Plant Physiology Preview. Published on September 17, 2008, as DOI:10.1104/pp.108.124420

The Pollen Receptor Kinase LePRK2 Mediates Growth-Promoting Signals and Positively Regulates Pollen Germination and Tube Growth

Dong Zhang[1], Diego Wengier[2, 3], Bin Shuai[3#], Cai-Ping Gui[1], Jorge Muschietti[2], Sheila McCormick[3,4], Wei-Hua Tang[1, 3,4*]

[1]SIBS-UC (Berkeley) Center of Molecular Life Sciences, National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, P.R. China.

[2]Instituto de Ingeniería Genética y Biología Molecular (INGEBI), CONICET Departamento de Fisiología y Biología Molecular y Celular FCEN, Universidad de Buenos Aires, Obligado 2490, 1428 Buenos Aires, Argentina

[3]Plant Gene Expression Center, United States Department of Agriculture/Agricultural Research Service, and Department of Plant and Microbial Biology, University of California at Berkeley, 800 Buchanan Street, Albany, California 94710

[4]Equal contribution.

[#]Present address: Department of Biological Sciences, Wichita State University, Wichita, KS67260, USA

*Corresponding author, e-mail whtang@sibs.ac.cn.

Summary

In flowering plants, the process of pollen germination and tube growth is required for successful fertilization. A pollen receptor kinase from tomato, LePRK2, has been implicated in signaling during pollen germination and tube growth as well as in mediating pollen (tube)-pistil communication. Here we show that reduced expression of *LePRK2* affects four aspects of pollen germination and tube growth. First, the percentage of pollen that germinates is reduced, and the time window for competence to germinate is also shorter. Second, the pollen tube growth rate is reduced both in vitro and in the pistil. Third, tip-localized superoxide production by pollen tubes can not be increased by exogenous calcium ions. Fourth, pollen tubes have defects in responses to STIL, an extracellular growth-promoting signal from the pistil. Pollen tubes transiently over-expressing LePRK2-fluorescent protein fusions had slightly wider tips, whereas pollen tubes co-expressing LePRK2 and its cytoplasmic partner protein KPP (a Rop-GEF) had much wider tips. Together these results show that LePRK2 positively regulates pollen germination and tube growth and is involved in transducing responses to extracellular growth-promoting signals.

Keywords: calcium ions, reactive oxygen species, signal transduction, style component, tip growth

Introduction

The main task for pollen is to deliver the sperm cells for fertilization. This is achieved by pollen germination and tube growth, which carries the sperm cells to the embryo sac. Pollen germination and tube growth requires directional transport of vesicles containing cell wall materials and is driven by a dynamic actin system that is regulated by a tip-localized Ca^{2+} gradient. These vesicles are deposited to a defined exocytosis zone very close to the tip, which is balanced by endocytosis at the tip and the shank to retrieve excess membrane (Cardenas et al., 2008; Zonia and Munnik, 2008; also see review by Krichevsky et al., 2007).

The pollen receptor kinase LePRK2 is one of the receptor-like kinases localized in the plasma membrane of tomato pollen tubes (Muschietti et al., 1998; Kim et al., 2002). Several pieces of evidence suggested a signaling role for LePRK2 during pollen germination and tube growth. Before pollen germination, the extracellular portion of LePRK2, which mainly contains 5 leucine-rich repeats, can interact with an extracellular protein from pollen, LAT52 (Tang et al., 2002). Tomato pollen expressing antisense *LAT52* RNA appeared to be normal at the mature pollen stage, but could not hydrate and germinate normally when cultured in vitro, and formed aberrant, twisted tubes on the stigma, failing to reach the embryo sac (Muschietti et al. 1994). LePRK2 can switch from interacting with LAT52 to interact with an extracellular protein from the stigma, LeSTIG1 (Tang et al., 2004). The addition of LeSTIG1 to germination medium promoted pollen tube growth in vitro (Tang et al., 2004). In the cytoplasm, the intracellular portion of LePRK2 interacts with KPP (Kaothien et al. 2005). KPP belongs to a family of plant-specific guanine nucleotide exchange factors (Berken et al., 2005) for Rop small GTPases. Pollen tubes that overexpressed nearly full-length KPP (missing the first eight amino acids at the N-terminus) were wider, especially at the tip (Kaothien et al., 2005), probably due to the partial loss of polarity, which is maintained by local activation of Rop (Zhang and McCormick 2007). Furthermore, the phosphorylated form of LePRK2, which is present in the plasma membranes of pollen tubes, could be specifically dephosphorylated by a 3-10kD fraction of style extracts (Wengier et al., 2003), showing that LePRK2 on the pollen tube responds to signals from the style. The biological functions of these extracellular and cytosolic binding partners suggest that LePRK2 plays a role in pollen germination and tube growth, but how LePRK2 regulates pollen germination and tube

growth was not known.

Here, using antisense constructs which reduce the expression level of *LePRK2* in pollen and pollen tubes, we provide evidence that LePRK2 positively regulates pollen germination and tube growth rate. Antisense *LePRK2* pollen tubes have mis-positioned large vacuoles and reduced spacing between callose plugs, suggesting a problem with turgor pressure. Furthermore, they do not increase reactive oxygen species (ROS) production or their lengths in response to exogenous calcium ions. We also show that the style extract component (STIL) that dephosphorylates LePRK2 (Wengier et al., 2003) and can increase pollen tube lengths (Wengier et al., submitted) can not increase the lengths of antisense *LePRK2* pollen tubes grown in vitro, indicating that this increase is dependent on LePRK2.

