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Kinase partner protein interacts with the LePRK1 and LePRK2 receptor kinases and plays a role in polarized pollen tube growth

Pulla Kaothien[1,2], Sung Han Ok[1,2,†], Bin Shuai[1,2], Diego Wengier[1,2,3], Robyn Cotter[1,2,‡], Dior Kelley[1,2], Stephanie Kiriakopolos[1,2], Jorge Muschietti[3] and Sheila McCormick[1,2,*]

[1]*Plant Gene Expression Center, United States Department of Agriculture/Agricultural Research Service, Albany, CA 94710, USA,*
[2]*Department of Plant and Microbial Biology, University of California at Berkeley, 800 Buchanan Street, Albany, CA 94710, USA, and*
[3]*Departamento de Fisiología, Biología Molecular y Celular, Instituto de Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Cientificas y Técnicas de Argentina, Universidad de Buenos Aires, Obligado 2490, 1428 Buenos Aires, Argentina*

Received 20 October 2004; revised 19 January 2005; accepted 31 January 2005.
[*]For correspondence (fax 510 559 5678; e-mail sheilamc@nature.berkeley.edu).
[†]Present address: School of Life Science and Biotechnology, Korea University, Seoul 136-701, South Korea.
[‡]Present address: Xoma (US) LLC, 2910 7th St, Berkeley, CA 94710, USA.

Summary

The pollen-specific receptor kinases LePRK1 and LePRK2 have localization and expression profiles that strongly suggest they play roles in pollen germination and tube growth. To identify downstream components of LePRK signaling, we used their cytoplasmic domains (CDs) as baits in yeast two-hybrid screens of a tomato pollen cDNA library. A pollen-specific protein we named kinase partner protein (KPP) interacted with the CDs of both LePRK1 and LePRK2 in yeast and in an *in vitro* pull-down assay, and with LePRK2 in a co-immunoprecipitation assay. KPP is a peripheral membrane protein and is phosphorylated in pollen. Pollen tubes over-expressing KPP developed balloon-like tips with abnormal cytoplasmic streaming and F-actin arrangements and plants over-expressing KPP exhibited impaired transmission of the transgene through the male. KPP-like genes are found only in plants; the 14 family members in *Arabidopsis thaliana* exhibit diverse expression patterns and potentially play roles in signaling pathways in other tissues.

Keywords: actin, cytoplasmic streaming, signaling, tomato, vacuole, yeast two-hybrid.

Introduction

Successful sexual reproduction of flowering plants requires precisely regulated pollen tube growth. Recent molecular analyses of pollen tube growth have revealed several components of signal transduction pathways thought to mediate interactions between the pollen tube and the pistil (stigma, style, and ovary). Extracellular components from the pistil include TTS (Cheung *et al.*, 1995; Wang *et al.*, 1993), pectin and SCA (Kim *et al.*, 2003; Mollet *et al.*, 2000), chemocyanin (Kim *et al.*, 2003), GABA (Palanivelu *et al.*, 2003), and LeSTIG1 (Tang *et al.*, 2004), and from pollen include LAT52 (Tang *et al.*, 2002) and SHY (Guyon *et al.*, 2004).

The tomato receptor kinases LePRK1 and LePRK2 (Muschietti *et al.*, 1998) are localized at the surface of pollen tubes. LePRK1 interacts with LePRK2 in pollen, and this interaction can be disrupted by component(s) present in style extract (Wengier *et al.*, 2003). Moreover, LAT52, a cysteine-rich protein secreted by pollen, interacts with LePRK2 (Tang *et al.*, 2002), as does LeSTIG1, a cysteine-rich protein in the stigma (Tang *et al.*, 2004). Pollen-specific receptor kinases, therefore, possibly function as bridges to transduce such signals from extracellular components into the pollen cytoplasm through interactions with specific cytoplasmic components. Many already identified cytoplasmic components important for pollen tube growth are involved in actin cytoskeleton arrangements and vesicle trafficking pathways. These components include Rop/Rac GTPases (Lin and Yang, 1997; Lin *et al.*, 1996; Zheng and Yang, 2000), Actin depolymerization factor (Chen *et al.*,

2002, 2003), profilin (Clarke *et al.*, 1998), formin (Cheung and Wu, 2004), and Rab GTPase (Cheung *et al.*, 2002).

Here, in an attempt to deduce potential cytoplasmic components of LePRK signaling, we used a yeast two-hybrid screen to identify proteins that interact with the cytoplasmic domains (CDs) of these kinases. One thus identified protein, which we named kinase partner protein (KPP), interacted with both LePRK1 and LePRK2 in yeast and in *in vitro* pull-down assays. Co-immunoprecipitation experiments confirmed that KPP interacts with LePRK2 in pollen. KPP is a peripheral-membrane protein and is phosphorylated in pollen. Over-expression of a KPP-eGFP fusion protein under the control of the pollen-specific LAT52 promoter (Twell *et al.*, 1991) caused depolarized pollen tube growth and an aberrant arrangement of the actin cytoskeleton, similar to the phenotypes seen in pollen tubes over-expressing either wild type or constitutively active Rop GTPases, key proteins controlling actin cytoskeleton dynamics in pollen tubes (Cheung *et al.*, 2003; Kost *et al.*, 1999; Li *et al.*, 1999). Reduced transmission of the linked *kanamycin-resistance* transgene in self-progeny of the KPP-eGFP primary transformants further suggests an important role for KPP in pollen tube growth *in vivo*. These results, together with those in our previous reports, suggest a link from the extracellular matrix to the pollen cytoplasm, via signals transduced through pollen receptor kinases. Moreover, because there are many *KPP-like* genes in plants (14 members in *Arabidopsis thaliana*, with diverse expression patterns), our results imply that other KPP-like proteins might play analogous roles in signaling in other tissues.

Results

KPP interacts with the CDs of LePRK1 and LePRK2

We used yeast two-hybrid screens to identify potential cytoplasmic components of LePRK signaling pathways. A cDNA library prepared from mature tomato pollen was separately screened with the CDs of LePRK1 and LePRK2. A majority of the positive clones corresponded to proteins annotated as unknown or hypothetical. In particular, one such protein was obtained eight times with the CD1 bait and two times with the CD2 bait. We named this protein KPP. A protein similar to KPP, therefore named KPP-like, was obtained two times with the CD1 bait. Re-transformation of yeast expressing CD1 and CD2 with clones encoding KPP confirmed that KPP interacts specifically with CD1 and CD2. The longest *KPP* clone obtained would encode a protein of 494 amino acid residues that lacked an initiating methionine. Database searches with KPP revealed several expressed sequence tag (EST) clones identical to KPP from wild tomato (*Lycopersicon pennellii*) pollen (GenBank accession numbers BG139634, BG137333, BG140917, and BG139009) and from tomato (*Lycopersicon esculentum*) flower buds (GenBank accession number BE354482). Although the amino acid sequences from these EST clones are shorter than KPP at the C-terminus, one of the clones had an additional eight amino acid residues at the N-terminus, including an initiating methionine. Therefore, we conclude that the full-length *KPP* encodes a protein of 502 amino acid residues. The shortest *KPP* clone we obtained in the yeast two-hybrid screen corresponds to only the last 115 amino acid residues of KPP, implying that KPP is capable of binding to CD1 and CD2 in yeast through a domain(s) located within its C-terminal region.

