and PRKs means it is possible that PRK proteins may also contribute to cytoskeleton regulation.

MATERIALS AND METHODS

Plant materials and growth condition

All wild-type plants were *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). Plants were grown in a growth chamber with a 16-h photoperiod at a constant temperature of 23°C. Tobacco plants, *Nicotianatata bacum*, were grown at 28°C under the same light-dark cycle.

RT-PCR analysis

In order to get the *AtPRK6* cDNA fragment, the total RNA from the flower tissue was extracted, and subsequently used as template to synthetize the first strand cDNA using Super-Script III first-strand synthesis system (Invitrogen, USA). Three independent clones were sequenced to confirm the accurate *AtPRK6* fragment. Primers (LAT52-PRK6-GFP-F/LAT52-PRK6-GFP-R) used in PCR reaction to clone PRK6 cDNA were listed in Table 1.

To analyze the expression pattern of AtPRK6, seedlings, roots, leaves, stems and flowers were collected from wild-type *Arabidopsis* plants. Total RNA was extracted from each tissue sample using the TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized using Super-Script III first-strand synthesis system (Invitrogen, USA). PCR amplification was performed with 2× easy-Taq PCR super-mix (TransGen, Beijing) using PRK6-RT-F/R primers, with *ACTIN2* as an internal reference gene. All primers are listed in Table 1.

Generation of *AtPRK6$_{Pro}$-GUS* transgenic plants

A 1,383-bp promoter region of *AtPRK6* was amplified from *Arabidopsis* genomic DNA and subsequently cloned into a plasmid (pBASTA-GUS-GW) harboring a *GUS* reporter gene using the Gateway cloning system (Invitrogen). The constructed plasmid was then transformed into the *Agrobacterium tumefaciens* strain *GV3101*. Transgenic plants were developed by the floral dip method using positively transformed *Agrobacterium* cells and Col-0 plants (Clough and Bent, 1998). The seeds from the transformed plants were collected and further selected using Basta resistance. The GUS activity was analyzed using the method described previously (Jefferson et al., 1987). To detect GUS activity in pollen tubes, the pollen grains were cultured on pollen germination media (Li et al., 1999) for 4 h and then stained with the X-gluc staining solution.

Subcellular localization of AtPRK6

For subcellular localization in *Arabidopsis* mesophyll protoplasts, the full-length coding sequence of AtPRK6 was PCR