Results

Generation of transgenic tomato plants with specific reductions in *LePRK2* expression in mature pollen and pollen tubes

In order to directly address the biological function of *LePRK2* in pollen, we generated transgenic tomato plants with a construct including a full-length *LePRK2* antisense DNA driven by a pollen-specific promoter (*LAT52*), a separate *GFP* gene driven by the *LAT52* promoter, and a kanamycin resistance gene driven by the *CaMV 35S* promoter. We used the *LAT52* promoter to drive antisense *LePRK2*, because both *LePRK2* and *LAT52* are expressed in mature pollen and in pollen tubes, and in immature pollen *LAT52* is expressed slightly earlier (Twell et al., 1989b) than *LePRK2* (Muschietti et al. 1998). Furthermore, *LAT52* mRNA is about 10-fold more abundant than *LePRK2* mRNA in mature pollen and pollen tubes (real-time PCR, data not shown), and therefore the *LAT52* promoter is likely stronger than the *LePRK2* promoter. Lastly, the *LAT52* promoter was used in generating antisense *Shy* in petunia pollen (Guyon et al. 2004) and in generating antisense *LAT52* in tomato pollen (Muschietti et al., 1994).

Seeds of 19 self-pollinated T_0 plants were collected and germinated on kanamycin medium. Judging by the KanR:KanS ratio, four lines had multiple insertions and were not further characterized. Thirteen lines showed a 3:1 ratio, while two lines showed a 1:1 ratio. We further

analyzed one line with a 1:1 ratio (line 1) and 5 lines with a 3:1 ratio (lines 2-6) (Supplemental table 1). We grew 6 kanamycin-resistant T1 seedlings of each of these lines to flowering.

We confirmed the presence of the *LePRK2* antisense construct in T1 plants of all six lines by genomic DNA PCR (data not shown). For each line, most of the T1 plants had about 50% GFP-expressing pollen, as expected for heterozygotes, but in at least one plant in each line, all pollen expressed GFP, consistent with these plants being homozygotes (Supplemental Table 1). We self-pollinated heterozygous T1 plants of 4 lines, and counted the Kanamycin resistance to sensitive ratio of their progenies. Lines 2, 4 and 6 again gave a 3:1 ratio, and line 1 again gave a 1:1 ratio (Supplemental Table 1). We also pollinated wild-type tomato pistils with pollen from heterozygous lines 2 and 4, but didn't see any male transmission defects (Supplemental Table 1). We pollinated wild-type tomato pistils with pollen from the putative homozygous antisense *LePRK2* lines 1-6 and germinated the resulting seeds on medium containing kanamycin. All seedlings were resistant to kanamycin, confirming that these plants were homozygous for the construct. We obtained homozygous transgenic plants for all six lines and all subsequent experiments used homozygous plants.

To determine whether *LePRK2* expression was reduced in these plants, we performed quantitative RT-PCR with *LePRK2*-specific primers, using total RNA of mature pollen as templates. Figure 1A shows that the *LePRK2* expression level was significantly reduced in all 6 lines, to 20-30% of wild-type levels in lines 1 and 6, and to less than 5% in lines 2-5. LePRK1 is the closest homolog for LePRK2 among known tomato genes, with 54% identity in overall protein sequence, and 64% identity in overall nucleotide sequence (Kim et al., 2002). The longest identical DNA fragment between *LePRK1* and *LePRK2* is 26 nucleotides. To address the specificity of the antisense construct, we checked whether the *LePRK1* expression level was also altered in *LePRK2* antisense plants. Figure 1A shows that the *LePRK1* mRNA level varied from 80% to 105% of the levels in wild-type pollen. We therefore concluded that *LePRK1* expression was not affected significantly, and that *LePRK2* expression was specifically reduced in mature pollen of lines 1-6.

To test whether the reduction of LePRK2 expression is maintained after pollen germination, we also examined the expression level of *LePRK2* in pollen that had been germinated in vitro, and found that *LePRK2* mRNA levels were reduced to about 10% of wild-type levels in line 1 and line

6, and were reduced to less than 5% in lines 2-5 (Fig. 1B). Figure 2 shows that the LePRK2 protein level was also significantly reduced, to less than 20% of wild-type levels in germinated pollen of lines 1-4.

Pollen with reduced expression of *LePRK2* has a lower germination percentage and a shorter germination time window

We examined the pollen phenotypes of all six transgenic lines. Mature pollen of each line showed no obvious differences from pollen of wild-type or of plants transformed with a *pLAT52::GFP* construct (data not shown). When placed in germination medium, antisense *LePRK2* pollen hydrated and germinated normally-shaped tubes. Although the germination percentage of wild-type or GFP-expressing pollen varied from day to day (within a range between 40-90%), the germination percentage of antisense *LePRK2* pollen was always 10-30% lower than the percentage for wild-type or GFP-expressing pollen germinated on the same day. To further understand why this was so, we plotted pollen germination percentage against time. Wild-type, GFP-expressing and antisense *LePRK2* pollen (lines 2, 3 and 4) all started to germinate ~30 min after transfer to germination medium, and within the first hour of germination the increase in germinating grains was similar for all samples. However, the numbers of wild-type and GFP-expressing pollen with tubes continued to increase during the first 120-150 min of incubation, while the numbers of antisense *LePRK2* pollen with tubes did not increase after 70-100 min (Fig. 3A shows two representative experiments). Thus the reduced germination percentage of antisense *LePRK2* pollen is not due to a delay in starting germination, but instead to a reduced time window for germination competence.

