Kinase Partner Protein Plays a Key Role in Controlling the Speed and Shape of Pollen Tube Growth in Tomato

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The rapid and responsive growth of a pollen tube requires delicate coordination of membrane receptor signaling, Rho-of-Plants (ROP) GTPase activity switching, and actin cytoskeleton assembly. The tomato (Solanum lycopersicum) kinase partner protein (KPP), is a ROP guanine nucleotide exchange factor (GEF) that activates ROP GTases and interacts with the tomato pollen receptor kinases LePRK1 and LePRK2. It remains unclear how KPP relays signals from plasma membrane-localized LePRKs to ROP switches and other cellular machineries to modulate pollen tube growth. Here, we biochemically verified KPP’s activity on ROP4 and showed that KPP RNA interference transgenic pollen tubes grew slower while KPP-overexpressing pollen tubes grew faster, suggesting that KPP functions as a rheostat for speed control in LePRK2-mediated pollen tube growth. The N terminus of KPP is required for self-inhibition of its ROPGEF activity, and expression of truncated KPP lacking the N terminus caused pollen tube tip enlargement. The C-terminus of KPP is required for its interaction with LePRK1 and LePRK2, and the expression of a truncated KPP lacking the C-terminus triggered pollen tube bifurcation. Furthermore, coexpression assays showed that self-associated KPP recruited actin-nucleating Actin-Related Protein2/3 (ARP2/3) complexes to the tip membrane. Interfering with ARP2/3 activity reduced the pollen tube abnormalities caused by overexpressing KPP fragments. In conclusion, KPP plays a key role in pollen tube speed and shape control by recruiting the branched actin nucleator ARP2/3 complex and an actin bundler to the membrane-localized receptors LePRK1 and LePRK2.

The delivery of nonmotile sperm to the embryo sac via a pollen tube is a key innovation that allowed flowering plants to carry out sexual reproduction without the need for water (Friedman, 1993; Lord and Russell, 2002). Both the speed and signal responsiveness of pollen tube growth are critical for successful fertilization (Johnson et al., 2019). The typical shape of a growing pollen tube cell protruding from a pollen grain is a cylinder with a dome-shaped tip (Geitmann, 2010). Maintaining such a typical tube shape during pollen tube growth is fundamental to support its ability for fast growth (Michard et al., 2017), and a plasticity range of tubular growth rates allows a pollen tube to optimize directional growth along its journey from the stigma to the ovule (Luo et al., 2017). The pollen tube cell extends mainly by tip growth, requiring huge amounts of secretion/exocytosis at the tip (McKenna et al., 2009; Grebnev et al., 2017). The newly secreted cell wall at the tip is mainly composed of esterified pectin, which is expandable, whereas cell wall remodeling at the lateral region (including pectin deesterification and callose deposition) limits expansion (Grebnev et al., 2017). The tip width of a growing pollen tube actually reflects the size of the secretion zone capped by an expandable membrane and cell wall, as a collective result of multiple pollen tube growth machineries (Luo et al., 2017).

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W.-H.T. and D.Z. conceived the research; H.-K.L. performed experiments on KPP transgenic plant construction and biochemical assays; Y.-J.L. performed experiments for phenotypic analyses; S.-J.W. performed KPLs’ biochemical assays; X.D. analyzed the expression of KPP and SCAR; T.-L.Y. analyzed parts of the BiFC results; J.-Q.P. performed the inhibitor assay; H.-K.L., X.-J.L., D.Z., W.-H.T., and S.M. provided constructs for tomato transformation; J.-Q.P. performed the inhibitor assay; H.-K.L., Y.-J.L., D.Z., W.-H.T., and S.M. analyzed data; W.-H.T., H.-K.L., and S.M. wrote the article; all the authors revised the article.

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The tip-localized exocytosis of a growing pollen tube is supported by a spatiotemporal tightly controlled actin cytoskeleton network (Hepler, 2016). The actin cytoskeleton configuration in a pollen tube includes highly dynamic fine actin filaments in the apical and subapical regions and parallel longitudinal actin bundles in the shank region (Qu et al., 2017). Various actin-binding proteins, such as actin nucleation factors, actin-severing proteins, and actin-bundling factors, are responsible for organizing the dynamic actin cytoskeleton network (Ren and Xiang, 2007). For example, the actin-bundling proteins fimbrin and LIM (Lin-1, isl1, Mec3) domain-containing proteins function in shank-localized actin bundles in pollen tubes (Zhang et al., 2019). For another example, the actin nucleator formin (formin3 in Arabidopsis [Arabidopsis thaliana] and formin1 in lily [Lilium longiflorum]) functions in actin polymerization in the pollen tube tip (Li et al., 2017; Lan et al., 2018). The branched actin nucleator Actin-Related Protein2/3 (ARP2/3) complex is an evolutionarily conserved, seven-subunit complex consisting of the actin-related proteins ARP2 and ARP3 (Machesky et al., 1994). The ARP2/3 complex initiates the formation of branches on the side of preexisting actin filaments, locally creating a force-generating branched actin network that underlies cellular protrusion and movement (Blanchon et al., 2000; Amann and Pollard, 2001; Molinie and Gautreau, 2018). The phenotypes of mutants in ARP2/3 in the moss Physcomitrella patens (Harries et al., 2003; Brembu et al., 2004; Deeks et al., 2004), in maize (Zea mays; Frank and Smith, 2002), and in tomato (Solanum lycopersicum; Chang et al., 2019) demonstrated the broad importance of the ARP2/3 complex and its activation during cellular morphogenesis, including tip-growing cells. Perhaps surprisingly, in Arabidopsis, null ARP2/3 alleles are transmitted normally through pollen and there is no obvious root hair phenotype (Le et al., 2003; Djakovic et al., 2006).

These cell growth machineries are tightly coordinated by multiple signaling pathways, including membrane-localized receptor kinases and Rho-of-Plants (ROP) GTases (Li et al., 2018). The tomato pollen-specific and membrane-localized receptor kinases LePRK1 and LePRK2 mediate signaling during pollen tube growth (Muschietti et al., 1998). LePRK2 perceives several extracellular growth-stimulating factors, including a Cys-rich extracellular protein (Late-Anther-Specific52 [LAT52]), a Leu-rich repeat protein from pollen, and two pistil/stigma molecules, Style Interactor for LePRKs and Stigma-Specific Protein1 (Tang et al., 2002, 2004; Wengier et al., 2003, 2010), which increase the speed of pollen tube growth (Zhang et al., 2008b; Huang et al., 2014). LePRK2 antisense and RNA interference (RNAi) pollen tubes grow slower (Zhang et al., 2008b), consistent with a positive role for LePRK2 in regulating the speed of pollen tube growth. LePRK1 binds LePRK2 (Wengier et al., 2003), but LePRK1 plays a negative role in pollen tube growth by controlling a switch from a fast tubular mode to a slow blebbing mode (Gu et al., 2014). LePRK1 RNAi pollen tubes burst more often than wild-type pollen tubes, implicating a role for LePRK1 in maintaining plasma membrane integrity (Gu et al., 2014). An Arabidopsis paralog of these LePRKs, PRK6, also localized on the tip membrane, perceives Arabidopsis attraction cues from the female, AtLURE1s, to guide pollen tube growth (Takeuchi and Higashiyama, 2016; Zhang et al., 2017).