KPP *and its homologs in Arabidopsis are expressed specifically in pollen*

We used RT-PCR to determine the expression profile of *KPP*. *KPP* is specifically expressed in pollen during late developmental stages (Figure 1a,b), and a *KPP-like* gene in tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR-1) is expressed specifically in pollen (Figure S1). Database searches with the amino acid sequence of KPP revealed a family of 14 *KPP-like* genes in *A. thaliana*. KPP and At1g79860 are the most similar, with 62% overall amino acid identity. KPP and all Arabidopsis KPP-like proteins contain a highly conserved domain of unknown function 315 (DUF315). The DUF315 motif and KPP-like proteins are found only in plants, in both dicots and monocots. One KPP-like protein (At4g38430) contains a potential N-myristoylation site.

Although most Arabidopsis KPP-like proteins were annotated as hypothetical, RT-PCR analysis (Figure 1c) indicates that most of them are expressed. We found that At1g79860, At1g52240, and At4g13240 were expressed specifically in pollen. Other Arabidopsis *KPP-like* genes were expressed in specific tissues; for example, At3g55660 was expressed only in pistil, At5g02010 was expressed in all green tissues tested, and At4g38430 was expressed in all tissues tested except pollen. Although expression was not detected in our RT-PCR analyses, database searches revealed that there are EST clones for At3g16130, from seedling hypocotyls, and for At1g01700, from mixed tissues. Collectively, our data and the data obtained *in silico* confirmed expression for 10 of the 14 Arabidopsis *KPP-like* genes.

KPP interacts with the CDs of LePRK1 and LePRK2 in vitro *and functional kinase domains are not required for the interactions*

To confirm that KPP could specifically interact with CD1 and CD2, and to determine if other proteins present in yeast might be required for the interactions, we tested the interaction between ^{35}S-Met-labeled T7-KPP fusion protein and GST-CD fusion proteins (GST-CD1 and GST-CD2) in an *in vitro* pull-down assay. As shown in Figure 2(a), T7-KPP was pulled down by GST-CD1 and GST-CD2 but not by the control GST.

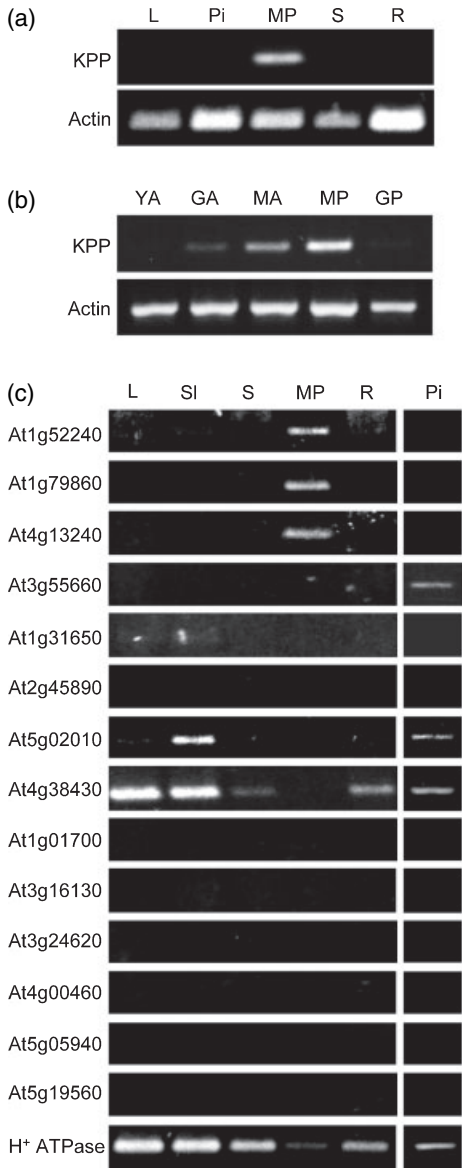

Figure 1. Expression of *KPP* and its homologs in Arabidopsis as assayed by RT-PCR.
(a) Expression profile of *KPP*. L, leaf; Pi, pistil; MP, mature pollen; S, seed; R, root.
(b) Expression profile of *KPP* in different developmental stages. YA, anthers from 10 mm buds; GA, anthers from green-petal flowers; MA, anthers from mature flowers; GP, germinated pollen; other abbreviations as in (a).
(c) Expression profiles of Arabidopsis *KPP-like* genes. SL, seedling, other abbreviations as in (a). An *actin* gene was used as control in (a) and (b) and an *H⁺ATPase* gene in (c).

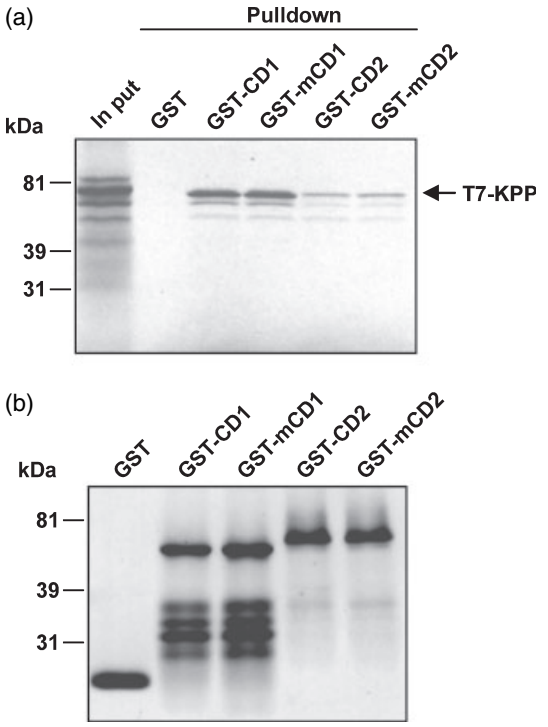

Figure 2. *In vitro* pull-down assay.
(a) Autoradiograph showing interaction between *in vitro* translated ^{35}S-Met-T7-KPP and GST fused to the cytoplasmic domains of LePRK1 (GST-CD1) and LePRK2 (GST-CD2), or to the mutated cytoplasmic domains lacking kinase activity (GST-mCD1 and GST-mCD2). GST was used as control. Molecular mass markers in kDa are indicated.
(b) SDS-PAGE gel stained with Coomassie to show amounts of GST and GST-fusion proteins used in the pull-down reactions in (a).

This result indicates that KPP directly interacts with CD1 and CD2 *in vitro*. Figure 2(b) shows the amount of protein used in each pull-down reaction. The *in vitro* pull-down assay suggested that KPP binds more strongly with CD1 than with CD2, and KPP was obtained more frequently from the CD1 screen. But the yeast two-hybrid screen with CD2 was less extensive, and when KPP was used as bait in a yeast two-hybrid screen of the same library we identified a clone that encodes the cytoplasmic domain of LePRK2 (data not shown). These findings confirm that CD2 does interact directly with KPP.