Reduced expression of *LePRK2* causes reduced growth rate of pollen tubes both in vitro and in vivo

Besides the reduction in germination, antisense *LePRK2* pollen tubes grown in vitro were shorter than wild-type pollen tubes, when observed at either 3 hours (Fig. 3B) or 10 hours after germination (Fig. 3C). The length difference at 3 hours was statistically significant in

double-blinded assays. After 10 hours the difference was readily apparent with the naked eye. Wild-type pollen formed long tubes and overnight growth resulted in interlocked pollen tubes resembling a mat of fungal hyphae, whereas the antisense *LePRK2* pollen tubes formed only small clumps (Fig. 3C). Figure 3B shows that pollen tubes of lines 1 and 6, which express ~10% of wild-type levels of *LePRK2* (Fig. 1B), were slightly longer than pollen tubes of lines 2-4, which express less than 5% of wild-type levels of *LePRK2* (Fig. 1B). The correlation between *LePRK2* expression level and pollen tube length supports a positive role of LePRK2 in regulating pollen tube growth. These results were confirmed in the T2 and T3 generations.

To determine whether the reduced tube length of antisense *LePRK2* pollen was due to a slower growth rate, to early termination of tube growth, or both, we measured pollen tube lengths after 2-4 hours, and calculated an average growth rate. The growth rate of line 4 pollen tubes was ~0.11mm/hour, half of the rate (~0.22mm/hour) for wild-type pollen tubes (Supplemental Fig. 1). Although many tubes of antisense *LePRK2* pollen continued to grow after 10 hours, many stopped growth much earlier. The reduction in growth rate is at least one of the causes for the reduction of tube length, but earlier termination of tube growth might also contribute.

To determine whether antisense *LePRK2* pollen tubes also grew slower in pistils, we pollinated wild-type pistils with pollen from lines 2 and 4 as well as with wild-type pollen, then checked pollen germination status on the stigmas and recorded the time pollen tubes arrived at the ovary. On the stigmas, the wild type pollen and antisense *LePRK2* pollen germinated 3-5 hours after pollination (Fig. 4A-D). Wild-type or GFP pollen tubes arrived at ovaries 7-9 hours after pollination (growth rate estimated at 1.2 mm/hour), while pollen tubes of lines 2 and 4 did not reach the ovaries until 10-12 hours after pollination, with growth rates estimated at 0.9 mm/hour (Table 1 and Fig. 4). Thus antisense *LePRK2* pollen tubes grew slower than wild-type tubes both in vitro and in vivo, although the difference was smaller in vivo.

Antisense *LePRK2* pollen tubes have vacuoles near the tip and more frequent callose plugs

To determine what downstream processes might account for the slower growth of antisense *LePRK2* pollen tubes, we observed the sub-cellular morphology of growing pollen tubes. Vacuoles perform multiple functions in plant cells, including the storage and degradation of cellular components, osmoregulation, and modulation of turgor (Bassham and Raikhel, 2000; Lew, 2004;

MacRobbie 2006). Growing wild-type pollen tubes usually have large vacuoles (>5 μm in diameter) at the very rear, while only thin tubular vacuoles are seen near the tip (Lovy-Wheeler et al., 2007). Many of the antisense *LePRK2* pollen tubes had large vacuoles near the tip (Fig. 5A) and sometimes the front edge of these vacuoles were as near as 10 μm from the tip. These large vacuoles were not present in early stages of tube growth, but were seen in about 50% of the tubes, starting as early as 5 hrs after germination. Upon further observation of such pollen tubes, larger and larger vacuoles moving closer to the tip were noted. If they reached the tip they should disrupt the normal organelle distribution of the tip and the tube should stop growth. However, continued observations of these pollen tubes showed that the large vacuoles sometimes moved away from the tip – this backward and forward movement kept the vacuoles near the front but away from the clear zone, allowing such pollen tubes to grow for quite a while (Supplemental movies 1-3).

Pollen tubes form periodic callose plugs to keep the cytosol and the sperm towards the front (Nishikawa et al., 2005). Figures 5B and 5C show that wild-type pollen grown in vitro had callose plugs that were spaced at regular intervals of ~350 μm, whereas callose plug placement in the antisense *LePRK2* pollen tubes was more frequent and the intervals were more variable, occurring from 220-250 μm.

Antisense *LePRK2* pollen tubes have impaired responses to Ca^{2+}

In tobacco, ROS production was detected at the pollen tube tip, and the ROS level was increased with exogenous Ca^{2+} (Potocký et al., 2007). To determine whether there was a difference in ROS levels between wild-type and antisense *LePRK2* pollen tubes, we stained growing pollen tubes with nitroblue tetrazolium (NBT), which reacts with O_2^- and forms a blue precipitate. NBT staining of wild-type tomato pollen tubes was darker in a medium with 1mM $CaCl_2$ than in a medium without $CaCl_2$, indicating an increase in the ROS level with exogenous Ca^{2+} (Fig. 6 A and B). However NBT staining for lines 1, 2 and 4 didn't change significantly upon adding Ca^{2+}, indicating that the antisense *LePRK2* pollen tubes had a diminished response to Ca^{2+} for ROS production.

Ca^{2+} is required for pollen germination and tube growth, and exogenous Ca^{2+} at an appropriate concentration promotes in vitro growth of pollen tubes (Steer and Steer 1989).