Rho family small guanine nucleotide-binding proteins called ROPs or RACs, which can switch between a GDP-bound inactive form and a GTP-bound active form, are regulators of polar growth in pollen tubes (Cheung and Wu, 2008; Yang, 2008). In Arabidopsis, ROP1-dependent signaling controls tip growth. Active ROP1 defines a cap region in the apical plasma membrane as an exocytosis zone (Luo et al., 2017). Overexpression of ROP1 or of a constitutively active version resulted in pollen tube tip swelling (i.e. increased tip width) and slower growth (i.e. reduced tube length), while overexpressing a dominant negative version of ROP1 inhibited pollen tube growth (i.e. shorter but normal width tubes). The size of the pollen tube tip reflects the aggregate activity of membrane-associated ROP at the tip (McKenna et al., 2009; Luo et al., 2017). Tomato ROPs have been reported to be associated with the LePRK1-LePRK2 complex (Wengier et al., 2003) and therefore presumably play similar roles as the Arabidopsis homologs in pollen tube growth, yet their biological roles have not been directly investigated.

Guanine nucleotide exchange factors (GEFs) activate ROPs by promoting the conversion of ROP/RAC GTases from a GDP-bound inactive form to a GTP-bound active form. Plants possess a plant-specific ROPGEF family whose members contain a highly conserved GEF catalytic domain, the PRONE (plant-specific ROP nucleotide exchanger) domain (Berken et al., 2005; Gu et al., 2006). The intracellular portions of LePRK1 and LePRK2 interact with Kinase Partner Protein (KPP; Kaothien et al., 2005), whose Arabidopsis homologs were later shown to belong to the PRONE-type ROPGEF family (Berken et al., 2005; Gu et al., 2006). Pollen tubes overexpressing nearly full-length KPP (missing eight amino acids at the N terminus) developed swollen tips with abnormal cytoplasmic streaming and F-actin arrangements (Kaothien et al., 2005). An Arabidopsis homolog of receptor kinase, AtPRK2a (also named AtPRK2), interacts with AtROPGEF12 (Zhang and McCormick, 2007) and with AtROPGEF1 (Chang et al., 2013) to affect ROP activity. Based on the in vitro catalytic activity of full-length and truncated AtROPGEF1, an autoinhibition conferred by the C-terminal variable region was proposed (Gu et al., 2006). AtROPGEF12 was also shown to interact with the guidance receptor kinase PRK6 (Takeuchi and Higashiyama, 2016).

Increased expression of full-length KPP increased the speed of pollen tube growth without significantly affecting pollen tube shape. We show biochemically that the PRONE domain of KPP does have ROPGEF activity on several class I ROPs, with highest activity on ROP4.
The N-terminal domain of KPP inhibits its own GEF activity, while its C-terminal domain enhances its own GEF activity. The C-terminal domain of KPP is also required for its interactions with LePRK1, LePRK2, and an actin-bundling protein, Pollen-expressed LIM2a (PLIM2a), while the C-terminal domain alone is sufficient to bind LePRK1 but insufficient to bind LePRK2. Furthermore, self-associated KPP colocalized with the actin nucleation proteins ARP2/3 complex during pollen tube growth and enriched the membrane localization of ARP2/3 in the pollen tube. Interfering with ARP2/3 activation by coexpressing a dominant negative version of ARP2 reduced the speed of pollen tube growth and alleviated the defects caused by the overexpression of truncated KPP. CK-666, a specific small molecule inhibitor of ARP2/3 activation, canceled the promotive effect of full-length KPP on the speed of pollen tube growth. These results indicate that during pollen germination and tube growth, KPP not only links pollen receptor kinase and ROP signaling but also links the actin network to the pollen tube plasma membrane, thereby directly affecting the cellular morphology and efficiency of pollen tube growth.

RESULTS

Overexpressing Full-Length KPP Increases Pollen Tube Growth Speed

We previously reported that increased expression of full-length KPP (Supplemental Fig. S1) resulted in pollen tubes with slightly increased width (Zhang et al., 2008b). More detailed statistical analyses showed that, in general, pollen tubes transiently overexpressing full-length KPP fused with enhanced GFP (eGFP) grew faster than wild-type pollen tubes (Fig. 1, A and B), based on the observation that KPP-expressing pollen tubes were ~25% longer than wild-type tubes when measured after 5 h of in vitro germination (Fig. 1C) and were ~50% longer when measured after 9 h (Fig. 1D). Except for a few pollen tubes with very high KPP-eGFP expression that showed significantly enlarged tips and short tubes, most KPP-eGFP-expressing tubes had a normal tubular shape, except for large vacuoles that were most often observed in the tip region (Fig. 1, A and B; Supplemental Figs. S2 and S3).

To clarify whether this growth promotion effect of KPP was shared by its close homologs, we also performed phenotypic analysis on pollen tubes transiently overexpressing the four pollen-preferential KPP-like proteins individually (Supplemental Fig. S4). KPP-like1 (KPPL1) and KPPL2 overexpression showed no significant difference in pollen tube length and tip width, while KPPL3 and KPPL4 overexpression resulted in shorter tubes with wider tips (Fig. 1, B and D). So, despite sharing the PRONE domain and the additional C-terminal domain that is conserved in the pollen-preferential clade (AtROPGEF8–AtROPGEF13; Zhang and McCormick, 2007), of the five pollen-preferential members, KPP is the only one that can increase the speed of pollen tube growth when overexpressed.

To verify these transient expression results, we constructed stable transgenic tomato lines that overexpressed full-length KPP fused with mRFP in pollen (Supplemental Data Set S1). Pollen tubes from two independent KPP-mRFP lines grew about 10% longer and about 10% to 20% wider tips than those expressing mRFP alone (Fig. 1, E and F). We also constructed RNAi tomato lines to reduce KPP expression in pollen (Fig. 2A; Supplemental Data Set S1; Supplemental Fig. S5). Pollen tubes from three independent transgenic lines with reduced KPP mRNA levels grew slower both in vitro and in the pistil (Fig. 2, B, D, and E), and the lengths of pollen tubes grown in vitro correlated with the KPP expression levels (Fig. 2, A and D). In addition, KPP RNAi pollen had poorer germination (Fig. 2C), and in those that did germinate, pollen tubes exhibited actin cable aggregation in the shank, close to the germination pore (Fig. 2F; Supplemental Fig. S6), as visualized by transiently coexpressing Lifeact-mRFP (Vidali et al., 2009). This suggests an impact of KPP on the spatial organization of the actin cytoskeleton in the pollen tube. The correlation between KPP expression level and the speed of pollen tube growth suggests that KPP functions like a rheostat in the tip growth machinery.