Table 1 Primers used in this article

Names of primer	Sequence (5′→3′)
PRK6-RT-F	CAACCAAGCAACGCATCGCAAG
PRK6-RT-R	ATGCGAGACCTGTTGTGTCTGC
ACTIN2-F	TCTTCCGCTCTTTCTTTCCAAGC
ACTIN2-R	ACCATTGTCACACACGATTGGTTG
NPPRK6-GUS-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCAATACCTTCAAAAGATCGAC
NPPRK6-GUS-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGATTGAAGAGAGGAGATG
GEF1-AD-F	CCGGAATTCATGGGGAGCTTATCTTCTGAGGAAGAC
GEF1-AD-R	CGCGGATCCTTAATCTCTTTCCGGCGTCACTCCC
GEF2-AD-F	GGGAATTCCATATGATGGAGAATTTGCCAAATCACGAAG
GEF2-AD-R	CGCGGATCCTCATTCTTCTCCTCTCATTGTTTTG
GEF3-AD-F	GGGAATTCCATATGATGGAGAATTTATCGAATCCAG
GEF3-AD-R	CGCGGATCCTTATTCACTACCTCTCATGGTTTTGTC
GEF4-AD-F	GGGAATTCCATATGATGGAGAGTTCTTCGAATTCCGAC
GEF4-AD-R	CGCGGATCCCTAATCATCTCTGTTTCTCACTGTTC
GEF5-AD-F	GGGAATTCCATATGATGGAGAATTTAGTGAAGAGCTGC
GEF5-AD-R	CGCGGATCCTTAAGAGACAGTGTACTTTGGAG
GEF6-AD-F	GGGAATTCCATATGATGGAGGATAATAGCTGTAT CGGG
GEF6-AD-R	CGCGGATCCTCAACCCCGGAGATAATTGGCCAATG
GEF7-AD-F	GGGAATTCCATATGATGGATGGTTCGTCGGAAAATTTGC
GEF7-AD-R	CGCGGATCCTCAAATCCCAGGATCAAGGTTC
GEF8-AD-F	GGGAATTCCATATGATGGTTGCAGCGTTGGAACGAG
GEF8-AD-R	CGCGGATCCTTAATGCCTATCTTTGGGACTTCTAAAAC
GEF9-AD-F	GGGAATTCCATATGATGGTTCCATCGTTGGAACGAG
GEF9-AD-R	CGCGGATCCTCAATGCCTATCTTTAGG
GEF10-AD-F	GGGAATTCCATATGATGTTCGATGGTCGGAACTCTGGAC
GEF10-AD-R	GGAAGATCTTCAGTGTCTGTCACTAGGGCTTCTC
GEF11-AD-F	GGGAATTCCATATGATGTTGGAAGGCAAAGCAATGGTG
GEF11-AD-R	CGCGGATCCTCAGGAGTATCTTGCGGTTGGACTC
GEF12-AD-F	GGGAATTCCATATGATGGTTCGTGCTTCGGAACAGAAC
GEF12-AD-R	CCGGAATTCTCAATGCCGTGCCGTTGGACTCTTG
GEF13-AD-F	CGCGGATCCATATGGTGAAAGCGAGTGAGAAAGAAC
GEF13-AD-R	CCGCTCGAGTCAAGAGTATCGCCCCGCGGTTG
GEF14-AD-F	CCGGAATTCATGAAGAATGATGAACACAGTT
GEF14-AD-R	CGCGGATCCTCAAGGAGAAGTATCAGAAGGC
PRK6-CD-BD-F	GGAATTCCATATGAATAAGAAGAAGAACCCTGA
PRK6-CD-BD-R	CGGGATCCCTCAAGTTTTTACTTGTTCTATCCTTC
PRK6-JM-BD-F	GGAATTCCATATGAATAAGAAGAAGAACCCTGA
PRK6-JM-BD-R	CGGGATCCCGATCCAAGGCTACCGTTGCC
PRK6-KD-BD-F	GGAATTCCATATGGGCAACGGTAGCCTTGGATC
PRK6-KD-BD-R	CGGGATCCCTCAAGTTTTTACTTGTTCTATCCTTC
PRK6-K412N-F	GATTATCCGTTGTGGTGAACAGGATTAGGGATAT
PRK6-K412N-R	GTTCACCACAACGGATAATCCAGTGGTCATCACC
GEF8-CT-AD-F	GGAATTCCATATGGAAGAAACATCAGATGGTGG
GEF8-CT-AD-R	CGGGATCCCTTAATGCCTATCTTTGGGACTT
GEF12-CT-AD-F	GGAATTCCATATGCCATTGGAAGCAGAGGAA
GEF12-CT-AD-R	CGGGATCCCTTAATGCCTATCTTTGGGACTT
LAT52-PRK6-GFP F	CTAGACTAGTATGGCTGCTGCTGTTCTGAATCC
LAT52-PRK6-GFP R	CGGGATCCCAGTTTTTACTTGTTCTATCCTTC
LAT52-PRK6ΔKD-GFP-F	CTAGACTAGTATGGCTGCTGCTGTTCTGAATCC
LAT52-PRK6ΔKD-GFP-R	CGGGATCCCATACCACCGCCTAGAGCTCC
LAT52-GEF8-GFP F	CTAGACTAGTATGGTTGCAGCGTTGGAACGAG
LAT52-GEF8-GFP R	CGCGGATCCCATGCCTATCTTTGGGACTTCTAAAAC

amplified and directionally cloned into the vector pCAMBIA1302 for GFP fusion at the *Nco* I/*Spe* I sites. PEG-mediated transformation was used to introduce the resultant plasmids into the *Arabidopsis* mesophyll protoplasts (Yoo et al., 2007). Transformed cells were incubated in W5 solution (Yoo et al., 2007) for 16–20 h in the darkness. A LSM780 confocal microscope (Zeiss, Germany) was used to observe and capture the fluorescence images.