The kinase activity of LePRK2 is required for its interaction with LePRK1 in yeast (Wengier *et al.*, 2003). To test whether active kinases were required for interactions with KPP, mCD1 and mCD2, which are mutant derivatives lacking kinase activity (Muschietti *et al.*, 1998), were used in an *in vitro* pull-down assay. Figure 2(a) shows that GST-mCD1 and GST-mCD2 pulled down the same amount of T7-KPP as did GST-CD1 and GST-CD2. This result indicates that the kinase activities of CD1 and CD2 are not required for their interactions with KPP.

KPP interacts with LePRK2 and is phosphorylated in pollen

To further characterize KPP by biochemical approaches, a mouse polyclonal antibody (anti-KPP) was raised against a recombinant T7-KPP fusion protein. We confirmed that anti-KPP specifically recognized *Escherichia coli*-expressed T7-KPP (Figure 3a, lane 1). We examined whether the amount of KPP varied during development by immunoblotting protein extracts prepared from mature and germinated tomato

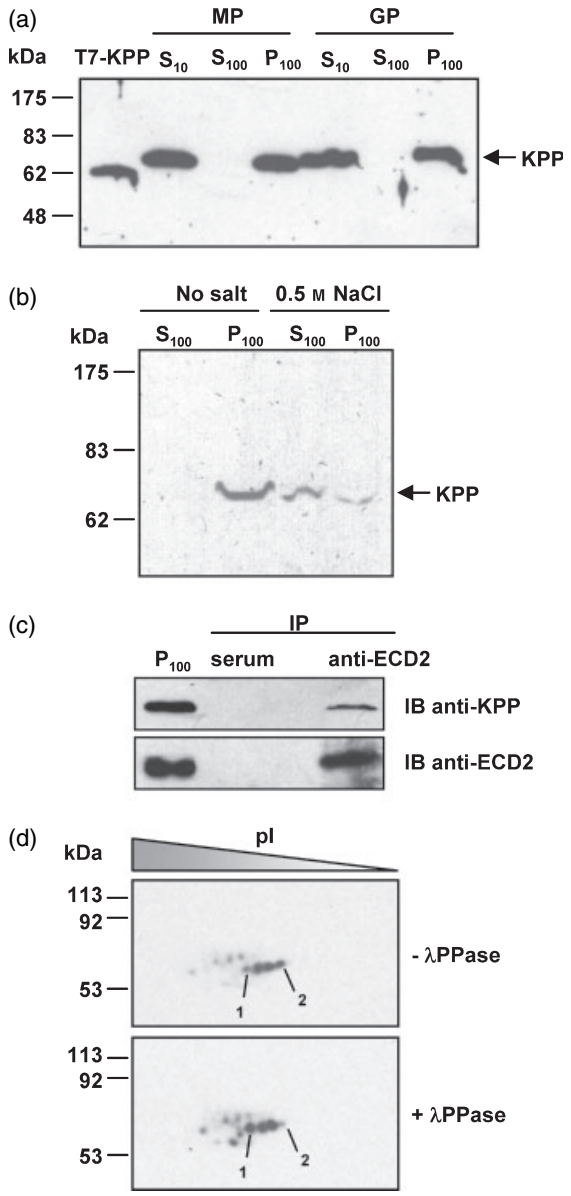

Figure 3. KPP interacts with LePRK2 and is phosphorylated in pollen.
(a) Purified *Escherichia coli*-expressed T7-KPP (1 ng) and total (S_{10}), soluble (S_{100}), and microsomal (P_{100}) proteins (60 μg/lane) extracted from mature (MP) and germinated (GP) tomato pollen were immunoblotted with anti-KPP.
(b) Dissociation of KPP from the microsomal protein fraction (P_{100}) of mature pollen with extraction buffer containing 0.5 M NaCl. Extraction buffer without salt was used as a control.
(c) Microsomal proteins (1.5 mg) from 5 h germinated pollen were immunoprecipitated with anti-ECD2 antibody or pre-immune serum. Separate membranes were immunoblotted with anti-KPP or with anti-ECD2. Microsomal proteins (20 μg) were loaded for reference.
(d) Mature pollen protein (200 μg) was incubated with (lower panel) or without (upper panel) λ-phosphatase, separated by 2-D gel electrophoresis, and immunoblotted with anti-KPP. The acidic end of the first-dimension gel is at the right of each panel. The numbers designate different phosphorylated forms of KPP.

pollen. As shown in Figure 3(a), anti-KPP recognized an approximately 72-kDa protein in both mature and germinated pollen extracts, and we found no substantial difference in the amounts. The predicted molecular weight of KPP is 56.7 kDa, so KPP might be subjected to post-translational modification in pollen. KPP was predominant in the microsomal protein fraction (P_{100}) of both mature and germinated pollen. Most peripheral membrane proteins can be extracted from the P_{100} fraction with salt (He *et al.*, 2001). KPP was present in the soluble fraction (S_{100}) after the P_{100} fraction of mature pollen proteins was treated with 0.5 M NaCl (Figure 3b), indicating that KPP is a peripheral membrane protein.

The presence of KPP in the microsomal fraction, despite its lack of a trans-membrane domain, is consistent with our hypothesis that KPP interacts with LePRK1 and/or LePRK2 in pollen. We used a co-immunoprecipitation assay to test this hypothesis. Membrane proteins from germinated pollen were immunoprecipitated with antibodies against the extracellular domains of LePRK1 (anti-ECD1) or LePRK2 (anti-ECD2), then immunoblotted with anti-KPP. Figure 3(c) shows that a band corresponding to KPP was detected when anti-ECD2 was used for immunoprecipitation; this result was obtained in three separate experiments. However, co-immunoprecipitation with anti-ECD1 was negative (data not shown).

Kinase partner protein contains more than 25 predicted phosphorylation sites. To determine whether KPP is phosphorylated in pollen, proteins from mature pollen were treated with λ-phosphatase, separated by two-dimensional electrophoresis, and immunoblotted with anti-KPP. Upon λ-phosphatase treatment, the most acidic form (2) disappeared and the most alkaline form (1) became more prominent (Figure 3d, lower panel). These results were obtained consistently in six separate experiments, indicating that there is at least one phosphorylated form of KPP in pollen. The other detected forms varied from experiment to experiment; we presume that they are other post-translational modifications or degradation products of full length KPP.

Pollen over-expressing KPP exhibits depolarized pollen tube growth in vitro *and* in vivo

If KPP is an essential component of pollen tube growth signaling, altering its expression might interfere with normal pollen tube growth. Delivering gene constructs by microprojectile bombardment is a quick and reliable method to alter gene expression and assay gene function in pollen (Twell *et al.*, 1989). For example, the function of Arabidopsis Rac-like GTPases (AtRacs) in pollen tube growth was inferred from the range of tube defects observed in pollen over-expressing a GFP-AtRac fusion protein (Cheung *et al.*, 2003). We used microprojectile bombardment to transiently over-express either a KPP-enhanced green fluorescent protein (KPP-eGFP) or a control (eGFP), both under the

control of the pollen-specific LAT52 promoter (Twell *et al.*, 1990), in tobacco pollen. We examined tube morphology of at least 150 eGFP-labeled pollen grains from three bombardment experiments for each construct, at 4 and 24 h after bombardment, and tabulated the germination percentage at each time. The images shown in Figure 4(a,b) are representative examples of the morphologies observed. At 4 h, 70% of pollen grains over-expressing the KPP-eGFP construct had germinated tubes, and 50% of those tubes had developed balloon-like tips. At 24 h, 93% of the tubes of pollen expressing KPP-eGFP had developed balloon-like tips (Figure 4b). This is in contrast to the 89% germination and 100% normal tubes observed 24 h after bombardment in pollen over-expressing eGFP (Figure 4a). To determine if the aberrant morphologies were due to the fusion of KPP to eGFP, we bombarded tobacco pollen with a plasmid in which the KPP expression cassette was separate from the eGFP expression cassette and examined the tube morphology of at least 150 eGFP-labeled pollen grains from two bombardment experiments. We found that 37% of the eGFP-labeled grains observed at 4 h had developed tubes with larger diameters toward the tips, or balloon-like tips. At 24 h, 74% of the pollen tubes had developed balloon-like tips (Figure 4c). Therefore, we conclude that KPP over-expression *per se* yields balloon-tipped tubes.