Expressing KPP-Truncated Versions Alters Pollen Tube Tip Morphology

We then asked how various domains of KPP contribute to the tip growth machinery. KPP possesses three parts, the N terminus (residues 1–45), the central PRONE domain (residues 46–403), and the C-terminal domain (residues 404–502). The PRONE domain of KPP has GEF activity on a ROP from tomato (Soly02g062020.1, named ROP3a in this report; Berken et al., 2005). Unlike its Arabidopsis homologs, which have a C terminus that is autoinhibitory (Zhang and McCormick, 2007), the activity of KPP’s PRONE domain alone is similar to that of full-length KPP (Löcke et al., 2010). The tomato genome encodes nine ROP GTPases including four type I ROPs expressed in pollen (ROP1, ROP3a, ROP3b, and ROP4; Supplemental Fig. S7). These four ROPs have higher GEF activity with KPP (Fig. 3A) than their intrinsic activities. Furthermore, KPP most potently activated ROP4, and an N-terminally truncated KPP showed higher GEF activity while a C-terminally truncated KPP showed reduced GEF activity (Fig. 3B), indicating that the N terminus might have an autoinhibition function while the C-terminal domain might enhance activity. Notably, the truncated KPPΔN8 (i.e. KPP lacking eight amino acids at the N terminus) represents the highest GEF activity version among the variants tested. GEF activity largely determines the abundance of active ROPs, which further might be reflected by the ability to cause the enlargement of pollen tube tips. Figure 3,
C to F, shows that expressing ROP4 alone only slightly increased pollen tube tip width, while coexpressing KPP or its N-terminal truncated variants with ROP4 resulted in greatly increased pollen tube tip widths.

We then examined the biological roles of the N-terminal, PRONE, and C-terminal domains of KPP by phenotypic analysis of pollen tubes overexpressing various truncated versions of KPP (Fig. 4A) in both transient and stable transformants. Overexpression of a construct in which KPP was N-terminally truncated by 45 amino acids (KPPΔN45-eGFP) yielded enlarged pollen tube tips (Fig. 4B), as did overexpression of KPPΔN8-eGFP (Kaothien et al., 2005), consistent with an overactivation of ROP GTPases causing increased secretion at the tip. Overexpression of a C-terminally truncated KPP (KPPΔC-eGFP) resulted in branching (Fig. 4B), as more than 40% of transformed pollen tubes had at least two tips. Overexpression of the PRONE domain (PRONEKPP-eGFP) also induced bifurcated tubes with enlarged tips (Fig. 4B). Overexpression of KPPΔC-mRFP or KPPΔN8&ΔC-mRFP also yielded bifurcated pollen tubes in stably transformed tomato plants (Fig. 4, C and D). In independent transgenic tomato lines, from 8% to 40% of the transformed pollen tubes had two tips (Supplemental Data Set S1). This is consistent with the expectation that lower ROP activity reduces the positive feedback loop maintaining a single tip, which leads to the appearance of additional tips (Luo et al., 2017). The plasma membrane was strongly deformed when any version lacking the C terminus was overexpressed (Fig. 4B). Notably, these branched tubes were often long, with visible actin cables in shanks of both the main and branch tubes (Fig. 4F). This suggests that the expression of KPP lacking the C terminus not only initiated additional tips but also employed machineries (such as actin cables) that sustain tip growth.

To show that these phenotypes were not due to fusion with eGFP, we also transiently expressed truncated versions of KPP and eGFP in separate expression cassettes; these pollen tubes exhibited phenotypes similar to the corresponding eGFP-fused truncated
Figure 2. Transgenic KPP RNAi tomato pollen tubes grew slower. Wild-type (WT) and homozygous transgenic plants carrying plAT52::eGFP (eGFP) are controls. A, Reverse transcription quantitative PCR of KPP mRNA levels, using total RNA of mature pollen as a template. The tomato actin gene was used as a control. Error bars indicate SE. n = 3 independent experiments. B and C, Representative images and measurements taken after 4 h of in vitro germination. A1, B1, and B2 are independent transgenic line.
versions of KPP (Supplemental Fig. S8), indicating that it is the truncated versions of KPP per se that caused the phenotypes.

The C-Terminal Domain of KPP Is Sufficient to Bind LePRK1 But Not LePRK2

We next tested if the different functions of the KPP domains might be achieved via the different proteins with which they interact. KPP can interact with the cytoplasmic domains (CD1 and CD2) of the pollen receptor kinases LePRK1 and LePRK2 (Kaothien et al., 2005) and can interact with the actin-bundling protein PLIM2a (Gui et al., 2014). In yeast two-hybrid assays, KPPΔN45 can interact with CD1 and CD2, whereas KPPΔC and PRONEKPP cannot (Fig. 5A). In particular, the C-terminal domain of KPP alone is sufficient for interaction with CD1 (Fig. 5A) but not for interaction with CD2. In bimolecular fluorescence complementation (BiFC) assays in pollen tubes, the full-length KPP and LePRK1 interaction was strong, while KPPΔC and LePRK1 interacted only weakly (Fig. 5B).

Based on the BiFC results, LePRK1-KPP and LePRK2-KPP interactors both localized on the pollen tube apical membrane, but not uniformly (see enlarged confocal image in Fig. 5B). Notably, pollen tube tip membranes with LePRK1-KPP localization showed dents (deformations), while those with LePRK2-KPP localization were smooth (normal with internal turgor). This further supports the idea that KPP links the actin cytoskeleton to the LePRK1-localized tip membrane, thereby enabling membrane deformation. However, the LePRK1-KPP BiFC signal on the membrane did not colocalize with the PLIM2a signal (Gui et al., 2014), suggesting that the linkage might involve components other than PLIM2a.

The LePRK1-KPP complex might constitute the majority of these two proteins, because in pollen grains expressing both LePRK1-eGFP and KPP-mRFP (obtained by crossing transgenic tomato lines LePRK1-eGFP×KPP-mRFP), LePRK1-eGFP and KPP-mRFP were highly colocalized both on the membrane and in the cytoplasm (Fig. 5C; Supplemental Fig. S9). In contrast, in pollen grains obtained by crossing LePRK1-eGFP and KPPΔN8&ΔC-mRFP, LePRK1-eGFP and KPPΔN8&ΔC-mRFP barely colocalized (Fig. 5C, right; Supplemental Fig. S9). This is also consistent with the idea that the C-terminus of KPP is required for the interaction.