Wild-type tomato pollen grew the longest tubes within a range of 1-3 mM exogenous Ca^{2+} (data not shown). They had much shorter tubes without additional Ca^{2+} in the medium, but still grew, probably due to the presence of endogenous Ca^{2+} in pollen grain. In contrast, the pollen tube lengths of antisense *LePRK2* lines 2 and 4 were not significantly different in medium with or without Ca^{2+} (Fig. 6C), indicating that antisense *LePRK2* pollen tubes have impaired responses to exogenous Ca^{2+}. Boric acid can also stimulate pollen tube growth (Johri and Vasil 1961). Both wild-type tomato pollen and antisense *LePRK2* pollen grew longer tubes in medium with 1.6 mM boric acid than in medium with 0.16mM boric acid (Supplemental Fig. 2), indicating that antisense *LePRK2* pollen still responds to boric acid.

A style component promotes pollen tube growth of wild-type pollen, but not of antisense *LePRK2* pollen

LePRK2 is specifically dephosphorylated by a component of style extract and this component can also cause dissociation of a complex that includes LePRK1 and LePRK2 (Wengier et al., 2003). We speculated that the style component triggers LePRK2 signaling during pollen tube growth through dephosphorylation and complex dissociation, but the nature and the biological function of this style component remained unknown. Recently STIL, the factor in the style component that is responsible for LePRK2 dephosphorylation, was purified and shown to stimulate pollen tube growth (Wengier et al., submitted). Figure 7 confirms that adding STIL to pollen germination medium increased the length of wild-type and GFP-expressing pollen tubes. However, the lengths of antisense *LePRK2* pollen tubes were not increased in the presence of STIL, suggesting that this stimulatory effect depends on *LePRK2* expression.

Pollen tubes transiently over-expressing LePRK2 have slightly swollen tips, but pollen tubes co-expressing LePRK2 and full-length KPP have much wider tips

Overexpression phenotypes are sometimes informative (Li et al., 1999; Kaothien et al., 2005). We attempted to obtain transgenic plants that over-expressed LePRK2, but were unsuccessful. Therefore we used microprojectile bombardment of pollen (Twell et al., 1989a) to transiently

overexpress a LePRK2-GFP fusion protein. The green pollen tubes were not shorter than untransformed pollen tubes, but their tips were 20% wider than tips of wild-type or GFP-expressing pollen tubes (Fig. 8 A and B). A similar swollen tip phenotype was seen with LePRK2-RFP-expressing pollen tubes (Fig. 8 C and D); GFP can form dimers but mRFP is a monomer (Campbell et al., 2002), so these similar phenotypes suggest that the swollen tip phenotype is not due to dimerization of the fusion protein via the fluorescent protein. The LePRK2-GFP and LePRK2-RFP proteins were mainly localized at the plasma membrane of the pollen tube, although some fluorescent protein aggregates, possibly due to over-expression, were also seen in the cytosol (Fig. 8B middle tube).

KPP is a cytoplasmic binding partner of LePRK2 (Kaothien et al., 2005). Here we show that pollen expressing a full-length KPP fused with GFP grew wavy tubes with slightly increased widths (Fig. 8F and Supplemental Fig. 3), while pollen tubes transiently co-expressing LePRK2 and full-length KPP had tips that were much wider than those on tubes expressing either LePRK2 or KPP fusion proteins alone (Fig. 8G and Supplemental Fig. 3).

Discussion

Homozygous plants were readily obtained from all 6 antisense *LePRK2* lines. This was surprising, especially from line 1, which had a 1:1 ratio for Kanamycin resistance to sensitivity, and from the lines with greatly reduced levels of LePRK2 mRNA. These results indicate that pollen with less than 5% of the normal level of *LePRK2* expression still can deliver sperm for successful fertilization. Nonetheless, pollen grains of the homozygous plants had reduced chances for germination (Fig. 3A) and had a reduced tube growth rate (Figs. 3B and C, Supplemental Fig. 1). Furthermore, the antisense *LePRK2* pollen tubes had morphological defects (Fig. 5) and were deficient in responses to some (Ca^{2+} and STIL, Figs. 6 and 7) but not all (Boric Acid, Supplemental Fig. 2) exogenous promotion factors. Therefore the main function for LePRK2 is to transduce specific external growth-promoting signals for the growing pollen tube.

Cytosolic turgor has to be maintained for pollen tube growth (Benkert et al., 1997). Among other functions, vacuoles modulate turgor during cell growth (Hicks et al., 2004; Lew, 2004). In normal growing pollen tubes only thin thread-like vacuoles are usually seen in the front, while large vacuoles are usually seen in the rear, close to callose plugs (Hicks et al. 2004; Lovy-Wheeler

et al., 2007). As growth proceeded in the antisense *LePRK2* pollen tubes larger vacuoles were seen more often closer to the tip region. The phenomenon might be interpreted as compensation for reduced cytosolic turgor in the front. The periodic formation of callose plugs that separate the growing front from the evacuated regions is thought to be useful for maintaining a manageable cytosolic volume for pollen tubes, because pollen tubes need to extend many times the grain diameter to reach ovules (Nishikawa et al. 2005). The antisense *LePRK2* pollen tubes had shorter intervals between callose plugs, giving a smaller cytosolic volume for the growing tube cell. Although the callose plug interval difference can be interpreted in many different ways, it might be a compensation for or a result of the reduced cytosolic turgor, because less turgor would be required to maintain the force needed for a smaller cell volume to grow forward. The presence of large vacuoles in front of the tube and the more frequent callose plugs both suggest that cytosolic turgor in the antisense *LePRK2* pollen tubes was reduced, and might explain why antisense *LePRK2* pollen tubes can not grow as fast as wild-type tubes.