To confirm the effects of KPP over-expression observed in transient expression experiments, we generated transgenic tomato plants expressing KPP-eGFP under the control of the LAT52 promoter; plants expressing eGFP under the control of the LAT52 promoter were generated as controls. Pollen counts showed that 50% of the pollen in the primary transformants had eGFP or KPP-eGFP expression, as expected for constructs under the control of the gametophytically expressed LAT52 promoter (Twell *et al.*, 1990). Transgenic pollen was germinated *in vitro* and pollen tube morphologies were observed. In 10 independent transgenic KPP-eGFP plants, pollen grains with strong KPP-eGFP expression developed balloon tip morphologies identical to those observed in the transient expression experiments (Figure 4e, lower panels). Pollen tubes with weaker KPP-eGFP expression sometimes developed balloon tip morphologies, although most tubes were normal (Figure 4e, upper panel). In both the transient expression experiments and in the stable transgenic lines, the severity of pollen tube defects correlated with the expression level of KPP-eGFP or KPP, i.e., pollen tubes with stronger GFP fluorescence were usually shorter and had larger balloon tips. In contrast, the balloon tip morphology was never detected in transgenic eGFP pollen tubes (Figure 4d).

The linked *kanamycin-resistance* transgene showed reduced transmission in self-progeny of the primary transformed KPP-eGFP plants (Table S2), suggesting that pollen tube growth *in vivo* might also be impaired. Despite the reduced transmission, we obtained homozygous plants from one of the transgenic lines (line no. 1, Table S2), and we used these to determine if the balloon tip phenotype was seen when pollen tubes grew in the pistil. We self-pollinated homozygous eGFP and KPP-eGFP plants and visualized pollen tubes 24 h later, by aniline blue staining to detect callose. As shown in Figure 4(f,g), some of the KPP-eGFP pollen had depolarized pollen tubes in the pistil.

The balloon tip morphologies seen in pollen over-expressing KPP (Figure 4) are similar to those seen in pollen over-expressing Rac/Rop GTPases (Chen *et al.*, 2003; Cheung *et al.*, 2003; Kost *et al.*, 1999; Li *et al.*, 1999) or in pollen treated with the proteasome inhibitor MG132 (Scoccianti *et al.*, 2003; Speranza *et al.*, 2001). Pollen tubes over-expressing Rac/Rop GTPases or treated with proteasome inhibitors also exhibited irregular actin cytoskeleton arrangements and abnormal cytoplasmic streaming. In a normal pollen tube, actin cables run along the shank of the tube and function as a roadway that transports vesicles packed with materials for wall construction to the tip region (Lord and Russell, 2002; Mascarenhas, 1993). This transport network creates a dynamic, reverse-fountain cytoplasmic streaming that stops and turns at a point just behind the apical region of the pollen tube, creating a clear zone lacking actin cables and cytoplasmic streaming at the tip apex (Lancelle and Hepler, 1992). In tubes over-expressing Rac/Rop GTPases, transverse actin bands and actin cables were detected in the area that should be the clear zone (Fu *et al.*, 2001; Kost *et al.*, 1999). Moreover, cytoplasmic streaming was detected in the apical region of pollen treated with MG132 (Scoccianti *et al.*, 2003). These phenotypic similarities led us to examine the cytoplasmic streaming and the arrangement of actin cables in pollen over-expressing KPP-eGFP.

Cytoplasmic streaming in a tube of tobacco pollen that was bombarded with the control eGFP expression construct is shown in Figure 5(a–c) (see also Figure S3,a). The tube had normal streaming in the shank and a well-defined clear zone in the apical region. However, cytoplasmic streaming was evident in the apical balloon tip region in tobacco pollen bombarded with the KPP-eGFP expression construct (Figure 5d–f; see also Figure S3,b). This abnormal cytoplasmic streaming was only found in association with the balloon tip morphology; i.e., a clear zone was detected in pollen tubes over-expressing KPP-eGFP that did not develop balloon tips. In normal pollen tubes, the tip region is always devoid of vacuoles (Lord and Russell, 2002). Here, we observed the presence of one or more vacuolar structures in the tips of pollen tubes over-expressing KPP-eGFP (Figure 5d–f,j–l). Highly vacuolated tubes were also detected in pollen over-expressing GFP-AtRac fusion proteins (Cheung *et al.*, 2003; Kost *et al.*, 1999). As in the transient expression experiments, transgenic eGFP pollen tubes had normal cytoplasmic streaming with a clear zone at the apical region (Figure 5g–i), while transgenic