Remarkably, transient expression of only the C-terminal portion (~100 amino acids) caused significantly slower in vitro growth (i.e. pollen tube lengths after 6 h were ~35% shorter than those expressing eGFP alone; Fig. 5D; Supplemental Fig. S8). This result is consistent with the idea that the C terminus of KPP negatively affects pollen tube growth and that the KPP C terminus is able to bind LePRK1. Furthermore, Figure 5E shows that, in comparison with LePRK1 overexpression, coexpression of the KPP-C-ter with LePRK1 yielded fewer pollen tubes with blebs and less enlargement at the tips, which can be interpreted as a KPP-C-ter interaction with LePRK1, therefore interfering with LePRK1 to cause pollen tube defects (including blebbing and tip enlargement); Figure 5F shows that coexpression of KPP-C-ter with LePRK2 did not reduce pollen tube length significantly, which can be interpreted as no interference with the pollen tube growth promotion effect from LePRK2 overexpression.

Self-Associated KPP Colocalizes with ARP2/3-Labeled Puncta in the Cytoplasm and Increases the Distribution of ARP2/3 at the Pollen Tube Apical Membrane

To understand the biological function of KPP in a spatial context, we examined its subcellular localization in pollen tubes. In transient overexpression experiments, pollen tubes weakly expressing KPP-eGFP were similar to wild-type tubes, and in these tubes, KPP-eGFP more likely performed similar to their native function. Therefore, we focused on pollen tubes weakly expressing KPP-eGFP. Figure 6A shows that KPP-eGFP is not only distributed in the cytosol but also enriched at the tip membrane, where the tip growth machinery (including actin polymerization and exocytosis) is organized. As assessed by BiFC after transient expression in pollen tubes, full-length KPP fused with split YFP (KPP-YN and KPP-YC) self-associated and also distributed to both the cytoplasm and the plasma membrane of the tip and shank (Fig. 6B). Interestingly, the BiFC signal of either version of N-terminally truncated KPP had more significant membrane enrichment and patched distribution as well as some punctate structures in the cytoplasm (Fig. 6, C and D). Given that the LePRK1 self-associated BiFC signal was uniformly distributed on the membrane (Gui et al., 2014), the patchy distribution of KPP on the membrane may not be due to LePRK1. This result suggests that KPP with released autoinhibition from its N terminus might form clusters.

The ARP2/3 complex was reported to provide a leading edge to yeast and animal cells as well as to plant
trichomes (Mathur, 2005; Chang et al., 2019), but its role at the pollen tube leading edge was not proved. We therefore examined the localization and phenotypic consequences of the expression of ARP2/3 complex components in pollen tubes. After identifying the tomato homologs of ARP2, ARP3, and ARPC4 by sequence similarity (Supplemental Fig. S10), they were fused with mRFP and transiently expressed in tobacco (Nicotiana tabacum) pollen. The ARP2-mRFP, ARP3-mRFP, and ARPC4-mRFP fusion proteins had an overall soluble cytoplasmic distribution but also localized in punctate structures (Fig. 6, E, I, K, and M) that moved with cytoplasmic streaming. This distribution is consistent with the expectation that ARP2/3 is mainly inactive in the cytosol and that only a very small portion spontaneously activates to initiate actin polymerization and attach to vesicles. More interestingly, the KPP BiFC signal partially colocalized with the punctate structures of the fusion proteins, indicating that KPP might interact with the ARP2/3 complex at the plasma membrane of pollen tubes.

Figure 3. KPP preferentially activates ROP4 and interacts with ROP4 at the plasma membrane of pollen tubes. A and B, GEF activity assay. A, Time course of exchanged guanine nucleotide amounts of various ROPs with or without KPPΔN8. B, Time course of exchanged guanine nucleotide amounts of ROP4 with various truncated fragments of KPP. Results shown are from one of three independent assays that gave similar results. Rate constants (Kobs) are shown in parentheses. Kobs values were obtained by monophasic exponential fits using PeakFit v4.01. C to E, Representative pollen tubes coexpressing BiFC constructs YC-ROP4 with KPP-YN (C), KPPΔN45-YN (D), and KPPΔN8-YN (E). F, Transient overexpression of eGFP-ROP4 in tobacco pollen tubes. BF, Bright field. Bars = 20 μm.
Figure 4. Overexpression of truncated fragments of KPP results in depolarized or bifurcated pollen tubes. A, Diagram of KPP fragments. Gray bars indicate the conserved PRONE domain. a.a., Amino acid. B to D, Partial deletions of KPP tagged with eGFP/mRFP were transiently expressed in tobacco pollen (B) or in transgenic tomato plants (C and D). E and F, Pollen tubes transiently coexpressing Lifeact-eGFP with mRFP (E) or with KPPΔN8&ΔC-mRFP (F). BF, Bright field; BP, bifurcated pollen tube percentage; G, grain of a pollen tube; T, tip of a pollen tube; TW, tip width of pollen tubes, the average from more than 50 pollen tubes. Bars = 20 μm.
with ARP2 both in the cytoplasm and plasma membrane (Fig. 6F): about 30% of the ARP2-labeled vesicles are colocalized with self-associated KPP, and the KPP D N45 BiFC signal colocalized with ARP2, ARP3, or ARPC4 patches more strongly both in the cytoplasm (80%) and plasma membrane (Fig. 6, F, G, J, L, and N). Particularly, KPPΔN8 BiFC signal colocalized with ARP2 mainly on membrane patches at the pollen tube tip (Fig. 6H). In addition, ARP2-eGFP and ARP3-mRFP were highly colocalized (Fig. 6, K and L; Supplemental Fig. S9), as were ARPC4-eGFP and ARP3-mRFP (Fig. 6, M and N; Supplemental Fig. S9). These colocalizations suggest that they are likely in a complex. Given that KPPΔN8 has higher ROPGEF activity than full-length KPP, we suspect that self-associated KPP with higher GEF activity might affect the distribution of ARP2/3 toward the tip membrane, the region of a leading edge-protruding actin network assembly.