Consistent with the idea that pollen tubes with reduced *LePRK2* expression have less cytosolic turgor, wild-type pollen tubes grew as slowly as antisense *LePRK2* pollen tubes when cultured in germination medium containing 32% PEG (data not shown), which increases the external osmotic pressure. Pollen germination also requires the accumulation of cytosolic turgor to a threshold level to start protruding from the pollen grain (Taylor and Hepler 1997). In antisense *LePRK2* pollen fewer pollen grains might have enough turgor to start germination, perhaps explaining the reduced germination percentage. Consistent with the idea that LePRK2 might regulate cytosolic turgor, overexpression of LePRK2-GFP caused slightly swollen tips, which could be interpreted as higher turgor. Swollen tips are also seen in depolarized growth, such as when a constitutively active ROP (Li et al., 1999) is overexpressed, but such depolarized growth is usually more extreme (so-called balloon tips), and also causes slower growth or arrest of pollen tube growth. The cell wall at the tip of pollen tubes is thin as it lacks callose or cellulose and contains mainly esterified pectins (Krichevsky et al., 2007). Under slightly higher cytolic turgor, the tip will be the first place to swell. In summary, we interpret the multiple morphological defects caused by altering *LePRK2* expression as affects on cytosolic turgor.

Antisense *LePRK2* pollen tubes did not increase production of extracellular O_2^- upon exogenous Ca^{2+}, while wild-type pollen tubes did, suggesting that LePRK2 might participate in

sensing extracellular Ca^{2+} and regulating O_2^- production. In plants, reactive oxygen species, including O_2^-, have been shown to play roles in mediating multiple physiological responses (Mori and Schroeder 2004). Extracellular O_2^- can be monitored by NBT staining (Rossetti and Bonatti, 2001), and in plants is thought to be produced by plasma membrane-localized NADPH oxidases (Mittler 2002). Plant NADPH oxidases are homologues of the mammalian neutrophil NADPH Oxidase $gp91^{phox}$, which catalyzes cytosolic NADPH and extracellular O_2 to cytosolic $NADP^+$, H^+ and extracellular O_2^- (Keller et al., 1998; Taylor et al., 1993). Root hairs and pollen tubes both grow by tip growth, and O_2^- plays important roles in root hair development (Foreman et al., 2003). In root hairs, localized ROS, along with a plasma membrane-localized NADPH oxidase RHD2 (for ROOT HAIR DEFECTIVE 2) and Ca^{2+}, have been shown to form a positive feedback loop to maintain polarity during root hair growth (Takeda et al., 2008). A similar mechanism might also work in pollen tubes, considering that tobacco pollen tubes produce tip-localized O_2^- that was increased by extracellular Ca^{2+}, and conversely, RNA interference of a tobacco NADPH oxidase gene impaired pollen tube growth (Potocký et al., 2007). Furthermore, cytosolic NADPH has been reported to oscillate in lily pollen tubes and to be correlated with tip growth (Cárdenas et al., 2006). In addition, the activity of NADPH oxidase can be regulated by extracellular Ca^{2+}. Although plant NADPH oxidases contain two Ca^{2+} binding EF-hand motifs, they are located in the cytoplasmic N-terminal portion of this transmembrane protein (Wong et al., 2007), and so extracellular Ca^{2+} would need to be sensed and transduced across the plasma membrane to increase NADPH oxidase activity. Our results support a role for LePRK2 in this transduction.

We showed that antisense *LePRK2* pollen tubes don't increase growth upon addition of calcium ions. Exogenous calcium ions of appropriate concentration increase pollen tube growth, while higher concentrations inhibit pollen tube growth (Steer and Steer 1989). However, how the exogenous Ca^{2+} signal is transduced and affects pollen tube growth is not clear. Our results suggest that LePRK2 is involved in transducing the extracellular Ca^{2+} signal.

For the antisense *LePRK2* pollen, the average reduction in vitro tube growth (50-70% less than wild type) was very significant, but the delay in arriving at ovaries was small (2-3 hours delay for a journey that normally takes 7-9 hours) and, except for line 1, the transmission ratio of the transgene was not significantly distorted. Considering that there is always more pollen on the stigma than the number of ovaries to be fertilized, only the earliest-arriving pollen tubes will

successfully deliver sperm. Given the variation of tube growth rate among pollen of same genetic background, it is possible that the fastest-growing antisense *LePRK2* pollen tubes had growth rates comparable to those of wild-type pollen. In self-pollinated flowers, pollen does not land on the stigma at the same time, and this might have further diminished the difference in average tube growth. It is also possible that other growth-promoting factors from the pistil helped pollen tube growth in ways that are independent of LePRK2. Furthermore, LePRK1, a similar pollen receptor-like kinase that can interact with LePRK2 (Muschietti et al. 1998), might compensate for LePRK2 when LePRK2 is knocked-down.

Mutations in ligands and receptors in the same signaling pathway have similar phenotypes, for example Clavata 1, 2 and 3 (see review Williams and Fletcher 2005). We showed here that antisense *LePRK2* pollen had a lower germination percentage and slower tube growth, and similar phenotypes were seen when two extracellular partners for LePRK2 were down-regulated. LAT52 is an extracellular partner of LePRK2 before pollen germination (Tang et al., 2002), and antisense *LAT52* pollen can not germinate at all in vitro (Muschietti et al., 1994). The tomato homolog of SHY is also an extracellular partner for LePRK2 (Tang et al., 2004), and in *Petunia hybrida* antisense *SHY* pollen has a lower germination percentage and slower tube growth (Guyon et al., 2004). Our results suggest that other factors (Ca^{2+}, STIL) and processes (turgor modulation) also act through LePRK2.