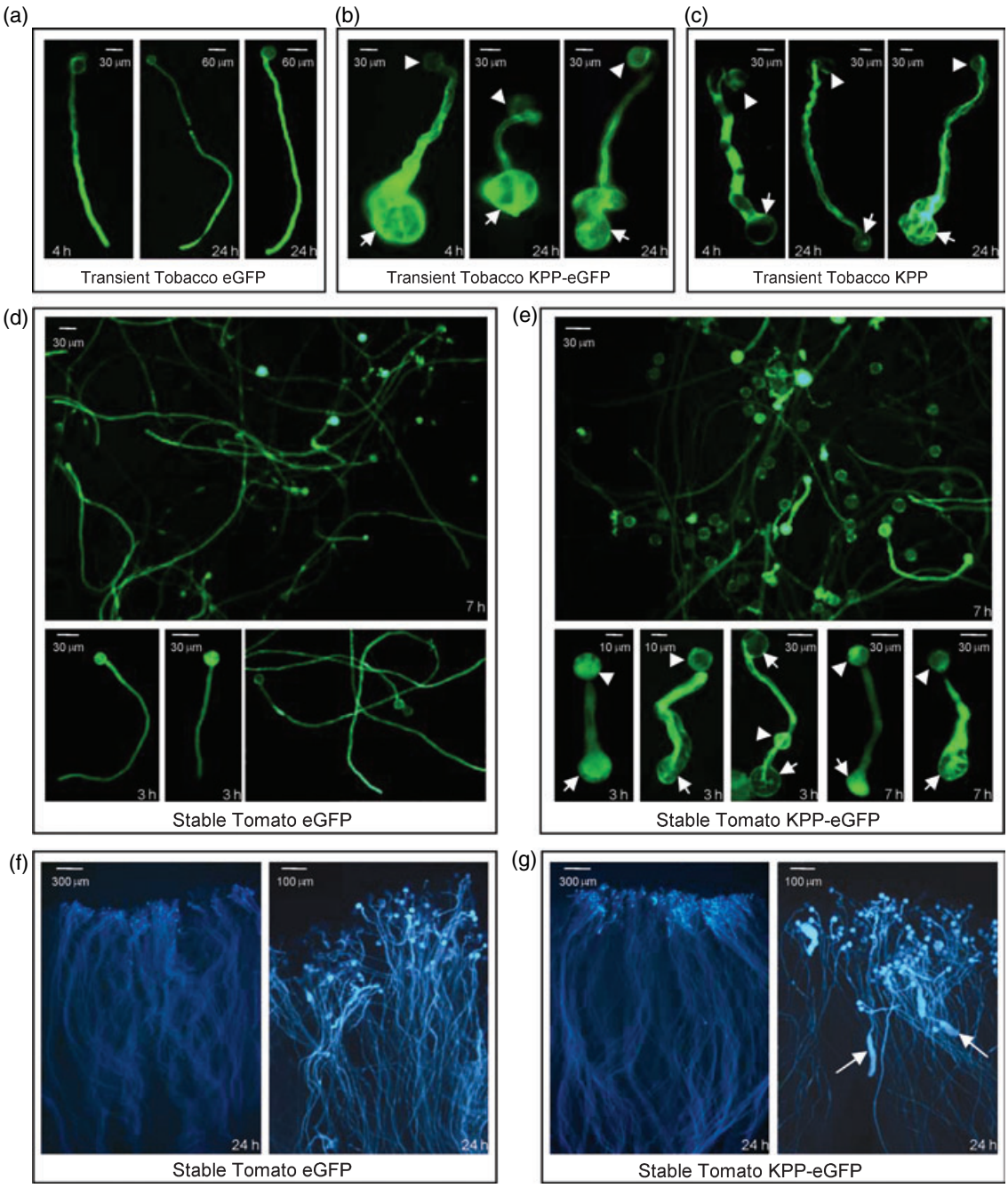

Figure 4. Over-expression of KPP results in depolarized pollen tube growth *in vitro* and *in vivo*.
(a) Tobacco pollen transiently expressing eGFP.
(b) Tobacco pollen transiently expressing a KPP-eGFP fusion.
(c) Tobacco pollen transiently expressing both KPP and eGFP.
(d) Transgenic tomato pollen expressing eGFP.
(e) Transgenic tomato pollen expressing KPP-eGFP.
(f) Pistil pollinated by eGFP pollen.
(g) Pistil pollinated by KPP-eGFP pollen. Pollen in (d) and (e) was from hemizygous primary transformants and in (f) and (g) was from homozygous T1 plants. Aniline blue staining was used to visualize pollen tubes in the pistil in (f) and (g). Arrowheads and arrows in (b), (c), and (e) point to the pollen grain and the tip of pollen tube, respectively. Arrows in (g) point to abnormal tubes. Bars (μm).

KPP-eGFP pollen tubes had abnormal cytoplasmic streaming and vacuolar structures in the apical region (Figure 5j–l).

To determine whether the organization of actin cables was altered in balloon tip tubes, we germinated transgenic eGFP and KPP-eGFP pollen, then fixed and stained them with

Figure 5. Cytoplasmic streaming extends into the apical region of pollen tubes over-expressing KPP-eGFP. Images were captured at 5-sec intervals to show vesicle movement (black arrows) as a result of cytoplasmic streaming.
(a–c) Cytoplasmic streaming in the shank of a tobacco pollen tube transiently expressing eGFP.
(d–f) Cytoplasmic streaming in the apical region of a balloon-tipped tube of tobacco pollen transiently expressing KPP-eGFP.
(g–i) Cytoplasmic streaming in the shank of a transgenic eGFP pollen tube.
(j–l) Cytoplasmic streaming at the apex of the balloon-tipped tube of a transgenic KPP-eGFP pollen. The images were captured at 24 h (tobacco) or 3 h (tomato) after the pollen grains were transferred to germination medium. White boxes indicate the clear zone. White arrows indicate movement of cytoplasm around the vacuolar structure in the tip region. V, vacuole. Bars (μm).

rhodamine–phalloidin to visualize F-actin. The eGFP pollen tubes showed a normal arrangement of actin cables (Figure 6a); i.e., longitudinal actin cables were observed in the shanks of the tubes but not in the clear zone. In contrast, longitudinal actin cables in KPP-eGFP-expressing pollen tubes extended into the apical region (white arrows, Figure 6b–d), similar to the actin arrangement observed in pollen tubes over-expressing Rop1At (Fu *et al.*, 2001). Because this aberrant actin arrangement was also detected in the KPP-eGFP pollen tubes that had not yet suffered severely depolarized growth (Figure 6b), the balloon tip morphology of KPP-eGFP over-expressing pollen is probably a consequence of the aberrant arrangement of actin cables in the tip region.

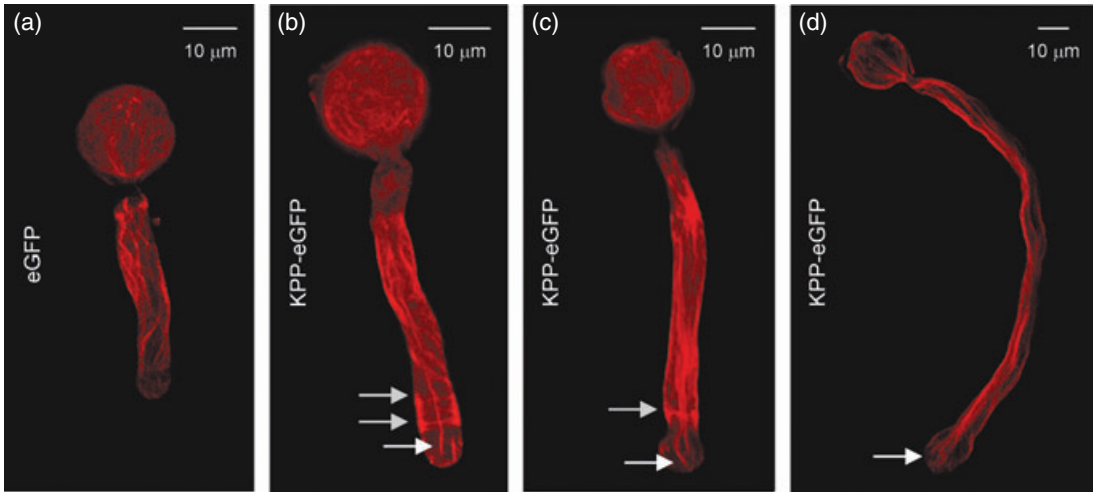

Figure 6. Aberrant arrangement of actin cables in the apical region of pollen tubes over-expressing KPP-eGFP. Transgenic pollen expressing eGFP (a) or KPP-eGFP (b–d) were fixed 5 h after pollen grains were transferred to germination medium and stained with rhodamine–phalloidin to visualize F-actin. Images were captured using confocal microscopy. White arrows indicate actin cables that extended into the tip. Gray arrows indicate transverse actin bands, possibly induced during the fixation process (Foissner *et al.*, 2002). Bars (μm).