The KPPΔN45-ROP4 BiFC signal showed plasma membrane localization (Fig. 3D), and therefore expressed Figure 5. KPP can interact with LePRK1 and LePRK2, and the C terminus of KPP is sufficient for interaction with LePRK1. A, Yeast two-hybrid analysis of interactions between KPP fragments and the cytoplasmic domains of LePRK1 and LePRK2. -W,-L, Synthetic dextrose medium lacking Trp/Leu; -W,-L,-H,-A, synthetic dextrose medium lacking Trp/Leu/His/adenine. B, Representative pollen tubes coexpressing the indicated BiFC constructs. C, Pollen grains of F1 plants from transgenic tomato expressing KPP-mRFP or KPPΔN8ΔC-mRFP crossed with transgenic tomato expressing LePRK1-eGFP. M0 and M4 are colocalization coefficients of RFP and GFP. D to F, Transient expression of the indicated constructs in tobacco pollen. BF, Bright field; G, pollen grain; T, pollen tube tip. Arrows in E point to the blebs growing out of pollen tube tips. Error bars indicate SE. n = 3 independent experiments. At least 20 pollen tubes were measured in each experiment. Asterisks indicate significant differences from the control (P < 0.05, Student’s t test). Bars = 10 μm (B and C) and 50 μm (D–F).
Figure 6. Self-associated KPP colocalized with ARP2/3 complexes. A to R, Representative pollen tubes expressing various constructs as indicated are shown. BF, Bright field; G, grain of pollen tube; T, tip of pollen tube. Insets are enlarged images focusing on tips. White arrows in R point to example points where ARP3-mRFP colocalizes with actin filaments. M_G and M_T are colocalization coefficients; PM ratio is the plasma membrane-localized signal average intensity versus cytoplasm-localized signal average intensity. Bars = 20 μm.
with ARP2, the KPP-ROP4 and KPPΔN8-ROP4 BiFC signals also colocalized with ARP2 on membranes (Fig. 6, O and P). These results suggest that ARP2 may form a complex with KPP and ROP4 (i.e. the ARP2/3 complex might be recruited by self-associated KPP and ROP4). Notably, when coexpressed with ARP2, self-associated KPP remained clustered, as indicated by patchy distribution on the plasma membrane, while interacting KPP-ROP4 uniformly distributed on the plasma membrane, indicating that both dimerized and monomeric KPP can interact with ROP4, consistent with the crystal structure showing that both monomeric and dimeric ROPGEF can catalyze ROP activation (Thomas et al., 2009).

We then asked whether the ARP2/3 complex participated in configuring the pollen tube actin cytoskeleton. When ARP3-mRFP was coexpressed with Lifeact-eGFP to label the actin filaments, pollen tubes exhibited a typical actin cytokinetic composed of cables at the shank and fine filaments at the tip (Fig. 6Q). When KPPΔN45 was coexpressed with ARP3-mRFP and Lifeact-eGFP, in addition to the tip swelled, which is typical for KPPΔN-expressing pollen tubes, ARP3-mRFP showed stronger accumulation at the tip plasma membrane and more actin filaments accumulated in the cytoplasm adjacent to the tip membrane (Fig. 6R). Noteworthy, coexpression with KPPΔN45 greatly increased the colocalization of ARP3-mRFP with Lifeact-eGFP (Fig. 6R), suggesting that active KPP causes ARP2/3 complex activation.

We also tried to assess ARP2/3 function by observing the phenotypic consequences upon overexpression in pollen tubes. Pollen tubes transiently overexpressing RFP fused to ARP2 or ARP3 exhibited normal growth in vitro, with tip widths similar to those of the wild type (Fig. 6, E and I). Expressing ARP2 or ARP3 caused no apparent pollen tube growth defects, which was expected given that the ARP2/3 complex needs to be activated to be functional in promoting actin polymerization (Zhang et al., 2013). We therefore tried to see if there were phenotypic consequences when ARP2/3 activation was altered. In Arabidopsis, a point mutation from a conserved Gly to Asp at position 151 in ARP2 (ARP-G151D) caused a pivotal change in the ARP2 structure leading to loss of ARP2/3 complex activity and a dominant negative phenotype in trichome cell expansion (Mathur et al., 2003). We therefore made a similar mutant of tomato ARP2 (ARP2-G151D) named DN-ARP2. Transient overexpression of DN-ARP2-mRFP in pollen yielded slower pollen tube growth in vitro (Fig. 7B).

The ARP2/3 complex needs to be activated to promote the branched actin network, and plants rely on a single family of ARP2/3 activators termed suppressor of cAMP repressor (SCAR) or WASP-family verprolin-homologous protein (Goley and Welch, 2006; Zhang et al., 2008a). The conserved SCAR homology domain (SHD) at the N terminus of SCAR is responsible for recruiting the ARP2/3 complex and is therefore required for ARP2/3 activation (Zhang et al., 2005). Overexpression of SHD alone (without the activation domains of SCAR) recruits ARP2/3 to a nonfunctional complex and yielded a dominant negative phenotype in transgenic Arabidopsis (Zhang et al., 2005). We cloned the SHD domain of SCAR2 (the only member of the SCAR family expressed in tomato pollen) and transiently overexpressed the SHD domain fused with mRFP in tobacco pollen. As shown in Figure 7C, there were no obvious morphological differences between the SHD-mRFP-transformed pollen tubes and mRFP-expressing pollen tubes (Fig. 7C), but pollen tubes expressing SHD-mRFP were shorter (Fig. 7D), suggesting slower growth. That overexpression of either the SHD domain of SCAR alone or of DN-ARP2, which affects ARP2/3 complex function, reduced the speed of pollen tube growth supports the idea that the ARP2/3 complex contributes to pollen tube growth.

To further examine whether the ARP2/3 complex directed the polymerization of actin filaments and thereby significantly supports pollen tube growth, we assayed the effect of a small molecule, CK-666, reported to specifically inhibit nucleation of actin filaments by the ARP2/3 complex (Nolen et al., 2009; Hetrick et al., 2013). We used a pollen tube growth assay (Huang et al., 2014) to determine the effects of the compound CK-666 and another small molecule, CK-689, which is structurally related to CK-666 but has no effect on actin polymerization, as a control. CK-666 slowed down pollen tube growth, as indicated by the size reduction of the pollen tube mat floating on the medium, while CK-689 at the same concentration had no effects on pollen tube growth (Fig. 7E). This result further supports that nucleation of actin filaments by the ARP2/3 complex significantly contributes to the fast growth of tomato pollen tubes.