Yeast two-hybrid analysis

Primers used to generate constructs for yeast-two-hybrid analysis are listed in Table 1. Sequences encoding the CD (290–659 aa), JMD (290–397 aa) and KD (397–659 aa) of AtPRK6 were cloned from cDNA into the pGBKT7 vector. The full-length and CT coding sequences of AtRopGEF8/12 were separately cloned into pGADT7. The successful constructs were co-transformed into the yeast strain AH109 using the lithium acetate method (Lin et al., 2012). Transformants were selected on SD/-Trp/-Leu medium and transferred onto selection medium (SD/-Trp/-Leu/-His). Measurement of the β-galactosidase activity was performed using filter-paper colony lift and a solution containing 10 mL Z-buffer (0.06 mol L^{-1} NaH_2PO_4; 0.04 mol L^{-1} Na_2HPO_4; 0.01 mol L^{-1} KCl and 0.01 mol L^{-1} $MgSO_4$) supplied with 0.27 mL β-mercaptoethanol and 1.67 mL X-gal following the previously reported procedures (Lin et al., 2012).

BiFC assay

For BiFC in *Arabidopsis* mesophyll protoplasts, pE3242 and pE3228 were used (Yang et al., 2015). The full-length cDNA sequence of AtPRK6 was cloned into pE3242 (cCFP-PRK6) in frame with cCFP, and AtRopGEF8/12 was separately cloned into the pE3228 (nVenus-RopGEF8/12) with nVenus. The cCFP-AtPRK6 and nVenus-AtRopGEF8/12 plasmids were co-transformed into mesophyll protoplasts isolated from 5-week-old *Arabidopsis* rosette leaves as described (Yang et al., 2015). Protoplasts co-transformed with cCFP-AtPRK6 and nVenus were used as negative controls. All assays were carried out with three independent replicates.

Transient overexpression in tobacco pollen tubes

To construct the *AtPRK6*::*GFP* plasmid for overexpression in tobacco pollen grains, the full-length coding sequence of *AtPRK6* from the cDNA from *Arabidopsis* flower tissues was fused with the GFP gene using primers listed in Table 1. The *AtPRK6*::*GFP* cassette was subsequently cloned into a vector harboring a pollen-specific *LAT52* promoter (Twell et al., 1991). The constructed plasmids were then delivered into mature tobacco pollen grains via particle delivery procedures as described previously (Twell et al., 1989). After transient expression and growth for 4 h on pollen growth medium (Chang et al., 2013; Gui et al., 2014), germinated pollen tubes were observed and images were captured with a confocal microscope, LSM780. At least three independent bombardment experiments were performed. For each experiment, more than 20 fluorescent pollen tubes were observed, and at least 30 pollen tubes were measured for length and width of tip area using ImageJ software. Cloning and transient overexpression of AtPRK6 K412N-GFP and AtPRK6 ΔKD-GFP were performed following the same methods.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

Acknowledgements *The authors are grateful to Dr. Jing Li for reading and giving comments to this manuscript. This work was supported by the National Natural Science Foundation of China (31300247).*

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SUPPORTING INFORMATION

Figure S1 Large vacuoles accumulated at the tips of pollen tubes overexpressing AtPRK6.

Figure S2 Pollengermination rate of *prk6* mutants. Pictures were taken after 5-h incubation on pollen germination medium (left). Percentage of germinated WT and *prk6* mutants (right).

Figure S3 AtRopGEF8 induced pollen tube depolarization when expressed in tobacco pollen tubes.

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