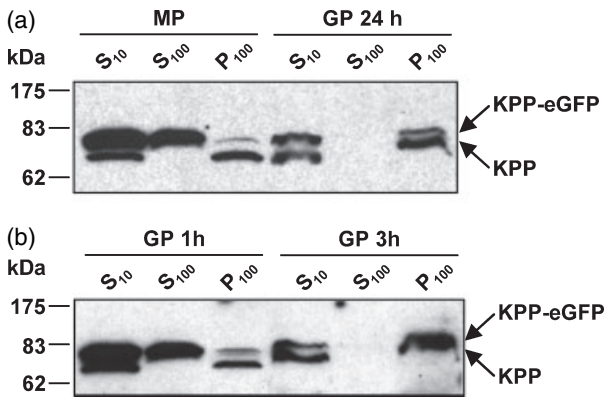

Figure 7. Endogenous KPP is correctly localized in KPP-eGFP transgenic pollen.
(a) Total (S_{10}), soluble (S_{100}), and microsomal (P_{100}) proteins were extracted from mature pollen or from 24-h germinated pollen, separated by SDS-PAGE, and immunoblotted with anti-KPP.
(b) Immunoblot of proteins fractionated as in (a), using 1- or 3-h germinated pollen. MP, mature pollen; GP, germinated pollen. Each lane contains 60 μg of the indicated protein extract.

Endogenous KPP is correctly localized to the pollen membrane in plants over-expressing KPP-eGFP

As the first step to determine the molecular basis of the balloon tip phenotype in pollen over-expressing KPP-eGFP, we determined the distribution of endogenous KPP and of the introduced KPP-eGFP in the transgenic KPP-eGFP pollen by immunoblot (Figure 7a). We found that over-expression of KPP-eGFP did not interfere with the membrane localization of endogenous KPP in mature and in germinated pollen; the membrane-associated amounts of KPP are comparable to those in untransformed plants (Figure 3). In primary transformed plants, only 50% of the pollen expresses KPP-eGFP. Thus we estimate that, in mature pollen, the amount of KPP-eGFP was at least 10-fold in excess of that of endogenous KPP, and it is striking that most KPP-eGFP is found in the soluble protein fraction. However, in germinated pollen KPP-eGFP was detected only in the microsomal protein fraction. From a time course (Figure 7b) we estimated that the excess KPP-eGFP in the soluble protein fraction disappeared between 1 and 3 h after germination.

It was surprising that KPP-eGFP was absent in the soluble protein fraction after germination (Figure 7), because the pollen tubes in Figure 4(a–e) are brightly fluorescent throughout. To seek an explanation, we immunoblotted proteins extracted from transgenic eGFP or KPP-eGFP pollen with anti-GFP antibody. A substantial amount of eGFP protein was found in the soluble protein fraction (Figure S2) of KPP-eGFP pollen. We do not know if this is due to proteolytic cleavage or to translational initiation at an internal methionine. We therefore concluded that the fluorescent signal of eGFP could not be used to monitor the localization of the KPP-eGFP fusion protein in pollen tubes.

Discussion

Kinase partner protein was obtained in a yeast two-hybrid screen as a cytoplasmic interactor of the pollen-specific receptor kinases LePRK1 and LePRK2. *In vitro* pull-down assays confirmed that the interactions between KPP and the CDs of LePRK1 and LePRK2 were direct and specific and showed that the kinase activities of LePRK1 and LePRK2 were not required for the interaction. Intact kinase activities of receptor kinases are often required for the interaction with cytoplasmic proteins (Gu *et al.*, 1998; Skirpan *et al.*, 2001; Stone *et al.*, 1998; Williams *et al.*, 1997), but some exceptions exist. For example, the kinase activity of SRK, a pollen receptor kinase involved in self-incompatibility signaling, is not required for its interaction with THL-1 and THL-2 (Bower *et al.*, 1996), even though THL-1 is phosphorylated by SRK (Gu *et al.*, 1998).

Kinase partner protein was detected in the membrane fraction of proteins extracted from mature and germinated pollen (Figure 3a), even though KPP lacks a trans-membrane domain or other motifs that might aid membrane localization. Moreover, because KPP was easily dissociated from the membrane fraction by salt treatment (Figure 3b), KPP probably associates with the microsomal fraction through its interaction with membrane protein(s) in pollen. The interaction between KPP and LePRK2 was proved by co-immunoprecipitation (Figure 3c). It is not clear why the co-immunoprecipitation with anti-ECD1 was unsuccessful; perhaps the co-immunoprecipitation with anti-ECD2 was successful because the amount of LePRK2 increases greatly upon germination (Muschietti *et al.*, 1998), whereas the amount of LePRK1 does not. KPP is phosphorylated in pollen (Figure 3d), possibly by LePRK2.

Over-expression of KPP resulted in aberrant balloon tip morphology in both tomato and tobacco pollen tubes (Figure 4). The cylindrical shape of normal pollen tubes is a result of polarized growth that is restricted to the apical region of the tubes (Speranza *et al.*, 2001). The balloon tip morphology detected in pollen tubes over-expressing KPP implies that growth is no longer restricted to the apical region. However, the range of morphologies, i.e., abnormally wide tips to irregularly shaped balloon tips, suggests that growth polarity in some tubes was not entirely abolished. Although only some KPP-eGFP pollen tubes developed balloon tips in the pistil (Figure 4g), the reduced transmission of the linked *kanamycin-resistance* transgene in self-progeny of the primary transformants and the difficulty in obtaining homozygous KPP-eGFP progeny indicates that KPP-eGFP pollen tubes are less successful.

The membrane-associated amount of endogenous KPP in plants over-expressing KPP-eGFP appears similar to that in wild type (Figure 7), suggesting that most of the endogenous KPP in the tube was not de-localized. However, de-localization of a small amount of endogenous KPP at the tip region,

where the phenotype is noted, might not be detectable by immunoblot. Thus the de-polarized growth phenotype might be due to: (i) de-localization of endogenous KPP at the tip, (ii) excess KPP-eGFP in the cytosol of mature pollen, or (iii) membrane-associated KPP-eGFP. We favor the first two possibilities: if KPP is needed to recruit to the LePRK complex a protein(s) important for the polarized growth, de-localization of endogenous KPP at the tip or excess cytosolic KPP-eGFP could interfere with membrane localization of such protein(s), by sequestering in the cytosol.

In pollen tubes over-expressing Rac/Rop GTPases, a network of irregular actin cables was detected in the apical region of pollen tubes (Fu *et al.*, 2001; Kost *et al.*, 1999), indicating that an aberrant arrangement of F-actin in the tip region caused de-polarized growth and the balloon tip morphology. We also observed irregular actin cables in the tip region of pollen over-expressing KPP-eGFP (Figure 6). Although a direct relationship has not been demonstrated, these results lead us to hypothesize that the function of KPP in the LePRK signaling pathway might involve the regulation of F-actin arrangements through Rac/Rop GTPases.