Interfering with ARP2/3 Activation Reduced the Pollen Tube Phenotypes Caused by Expressing Various Versions of KPP

ARP2/3 complexes typically contribute to the configuration of the actin cytoskeleton. We showed that KPP colocalized with ARP2/3 (Fig. 6) and that KPP RNAi altered the actin cytoskeleton configuration (Fig. 2F). To determine whether truncated versions of KPP caused pollen tube phenotypes by acting through ARP2/3, we transiently coexpressed DN-ARP2 or the SHD domain of SCAR alone with KPPΔC or KPPΔN45 in tobacco pollen (Fig. 7, G–I). Overexpression of KPPΔC alone resulted in branched pollen tubes: more than 50% of the transformed pollen tubes had at least two tips (Fig. 7G). However, coexpression of DN-ARP2 or SHD with KPPΔC yielded mostly normal tubes (Fig. 7, H and I), with less than 25% bifurcated pollen tubes (Fig. 7I). Overexpression of N-terminally truncated KPP disturbed pollen tube growth, and the transformed tubes had wider tips (Fig. 7K). Coexpression of DN-ARP2 or SHD with KPPΔN45 yielded mostly normal tubes (Fig. 7, L and M), as tips were only slightly wider (Fig. 7N). These data show that interfering with ARP2/3 activation indeed alleviated the defects caused by the overexpression of truncated KPP versions.
Figure 7. Overexpression of constructs interfering with ARP2/3 activation, and application of an ARP2/3 activation inhibitor suppressed the impacts on pollen tube morphology or growth speed caused by overexpression of KPP variants. A to C, Representative pollen tubes expressing mRFP (A), DN-ARP2-mRFP (B), or SHD-mRFP (C). D, Chart of pollen tube lengths measured after 6 to 7 h of in vitro culture. E, Tomato pollen tube growth assay. Representative images of pollen tubes at two different culture times and measurements of the area of pollen tube mats taken after 14 or 20 h of culturing are shown. F, Measurements of the length of tobacco pollen tubes overexpressing eGFP or KPP-eGFP treated with the indicated chemicals for 3 h. G to I, Representative pollen tubes expressing KPPΔC-eGFP with the indicated constructs. J, The percentage of bifurcated pollen tubes was calculated after 6 to 7 h of in vitro culture. K to M, Representative pollen tubes expressing KPPΔN-mRFP with the indicated constructs. N, Tip width was measured after 6 to 7 h of in vitro culture. BF, Bright field; G, grain of pollen tube; T, tip of pollen tube. For each sample in each experiment in D, H, and L, more than 50 pollen tubes were measured. n = 3 independent experiments. Error bars indicate SE. Asterisks indicate significant differences from the control by Student’s t test (*P < 0.05). Bars = 20 μm.
If the ARP2/3 complex contributes to KPP-mediated modulation of pollen tube growth, then inhibiting ARP2/3 complex activation should impair KPP’s effect on the speed of pollen tube growth. We therefore used CK-666 to treat pollen tubes overexpressing full-length KPP. The lengths of KPP-eGFP-expressing pollen tubes were similar to those of eGFP-expressing pollen tubes treated with CK-666 (Fig. 7F; Supplemental Fig. S11). In contrast, KPP-eGFP-expressing pollen tubes treated with CK-689 were longer than eGFP-expressing pollen tubes (about 20% longer; Fig. 7F). This result indicates that CK-666 suppressed the KPP promotion effects on pollen tube growth rate. Taken together, we conclude that KPP affects both the speed and shape of pollen tube growth, at least partially through the function of ARP2/3.

**DISCUSSION**

Here, we used biochemical assays, various truncated gene constructs, and cellular imaging to show that KPP links the membrane-localized pollen receptor kinases LePRK1 and LePRK2 with activation of the small GTPase ROP4 and regulators of the actin cytoskeleton, thereby connecting the changes of pollen tube tip shape and growth speed to extracellular signals in the pistil. The full-length KPP acts as a rheostat, whose protein abundance linearly corresponds to the speed of pollen tube growth without disrupting pollen tube shape (Figs. 1 and 2). The plasticity to control speed built in with the pollen tube growth machinery provides a foundation for fine adjustment of the speed of pollen tube growth according to diverse environmental cues in the journey to achieve fertilization, because tubes growing too fast can be less responsive or completely miss targets (Luo et al., 2017) while growing slightly slower might compromise its cargo sperm’s chances to be delivered to the ovule (Delph et al., 1998; Zhong et al., 2019).

The dissection of the N-terminal and C-terminal domains of KPP relative to GEF activity, protein interactions, and phenotypic consequences helped us understand how KPP works. Here, we show that KPP activation has selectivity on ROPs, since it most potently activated ROP4 (Fig. 3), and that the N terminus of KPP partially inhibits its GEF activity, which allows the regulation of GEF activity through releasing N-terminal inhibition. We also showed that the C-terminal domain of KPP binds LePRK1 but not LePRK2 (Fig. 5). That overexpressing the C-terminal domain of KPP alone resulted in a drastically reduced speed of growth (Supplemental Fig. S6) suggests that KPP might bring growth inhibitors to LePRK1 through interaction with its C terminus. Furthermore, overexpressing the C-terminal domain of KPP with LePRK1 alleviated the LePRK1 overexpression-caused defects, including blebbing and tip enlargement, probably by competition of the KPP C terminus in the LePRK1 interaction. In contrast, overexpressing the C-terminal domain of KPP with LePRK2 did not impair the speed of pollen tube growth, probably because the C terminus of KPP does not interact with LePRK2, while LePRK2’s promotion effect on pollen tube growth canceled the negative effect of the KPP C terminus on pollen tube growth.

The actin cytoskeleton in a growing pollen tube is spatiotemporally organized as a result of regulating many diverse actin-binding proteins. Through interactions with the actin bundler PLIM2a and the actin nucleator ARP2/3, KPP has impacts on the actin cytoskeleton organization. In KPP RNAi pollen tubes, more actin cables accumulated in the pollen tube neck region close to the pollen grain (Fig. 2F), probably because a reduction in KPP expression leads to alteration of actin bundling and network forming in the pollen tube tip and then causes a secondary balancing effect, resulting in enhancement in actin bundling in the neck region. The reduction of LePRK1-KPP interaction and the following KPP-PLIM2a interaction also contribute to the compromised pollen tube membrane integrity maintenance in transgenic LePRK1 RNAi pollen tubes (Gui et al., 2014).

By linking the transmembrane LePRKs and regulators of the actin cytoskeleton, KPP and its variants affect the shape at the leading edge of the growing pollen tube. As a walled cell, cell shape is usually thought to be controlled mainly by the rigid cell wall (Mathur, 2005). However, due to the expandable feature of the pollen tube cell wall, the tip shape can be determined by the plasma membrane and the tightly linked cortical actin cables. It is notable that manipulating expression levels of KPP fragments resulted in a huge deviation in pollen tube shape without a complete disruption of expansion at the leading edge. For example, about half of the pollen tubes that stably expressed KPPAC bifurcated and many of those tubes maintained growth at both tips (for representative images, see Fig. 4; for statistics, see Supplemental Data Set S1). Bifurcated pollen tubes are seen in gymnosperms such as pine (Pinus spp.; Çetinbaş-Genç and Vardar, 2020) but are not common in angiosperms, except in pollen tubes with targeting defects (Meng et al., 2020). Pollen tubes expressing KPP lacking the N terminus had enlarged tips (Fig. 4). Pollen tubes expressing KPP lacking both the N and C termini often bifurcated and had enlarged tips (Fig. 4). Even in slow-growing pollen tubes that coexpressed LePRK1 and PLIM2a, the addition of KPP expression led pollen tubes to extend by blebbing (Gui et al., 2014).