Pollen over-expressing a nearly full-length version of KPP (missing eight amino acids at the N-terminus) showed depolarized tube growth (Kaothien et al., 2005). However, overexpression of a full-length AtRopGEF12, the closest Arabidopsis homolog for KPP, only resulted slightly increase on pollen tube width (Zhang and McCormick 2007), suggesting that those 8 amino acids were important for the phenotypic differences. Here we confirmed that, since over-expressing a full-length version KPP caused only a slight increase of pollen tube width (Fig. 8F). Co-expressing AtRopGEF12 and a LePRK2 homolog in Arabidopsis (AtPRK2a) gave much wider pollen tube tips than when over-expressing AtRopGEF or AtPRK2a alone (Zhang and McCormick, 2007). Here, we showed similar situation in tomato for KPP and LePRK2 (Fig. 8G). This supports the model proposed by Zhang and McCormick (2007) that pollen-enriched RopGEFs serve as a link between pollen receptor kinases and Rop-mediated intracellular responses. It has been reported that ROP1 activation controls the polar accumulation and exocytosis of vesicles at pollen

tube tips, through RIC3 and/or RIC4 pathways (Lee et al., 2008). It is possible that antisense LePRK2 reduced the activation of KPP, which in turn reduced the activation of Rop, thus leading to reduced exocytosis, which slowed tube growth. It was also reported that a rice Rop (OsRac1) can interact and regulate OsRboHB, which is a plasma membrane-localized NADPH oxidase that produces extracellular O_2^- (Wong et al., 2007). It is plausible that the defects of antisense *LePRK2* pollen tubes in extracellular O_2^- production might be mediated by Rop.

Materials and Methods

In vitro pollen germination and measurements

Open flowers of tomato (*Solanum lycopersicon* cv VF36) were picked in the afternoons. Mature pollen was obtained by vibrating anthers of open flowers with a bio-vortexer (BioSpec Products). Pollen was used directly for germination or bombardment experiments, or was stored at -80°C for protein extraction. Pollen was germinated in optimized 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 4000], in dishes that were rotated horizontally at 60 rpm, at a concentration of 1mg pollen/ml medium, unless otherwise specified. Pollen tube images were captured with a digital camera attached to an epifluorescence microscope. Pollen tube length and width were measured using ImageJ (Rasband, 1997-2007). For measuring tube lengths, pollen was cultured in 5 individual wells for 3 hours, and fixed in FAA (10% formaldehyde, 5% glacial acetic acid, 50% ethanol (v/v)) before images were taken.

Pollen bombardment assay

The pollen-specific *LAT52* promoter (Twell et al., 1991) was used to replace the *CaMV 35S* promoter in a *35S::GFP* plasmid (pPK100, a kind gift from Robert Blanvillain) to obtain a *pLAT52::GFP* plasmid. Full-length *LePRK2* cDNA was inserted in-frame at the 5'-end of the GFP coding sequence in the *pLAT52::GFP* plasmid. The *mRFP* coding region was amplified from the *pMT-mRFP1* plasmid (Toews et al., 2004) to replace *GFP* in the *pLAT52::GFP* and *pLAT52::LePRK2-GFP* plasmids to obtain *pLAT52::RFP* and *pLAT52::LePRK2-RFP*. The plasmids *pLAT52::GFP*, *pLAT52::LePRK2-GFP*, *pLAT52::RFP* and *pLAT52::LePRK2-RFP* were

used for pollen bombardment. Tomato pollen bombardment assays were as described in Kaothien et al. (2005) for tobacco pollen bombardment except that pollen was cultured with shaking at 60 rpm. We observed pollen tubes four to seven hours after bombardment.

Transgenic tomato development

A GFP expression cassette and an antisense full-length *LePRK2*, both driven by the *LAT52* promoter, were individually inserted into pCAMBIA2300 to obtain the pCAMBIA-antisense LePRK2 plasmid. *Agrobacterium tumefaciens* strain *LBA4404* (Hoekema et al., 1983) carrying this plasmid was used to transform tomato (*Solanum lycopersicon* cv. VF36) as described (McCormick, 1991).

NBT and DAB staining of pollen tubes

Production of O_2^- was determined by its ability to reduce nitroblue tetrazolium (NBT) (Rossetti and Bonatti, 2001). Pollen tube staining was slightly modified from the method described in Potocky et al. (2007). To avoid nitrate interference with the NBT reaction, tomato pollen was cultured in a simplified medium (20 mM MES, pH 6.0, 13% (w/v) sucrose, 20% (w/v) polyethylene glycol 4000, 1.6mM Boric Acid, 1mM KCl) with or without 1mM $CaCl_2$, as indicated, for 1-4 hours before the addition of 1mM NBT. Each treatment had at least three replicates. Images were captured using an Olympus BX51 microscope fitted with an Olympus DP71 digital camera. The intensity of formazan precipitation in pollen tube tips was quantified using ImageJ (Rasband, 1997-2007). Minimal pixel intensity was calculated within each single image and inside regions of interest fitted to the outline of the pollen tube tip. Less intensity indicates more NBT stain. At least 15 pollen tubes from each replicate were measured. Callose of pollen tubes in the pistil was stained with decolorized aniline blue (DAB) directly after dissection of pistils without fixation, as described (Johnson-Brousseau and McCormick 2004).