The mechanisms controlling pollen tube and root tip growth in plants are thought to be analogous to those controlling hyphal growth in fungi and axon growth in animals (Palanivelu and Preuss, 2000; de Ruijter and Malho, 2000). Because KPP and KPP-like proteins are found only in the plant kingdom, plants might possess signaling pathways for tip growth with unique features, in addition to some conserved with tip growth pathways in fungi and animals. The diversity of expression patterns of the Arabidopsis *KPP-like* genes also implies that Arabidopsis KPP-like proteins might similarly interact with receptor kinases in diverse signaling pathways in different parts of the plant.

Experimental procedures

Plant material and in vitro *pollen germination*

Mature pollen grains were collected by vibrating mature tomato (*L. esculentum* cv. VF36) flowers. The pollen grains were used immediately for germination or bombardment experiments or were stored at −80°C for protein extraction. Pollen grains were germinated *in vitro* in pollen germination medium [20 mM MES pH 6.0, 3 mM $Ca(NO_3)_2$, 1 mM KCl, 0.8 mM $MgSO_4$, 1.6 mM boric acid, 2.5% (w/v) sucrose, and 24% (w/v) polyethylene glycol (PEG) MW 3350] at 25°C on a rotary shaker (40 rpm).

Yeast two-hybrid screens

To construct the baits, cDNAs encoding the CDs of LePRK1 and LePRK2 (CD1 and CD2) were individually cloned into the phagemid vector pBD-GAL4 Cam (Stratagene, La Jolla, CA, USA) to generate in-frame fusions of the GAL4 binding domain and the CDs (pBD-CD1 and pBD-CD2). Constructs were sequenced to confirm correct orientation and sequence of the insert.

The yeast strain PJ69-4A (James *et al.*, 1996) was transformed with one of the bait plasmids (pBD-CD1 or pBD-CD2), and then with a pAD-GAL4 pollen cDNA library (Tang *et al.*, 2002) by a modified lithium acetate method (Gietz *et al.*, 1992). Initial selection was made on synthetic complete (SC) medium lacking Leu, Trp, and Ade. Colonies were tested subsequently for growth on SC medium containing 3 mM 3-amino-1,2,4-triazole and lacking Leu, Trp, and His. Colonies that grew were assayed for β-galactosidase activity using an X-gal filter lift method (Breeden and Nasmyth, 1985). DNA from colonies that passed all three tests was extracted and transformed into *E. coli* XL1Blue (Stratagene) for amplification of pAD-GAL4 plasmids with cDNA inserts. For the CD1 bait, we screened approximately four million yeast transformants and sequenced cDNA inserts from 108 positive clones. For the CD2 bait, we screened fewer than one million transformants and sequenced 24 positive clones.

DNA sequence analysis

Database searches were conducted with the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), with the TIGR tomato gene index (http://www.tigr.org/tdb/tgi/lgi/), and with the MIPS Arabidopsis thaliana database (http://mips.gsf.de/proj/thal/db/index.html). Potential phosphorylation sites were predicted by the NetPhos 2.0 program (http://www.cbs.dtu.dk/services/NetPhos/). Cellular localization and post-translational modifications were predicted by the PSORT II program (http://psort.nibb.ac.jp/form2.html), the Plant Specific Myristoylation Predictor program (http://plantsp.sdsc.edu/myrist.html) and by eye.

RT-PCR

Total RNA from tissues (pollen, pistil, leaf, root, seed, and seedling) of tomato (*L. esculentum* cv. VF36), tobacco (*N. tabacum* L. cv. Petit Havana SR-1), and *A. thaliana* ecotype Columbia (Col-0) was isolated as described (Logemann *et al.*, 1987). Random-primed cDNA was synthesized from DNase-treated total RNA using Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Gene-specific primers designed to span an intron were used for RT-PCR of Arabidopsis *KPP-like* genes. Control PCR reactions to amplify tomato *actin* or an Arabidopsis *H^+ATPase* gene were performed to ensure the quality of the cDNA templates. PCR reactions using genomic DNA as a template were performed to test the quality of the primers. Sequences of the primers are listed in Table S1.

Expression and purification of recombinant proteins

For GST-fusion proteins, the cDNA encoding CD1 and CD2 and their K to R mutation constructs (mCD1, mCD2) were cloned in-frame at the 3′-end of the GST coding sequence in pGEX-4T3 vector (Amersham Pharmacia Biotech, Bucks, UK). The resulting constructs were transferred into *E. coli* strain BL21-DE3 (Stratagene), and production of the fusion proteins (GST-CD1, GST-CD2, GST-mCD1, and GST-mCD2) was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM.

For the T7-fusion protein, a 1.4-kb cDNA encoding KPP (missing the first eight amino acids at the N-terminus) was cloned in-frame at the 3′-end of T7 coding sequence in pET-17b vector (Novagen, Darmstadt, Germany). The resulting pT7-KPP plasmid was transferred into *E. coli* strain BL21-DE3, and production of the fusion protein was induced by addition of IPTG to a final concentration of

0.5 mM. The T7-KPP fusion protein was purified with T7•tag® Antibody Agarose (Novagen) and used as an antigen for raising the anti-KPP antibody.

In vitro *translation and* in vitro *pull-down assay*

The pT7-KPP plasmid was used as a template for *in vitro* translation with the TNT T7 Quick Coupled Transcription/Translation System kit (Promega, Madison, WI, USA). For the *in vitro* pull-down assay, *in vitro* translated ^{35}S-Met-T7-KPP was added to the binding reaction containing GST-fusion proteins pre-bound to Glutathione Sepharose® 4B beads in 300 μl binding buffer [50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40 (Sigma, St. Louis, MO, USA), 1x protease inhibitor cocktail Complete™ (Roche, Indianapolis, IN, USA)]. The reaction was incubated in a 1.5-ml sample tube on an orbital shaker for 3 h at 4°C, and then the beads were collected by centrifugation at 500 ***g*** for 5 min. The beads were washed three times with binding buffer without Nonidet P-40, resuspended in 1x SDS-PAGE sample buffer (Laemmli), and heated at 95°C for 2 min. The proteins bound to the beads were separated by SDS-PAGE.

Pollen protein extraction

Protein extraction and fractionation protocols were as described (Muschietti *et al.*, 1998). Extracts were centrifuged at 10 000 ***g*** for 30 min at 4°C, and the supernatant (S_{10}, total protein) was centrifuged at 100 000 ***g*** for 3 h at 4°C. The resulting supernatant (S_{100}) contained soluble proteins and the pellet (P_{100}) contained microsomal membranes and membrane-associated proteins. The P_{100} was re-suspended in extraction buffer (50 mM Tris, pH 7.5, 1 mM DTT, and 1x protease inhibitor cocktail Complete™) containing 0.5% (v/v) Nonidet P-40 (Sigma). Protein concentrations were quantified by a colorimetric method (Bradford, 1976) using BSA as a standard. Protein samples were stored at −20°C until use.

Co-immunoprecipitation

The P_{100} fraction (1.5 mg of protein) extracted from pollen that had been germinated for 5 h was mixed with 4 μl of anti-ECD2 antibody or 2 μl pre-immune serum, the volume was adjusted to 400 μl with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Nonidet P-40, and 1x Complete™) and was incubated for 2 h at 4 °C. The protein/antibody complex was bound to protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by rotating at 4 °C for 1 h. The agarose beads were washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.2% Nonidet P-40) and resuspended in 30 μl of 1x SDS-PAGE loading buffer. The protein samples were denatured at 70°C for 30 min, then subjected to immunoblot analysis.