The reason behind these shape diversities is that KPP links multiple actin cytoskeleton regulators, including the F-actin bundler PLIM2a and actin polymerization nucleator ARP2/3 complexes, to transmembrane proteins at the leading edge of the pollen tube. Under high concentrations of Ca$^{2+}$, KPP bound to PLIM2a and enhanced its effect on actin filament bundling (Gui et al., 2014). Given that Ca$^{2+}$ concentration oscillates in growing pollen tubes (Rounds and Bezanilla, 2013) and that connecting thick actin bundles to the membrane will confer more stiffness to the membrane, the...
LePRK1/2-KPP-PLIM2a interaction might periodically enhance local membrane stiffness, thereby contributing to the documented speed oscillations of pollen tube growth.

ARP2/3 complexes are thought to shape the leading edge, providing a protrusion force and connecting membranes by promoting actin network formation upon activation (Mathur, 2005; Akin and Zipursky, 2014; Molinie and Gautreau, 2018). However, a dominant negative mutant of Arabidopsis ARP2 has normal male transmission (Mathur et al., 2003), implying that ARP2/3 might not be important in pollen tube growth. We showed that, without coexpressing KPP, ARP2/3 mainly localized in the cytosol, presumably in its inactive form, while with coexpression with KPP, ARP2/3 changed localization to the tip membrane and/or association with actin filaments (Fig. 6). We further showed that treatment with CK-666 slowed down pollen tube growth (Fig. 7). Therefore, ARP2/3 complexes contribute to pollen tube tip growth, although perhaps not to a level to cause male transmission defects. Bringing the ARP2/3 complex to regions adjacent to the tubular pollen tube tip membrane allows local formation of actin filament networks that generate a pushing force to the expandable pollen tube tip membrane. The regulatory scenario in which KPP, as a ROPGEF, recruits ARP2/3 actin nucleation is reminiscent of the leaf cell shape regulation by SPIKE1, a Dedicator of Cytokinesis family ROPGEF (Basu et al., 2008). In summary, KPP is one of the key linkages modulating pollen tube growth, which, when modulated, can convert tubular growth to enlarged tip growth, to bifurcated growth, or to blebbing.

MATERIALS AND METHODS

Generation of Transgenic Tomatoes

Tomato (Solanum lycopersicum) 'VF36' was used for transgenic construction. For overexpression lines, fragments of KPP fused with mRFP driven by the LAT52 promoter were inserted into pCAMBIA2300. For RNAi lines, a KPP RNAi cassette, which includes two copies of 500 bp from KPP in a head-to-head configuration, and a GFP expression cassette, both driven by the LAT52 promoter, were individually inserted into pCAMBIA to obtain the pCAMBIA-KPP RNAI plasmid. Agrobacterium tumefaciens strain LBA4404 (Hoekema et al., 1983) carrying these plasmids was used to transform tomato as described (McCormick, 1991). Detailed construction information is provided in Supplemental Data Set S1.

Reverse Transcription Quantitative PCR

Total RNA from mature pollen was extracted using RNAiso plus (TaKaRa) according to the manufacturer’s protocol. cDNA was generated by Moloney murine leukemia virus (TaKaRa). Quantitative PCR was performed using SYBR Green on an iCycler (Bio-Rad). The primers used in this assay are listed in Supplemental Table S1. For each assay, amplification cycles at 95°C for 5 s followed by 40 amplification cycles at 95°C for 5 s and 60°C for 30 s.

In Vitro and in Vivo Pollen Germination

Tomato and tobacco (Nicotiana tabacum 'Gexin No. 1') were grown under standard greenhouse conditions as described (Huang et al., 2014). Mature pollen grains were collected and germinated as described (Zhang et al., 2008).

For in vitro germination, freshly collected mature pollen grains were obtained by vibrating anthers of open flowers with a biovortexer (BioSpec Products) and then germinated in vitro in pollen germination medium [PGM; 20 mM MES, pH 6, 3 mM Ca(NO3)2, 1 mM KCl, 0.8 mM MgSO4, 1.6 mM boric acid, 2.5% (w/v) Suc, and 24% (w/v) polyethylene glycol, molecular weight 4000] at 25°C on six- or 24-well plates rotated horizontally at 60 rpm (tomato) or 150 rpm (tobacco). For pollen germination in vivo, freshly collected pollen was applied to tomato pistils, in flowers whose anthers were removed in advance. The pollinated pistils were collected for microscopic analysis after 6 to 8 h. Decolorized Aniline blue staining of pollen tubes in pistils was performed as described (Muschietti et al., 1998).

Pollen Bombardment and Image Analyses

The pollen-specific LAT52 promoter (Twell et al., 1990) was used in all pollen bombardment assays. All target genes were inserted in frame at the 5’ end of the GFP/RFP/Y/N/YC coding sequence in the pLAT52::GFP and pLAT52::RFP plasmids and BiFC plasmids, except for ROP4, which was inserted at the 3’ end of the GFP or RFP. Pollen bombardment was carried out as described (Zhang et al., 2008b) using 10 μg of plasmid for each bombardment. After a 4-h incubation, bombarded pollen tubes were observed and images were captured using an Olympus BX51 microscope fitted with an Olympus DP71 digital camera or a confocal microscope (Olympus Fluoview FV10i). For confocal microscopy, eGFP was excited with a laser at 473 nm and detected at 500 to 540 nm, and mRFP was excited with a laser at 591 nm and detected at 670 to 690 nm. The pollen bombardment experiments for each construct or combination of constructs were performed at least three independent times, and each time at least 20 fluorescent pollen tubes were observed; representative images for each phenotype are shown. For each experiment, pollen tube lengths and tip widths of at least 20 pollen tubes in each category were measured using ImageJ (Schneider et al., 2012).

Colocalization Threshold in ImageJ was used for the quantitative colocalization analysis of fluorescence signals. Mf and Mm are Manders’ colocalization coefficients (Dunn et al., 2011). Mf is the proportion of the red fluorescence colocalized with green fluorescence in the total red fluorescence of the selected region of interest area. Mm is the proportion of the green fluorescence colocalized with red fluorescence in the total green fluorescence of the same selected region of interest area. Based on confocal images, the BiFC index was calculated by ImageJ as the average YFP intensity divided by the coexpressed mRFP intensity. At least 10 pollen tubes per treatment were measured, and the average BiFC index was recorded. The plasma membrane ratio was calculated by the pollen tube plasma membrane-localized signal average intensity relative to the cytoplasm-localized signal average intensity.