RNA extraction and Quantitative PCR

Total RNA extraction from mature or germinated pollen of tomato was according to a modification of the Qiagen protocol (http://www.pgec.usda.gov/McCormick/McCormick/mclab.html). We performed quantitative

real-time PCR reaction of reverse-transcribed RNA with SYBR Green detection on an ABI PRISM 7000 sequence detector (Applied Biosystems) as described in Tang et al. (2006). The primers used to amplify a 100-bp fragment of *LePRK2* were 5'-CTTGCCTGATTTGTTGAAAGCC-3' and 5'-AACAACCATGACAGGGCCA-3'. The primers used to amplify a 122-bp fragment of *LePRK1* were: 5'-GGCCTGAAGTACAAGCAGTACAACA-3' and 5'-CGAACCAAACACACCGCTA-3'. The primers used to amplify a 123-bp fragment of a tomato actin gene (BG140412) were: 5'-GCGAGAAATTGTCAGGGACGT-3' and 5'-TGCCCATCTGGGAGCTCAT-3'.

Pollen protein extraction and immunobloting

Tomato pollen protein extraction was as described by Tang et al. (2002) with a TED buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM DTT, and 1 x Protease Inhibitor Cocktail Complete (Boehringer Mannheim)). The P_{100} (membrane-associated) protein fraction was used for immunoblots with polyclonal anti-ECD1 or anti-ECD2 antibodies (Muschietti et al., 1998).

Style component preparation and treatment

STIL was purified as in Wengier et al. (submitted). Briefly, a pistil exudate was obtained by cutting 100 tobacco styles and stigmas transversely in 5 mm segments and incubating overnight in 50 mM ammonium bicarbonate (25 ml) at 4°C with gentle agitation. The pistil exudate was filtered through miracloth and filter paper and then subjected to chloroform-methanol extraction. The aqueous phase was dried by rotary evaporation and the pellet was dissolved in water. The dissolved pellet was centrifuged 10 min at 10,000 x g in a tabletop centrifuge and the supernatant was fractionated by FPLC in a Mono Q 5/50 GL Monobead™ column (GE Healthcare Life Sciences). The presence of eluted STIL in fractions was assayed by LePRK2-specific dephosphorylation in mature pollen protein extracts, as in Muschietti et al. (1998). Fractions that showed LePRK2 dephosphorylation were pooled, freeze-dried and subjected to solid-phase extraction in a Sep-Pak™ Plus C18 cartridge (Waters). The percolate was freeze-dried in order to eliminate acetonitrile and the STIL fraction was obtained by dissolving the pellet in MilliQ water.

For germination assays, freshly collected pollen from each line (wild-type, GFP-expressing, antisense *LePRK2* lines 2 and 3) was pre-hydrated in PGM without sucrose for 30 min at room

temperature with occasional gentle agitation. After incubation, the pollen suspension was centrifuged for 5 min at 3000 x g and resuspended to a final concentration of 1 mg pollen/ml in complete PGM ("no STIL" treatment) or supplemented with 0.0003 Abs units (280 nm) of STIL/µl of PGM ("+STIL" treatment). In every experiment, each line included 3 replicates for each treatment. Pollen germination was carried out on a rotating shaker (50 rpm) for 3 hours at 28°C, in 24-well microplates and each well contained 400 µl of the pollen suspension. After germination, the pollen suspension was transferred to 1.5 ml microtubes and 10X fixing solution (5.6% formaldehyde, 0.5% glutaraldehyde and 25% PEG 3350) was added to a final concentration of 1X. Samples were incubated with gentle agitation at 50 rpm for 30 min at 4°C. Fixed pollen tubes were observed using an Axiovert microscope (Zeiss) and 50 pictures were taken for each replicate with a digital camera (Diagnostic Instruments). Fifteen pictures were randomly selected and the lengths of all the pollen tubes in each picture were determined using AxioVision software (Zeiss), and averaged. Pollen tube lengths for each replicate were calculated as the average from all 15 values previously obtained. Control and STIL treatments were analyzed with the Student t-test using Prism version 4.03 for Windows (GraphPad). Germination experiments were repeated twice and since variances did not differ, data were pooled.

Accession numbers: *LePRK2* (U58473), *LePRK1*(U58474), *KPP*(AY730762).

Supplemental Material

Supplemental Table 1. Summary of antisense *LePRK2* plants

Supplemental Figure 1. Growth rate of pollen tubes germinated in vitro for 2-4 hours. This experiment was done only with antisense *LePRK2* line 4 and wild-type (WT).

Supplemental Figure 2. Pollen tube lengths for wild-type (WT) and antisense *LePRK2* lines 2 and 4 after 3 hour culture, with indicated concentrations of boric acid (BA). Error bar=standard deviation. Asterisks indicate a significant difference between different concentrations of BA at the same data point ($P<0.05$; t test).

Supplemental Figure 3. Relative tip widths of tobacco pollen tubes transiently expressing either RFP alone, LePRK2-RFP alone, KPP-GFP alone, or co-expressing LePRK2-RFP and KPP-GFP.

Supplemental movie 1. The growth of a wild-type tomato pollen tube. For all movies, frames were

taken every 2 seconds.

Supplemental movie 2. The growth of a pollen tube of antisense *LePRK2* line 2, note the vacuole moving towards the tip.

Supplemental movie 3. The growth of the pollen tube shown in movie 2, but taken two minutes later; note the vacuole moving away from the tip.