Phosphatase treatment and two-dimensional gel electrophoresis

An extract containing cytoplasmic and membrane proteins was prepared by grinding mature pollen grains in a Dounce homogenizer (Fisher Scientific, Santa Clara, CA, USA) at 4°C in extraction buffer containing 0.5% Triton X-100. The resulting homogenate was stirred on a magnetic stirrer for 1 h at 4°C and then centrifuged at 10 000 ***g*** for 15 min at 4°C. The supernatant was centrifuged at 100 000 ***g*** for 3 h at 4°C, and the resulting supernatant was used in the phosphatase reaction.

Two hundred micrograms of the supernatant in 1x reaction buffer supplemented with 2 mM $MnCl_2$ and 400 units of λ-protein phosphatase (New England Biolabs, Beverly, MA, USA) or water, was incubated for 3 h at 37°C. The reaction was stopped by adding SDS followed by acetone precipitation. Briefly, 4x SDS buffer was added to the reaction to a final concentration of 1x (3% SDS, 10% glycerol, 40 mM Tris-HCl, pH 6.8, 50 mM DTT) and incubated for 5 min at 80°C. After agitating the tubes, 4 volumes of cold (−20°C) acetone were added and the solution was incubated for 20 min at −20°C. After centrifugation, the precipitate was washed with cold 80% (v/v) acetone, and the pellet was dried in a speed vacuum for 5 min. Pellets were resuspended in 60 μl of fresh IEF buffer [0.57 g urea, 200 μl 10% Triton X-100, 16 μl Pharmalyte, pH 4–6.5 (Sigma), 4 μl Bio-Lyte Ampholytes, pH 3–10 (Bio-Rad, Hercules, CA, USA), 50 μl 2-mercaptoethanol, final volume 1 ml in distilled water] by mixing periodically for 1 h at room temperature. Samples were centrifuged at 10 000 ***g*** for 5 min and supernatants were loaded in the first-dimension gel.

First- and second-dimension gels were run as described (Coligan *et al.*, 1995). Briefly, IEF was performed in Model 175 Tube Gel Cell (Bio-Rad) until $V \times h = 12\,000$. Gels were extruded from capillary tubes into a vial containing 0.6 ml equilibration buffer (3% w/v SDS, 0.4 mM EDTA, 10% v/v glycerol, 20 mM Tris-HCl, pH 8.8), 10 μl of 2-mercaptoethanol was added, and each vial was immediately frozen in dry ice. Vials were stored at −70°C. Before second-dimension electrophoresis, vials were thawed at room temperature until the solution was clear, then gel tubes were placed on top of the stacking gel and covered with a 1:1 dilution of 2% agarose and equilibration buffer. Runs were performed at 10 mA until samples reached the resolving gel, then at 25 mA constant current.

Immunoblotting

Protein samples were separated by SDS-PAGE and transferred onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were incubated with the anti-KPP antibody, anti-ECD2 antibody (Tang *et al.*, 2002), or anti-GFP antibody [GFP (B-2), Santa Cruz Biotechnology] and then with a sheep anti-mouse secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Membranes were incubated with SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to X-ray film for 2–20 min before development.

Pollen microprojectile bombardment

The pollen-specific LAT52 promoter (Twell *et al.*, 1991) was used to drive the expression of eGFP, KPP-eGFP, or KPP. A plasmid carrying an eGFP expression cassette (pPK100a2) was used as a control for every bombardment experiment. For the KPP-eGFP expression construct, a 1.4-kb cDNA encoding KPP (missing the first eight amino acids at the N-terminus) was inserted in-frame at the 5′-end of the eGFP coding sequence in pPK100a2. For the KPP expression construct, an eGFP expression cassette excised from pPK100a2, and a KPP expression cassette containing 1.4 kb cDNA of KPP were inserted into the binary vector pCAMBIA2300 (Jefferson, 1993).

Tobacco pollen grains were freshly harvested, suspended in pollen suspension medium [20 mM MES, pH 6.0, 3 mM $Ca(NO_3)_2$, 1 mM KCl, 0.8 mM $MgSO_4$, 1.6 mM boric acid, and 18% w/v sucrose], and spread on nylon membranes for microprojectile bombardment (Twell *et al.*, 1989). Approximately 6 mg of pollen was bombarded with 4 μg of plasmid DNA coated on 1 μm (diameter) gold particles,

using the BIOLISTIC®PDS-1000/He particle delivery system (Bio-Rad) at a pressure of 1100 psi. The bombarded pollen grains were immediately transferred to pollen germination medium and incubated at 25°C in the dark until observation.

Plant transformation

Agrobacterium tumefaciens strain LBA4404 (Hoekema *et al.*, 1983) carrying an eGFP or a KPP-eGFP expression cassette driven by the LAT52 promoter was used to transform tomato (*L. esculentum* cv. VF36) as described (McCormick, 1991). The presence of the transgenes in the resulting transgenic plants was confirmed by PCR on genomic DNA, using primers that amplify the eGFP or the KPP-eGFP fusion constructs.

Pollen tube imaging

For visualization of actin cytoskeleton in pollen tubes, pollen was geminated for 5 h in pollen germination medium, then fixed for 30 min in 1× vol. of pollen suspension medium with the addition of 0.1% NP-40, 600 μM 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) and 0.5% glutaraldehyde. Fixed pollen was rinsed twice in pollen suspension medium with 0.05% NP-40 and gradually exchanged into TBST (50 mM Tris, pH 7.4, 200 mM NaCl, 0.05% Tween-20) with 300 mM sucrose and 5 mM DTT. Pollen was stained in TBST with 300 mM sucrose and 0.5 μM rhodamine–phalloidin (Molecular Probe Inc., Eugene, OR, USA) for 1 h, washed twice with TBST with 300 mM sucrose and mounted in the same medium. Fluorescence of F-actin was observed with a confocal laser-scanning microscope (Zeiss, Jena, Germany), using the 514 nm excitation line of an argon laser. All other pollen tube images were captured with a digital camera attached to an epifluorescence microscope. Pollen tubes in the pistil were visualized by staining callose with aniline blue as described (Muschietti *et al.*, 1994).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2388/TPJ2388sm.htm

Figure S1. RT-PCR expression profiles of the KPP homolog in tobacco (*NtKPP*). The expression profile of an *actin* gene was used as a control. L, leaf; Pi, pistil; Po, pollen; S, seed; R, root.

Figure S2. The soluble protein fraction of KPP-eGFP pollen contains eGFP. Total (S_{10}), soluble (S_{100}), and microsomal (P_{100}) proteins extracted from transgenic eGFP or KPP-eGFP pollen were immunoblotted with anti-GFP. Each lane contains 60 μg of the indicated protein extract.

Figure S3. Movies showing cytoplasmic streaming in tobacco pollen tubes transiently expressing eGFP or KPP-eGFP. (a) Normal cytoplasmic streaming in the shank of a pollen tube expressing eGFP. (b) Aberrant cytoplasmic streaming at the tip of a pollen tube expressing KPP-eGFP.

Table S1 Primer sequences used for RT-PCR.

Table S2 Segregation of the linked *kanamycin-resistance* transgene in self-progeny of primary transformants.

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