Pharmacological Treatments in Pollen Tube Growth Assay

CK-666 (HY-16926; MCE; Hetrick et al., 2013) and CK-689 (182517; Sigma; Nolen et al., 2009) were dissolved in 18% (v/v) dimethyl sulfoxide, 72% (v/v) polyethylene glycol, molecular weight 4000 at 25°C on six- or 24-well plates rotated horizontally at 60 rpm. For each construct or combination of constructs were performed at least three independent times, and each time at least 20 pollen tube experiments were measured using ImageJ.

Pollen tubes were cultivated in 500 μL of PGM per well for 2 h after the bombardment experiment. Then, 10 μL of stock solution of CK-666, CK-689, or equal volume of solvent was applied to each well for a further 3 h of culture. The pollen culture was incubated at 25°C in the dark on 24-well plates rotated horizontally at 60 rpm and visualized at the indicated time points after culture (Huang et al., 2014). The sizes of the pollen tube mats were measured by ImageJ.

Tobacco pollen tubes were cultivated in 500 μL of PGM per well for 2 h after the bombardment experiment. Then, 10 μL of stock solution of CK-666, CK-689, or equal volume of solvent was applied to each well for a further 3 h of culture. The pollen culture was incubated at 25°C in the dark on 24-well plates rotated horizontally at 125 rpm. A fluorescence microscope was used to capture the photographs of tobacco pollen tubes. The length of pollen tubes was measured by ImageJ software.

Protein Expression and Purification

For protein expression in Escherichia coli, fragments of KPP and ROPs were fused with GST to get GST-tagged proteins. All GST-tagged proteins were expressed in Rosetta bacteria and purified using Glutathione Sepharose 4B (GE Healthcare) following procedures described by the manufacturer. Briefly, fusion proteins were expressed at 18°C for 8 h with 1 mM isopropyl-β-D-thiogalactoside after 2 h of growth at 37°C. Cell cultures were spun down at 5,000 rpm.
for 10 min. Cell pellets were resuspended in a GST-binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.5) and sonicated 50 times using 10 s pulses. The supernatant was collected by centrifugation at 12,000g for 20 min. The supernatant was mixed with Glutathione Sepharose 4B (GE Healthcare). Before the addition of 15 gL−1 HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl2, and 1 mM DTT) using a 10 KDa dialysis cassette (Amicon), and stored on ice. Nucleotide exchange was induced at 18°C for 8 h with 1 mM isopropyl-β-D-thiogalactoside after 2 h of growth at 37°C. Cells were harvested and resuspended in buffer A (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM MgCl2, and 100 mM GDP). After sonication using 50 pulses of 10 s, the supernatant was obtained by centrifugation at 12,000g for 20 min. The supernatant was mixed with Glutathione Sepharose 4B (GE Healthcare) for 2 h. The mixture was applied to a column and washed with 10 ml of buffer B (20 mM Tris, pH 8, 100 mM NaCl, 5 mM MgCl2, 2.5 mM CaCl2, and 40 mM GDP) and eluted with elution buffer (50 mM Tris-HCl and 10 mM reduced glutathione, pH 8). GDP-bound ROPs were concentrated in a centrifugal filter (Amicon), buffer exchanged (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl2, and 1 mM DTT) using a 10 KDa cutoff dialysis cassette (Amicon), and stored on ice. Nucleotide exchange was monitored as the increase in relative fluorescence of a fluorescent GDP analog (mamb-GDP) upon binding to ROPs. Before the addition of 1 μM KPP, 2 mM GDP-RPops was incubated in the guanine nucleotide exchange buffer containing 0.75 μM mamb-GDP (Molecular Probes). After equilibration, fluorescence was measured every 10 s for 600 s using a spectrophotometer with excitation wavelength at 360 ± 5 nm and emission wavelength at 440 ± 5 nm. The Kobs values (pseudo-first-order rate constant) were obtained by monophasic-exponential fits using PeakFit v4.01 (Jandel Scientific Software).

**Measurement of Guanine Nucleotide Exchange Activity**

The guanine nucleotide exchange activities of KPP and its variants were assessed by the rate of exchange of prebound fluorescent GDP to fluorescent-upto-binding mamb-GDP on individual ROP GTases according to Gu et al. (2006) with minor modifications. A 5-mL overnight culture of E. coli expressing GST-ROPs was used to inoculate 200 mL of Luria-Bertani medium (10 gL−1 tryptone, 5 gL−1 yeast extract, and 10 gL−1 NaCl, pH 7). Protein expression was induced at 38°C for 4 h with 1 mM isopropyl-β-D-thiogalactoside after 2 h of growth at 37°C. Cells were harvested and resuspended in buffer A (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM MgCl2, and 100 mM GDP). After sonication using 50 pulses of 10 s, the supernatant was obtained by centrifugation at 12,000g for 20 min. The supernatant was mixed with Glutathione Sepharose 4B (GE Healthcare) for 2 h. The mixture was applied to a column and washed with 10 ml of buffer B (20 mM Tris, pH 8, 100 mM NaCl, 5 mM MgCl2, 2.5 mM CaCl2, and 40 mM GDP) and eluted with elution buffer (50 mM Tris-HCl and 10 mM reduced glutathione, pH 8). GDP-bound ROPs were concentrated in a centrifugal filter (Amicon), buffer exchanged (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl2, and 1 mM DTT) using a 10 KDa cutoff dialysis cassette (Amicon), and stored on ice. Nucleotide exchange was monitored as the increase in relative fluorescence of a fluorescent GDP analog (mamb-GDP) upon binding to ROPs. Before the addition of 1 μM KPP, 2 mM GDP-RPops was incubated in the guanine nucleotide exchange buffer containing 0.75 μM mamb-GDP (Molecular Probes). After equilibration, fluorescence was measured every 10 s for 600 s using a spectrophotometer with excitation wavelength at 360 ± 5 nm and emission wavelength at 440 ± 5 nm. The Kobs values (pseudo-first-order rate constant) were obtained by monophasic-exponential fits using PeakFit v4.01 (Jandel Scientific Software).

**Supplemental Data**

The following supplemental materials are available:

- **Supplemental Figure S1.** Integrated Genome Browser view of the tomato pollen RNA sequencing reads mapping to KPP/Solyc03g120650, to confirm the extent of full-length KPP.
- **Supplemental Figure S2.** Overall view of pollen tubes expressing KPP-eGFP or eGFP.

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