Acknowledgements

We thank undergraduates Xin Huang, Wei-Jie Huang, Hai-Kuan Liu, Vivian Ng, Yuan-Sheng Yu and Lin Zhang for assistance. We thank the Fungal Genomics Stock Center for the *pMT-mRFP1* plasmid. This work was supported by U.S. Department of Agriculture Current Research Information System grant 5335-21000-030-00D to SM. WT was supported by Ministry of Science and Technology of China Grants 2007CB108700, 2007AA10Z187, 2006AA10A102, the Knowledge Innovation Program of the Chinese Academy of Sciences KSCX2-YW-N-058 and National Natural Science Foundation of China 30770196.

References

Bassham DC, Raikhel NV (2000) Unique features of the plant vacuolar sorting machinery. Curr Opin Cell Biol 12: 491–495.

Benkert R, Obermeyer G, Bentrup FW (1997) The turgor pressure of growing lily pollen tubes. Protoplasma 198:1–8.

Berken A, Thomas C, Wittinghofer A (2005) A new family of RhoGEFs activates the Rop molecular switch in plants. Nature 436:1176-1180.

Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird BS, Zacharias DA, Tsien RY (2002) A monomeric red fluorescent protein. Proc Natl Acad Sci USA 99:7877–7882.

Cárdenas L, Lovy-Wheeler A, Kunkel JG, Hepler PK (2008) Pollen tube growth oscillations and intracellular calcium levels are reversibly modulated by actin polymerization. Plant Physiol 146:1611-1621.

Cárdenas L, McKenna ST, Kunkel JG, Hepler PK (2006) NAD(P)H oscillates in pollen tubes and is correlated with tip growth. Plant Physiol. 142:1460-1468.

Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P, Costa S,

Brownlee C, Jones JDG, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422: 442–446.

Guyon V, Tang W, Monti M, Raiola A, DeLorenzo G., McCormick S, Taylor L (2004) Antisense phenotypes reveal a role for SHY, a pollen-specific leucine-rich repeat protein, in pollen tube growth. Plant Journal 39:643-654.

Hicks G, Rojo E, Hong S, Carter D, Raikhel N (2004) Geminating pollen has tubular vacuoles, displays highly dynamic vacuole biogenesis, and requires VACUOLELESS1 for proper function. Plant Physiol. 134:1227-1239.

Hoekema A, Hirsch PR, Hooykaas P, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir and T-region of *Agrobacterium tumefaciens* Ti plasmid. Nature 303:179–180.

Johri BM, Vasil IK. (1961) Physiology of pollen. Bot Rev 27:325–81

Johnson-Brousseau S, McCormick S (2004) A compendium of methods useful for characterizing Arabidopsis pollen mutants and gametophytically-expressed genes. Plant J 39:761-775.

Kaothien P, Ok SH, Shuai B, Wengier D, Cotter R, Kelley D, Kiriakopolos S, Muschietti J, McCormick S (2005) Kinase partner protein interacts with the LePRK1 and LePRK2 receptor kinases and plays a role in polarized pollen tube growth. Plant J 42:492-503.

Keller T, Damude H, Werner D, Doerner P, Dixon R, Lamb C (1998) A plant homolog of the neutrophil NADPH oxidase $gp91^{phox}$ subunit gene encodes a plasma membrane protein with Ca^{2+} Binding Motifs. Plant Cell 10:255–266.

Kim H-Y, Cotter R, Johnson S, Senda M, Dodds P, Kulikauskas R, Tang W, Ezcurra I, Herzmark P, McCormick S (2002) New pollen-specific receptor kinases identified in tomato, maize and Arabidopsis: the tomato kinases show overlapping but distinct localization patterns on pollen tubes. Plant Mol Biol 50:1-16.

Krichevsky A, Kozlovsky SV, Tian GW, Chen MH, Zaltsman A, Citovsky V (2007) How pollen tubes grow. Dev Biol 303:405-420.

Lee YJ, Szumlanski A, Yang Z (2008) Rho-GTPase-dependent filamentous actin dynamics coordinate vesicle targeting and exocytosis during tip growth. J Cell Biol 181:1155-1168.

Lew R (2004) Osmotic effects on the electrical properties of Arabidopsis root hair vacuoles in situ. Plant Physiol 134:352-360.

Li H, Lin Y, Heath RM, Zhu MX, Yang Z. (1999) Control of pollen tube tip growth by a Rop GTPase–dependent pathway that leads to tip-localized calcium influx. Plant Cell 11:1731–1742.

Lovy-Wheeler A, Cárdenas L, Kunkel JG, Hepler PK (2007) Differential organelle movement on the actin cytoskeleton in lily pollen tubes. Cell Motil Cytoskeleton 64:217-232.

MacRobbie E (2006) Osmotic effects on vacuolar ion release in guard cells. Proc Natl Acad Sci USA 103:1135-1140.

McCormick S (1991) Transformation of tomato with *Agrobacterium tumefaciens*.in Plant Tissue Culture Manual, Fundamentals and Applications, K. Lindsey (ed), Kluwer, Vol B6: 1-9.

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends in Plant Sci. 7:405-410

Mori IC, Schroeder JI (2004) Reactive oxygen species activation of plant Ca^{2+} channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. Plant Physiol 135:702-708.

Muschietti JP, Dircks L, Vancanneyt G, McCormick S (1994) LAT52 protein is essential for tomato pollen development: Pollen expressing antisense *LAT52* RNA hydrates and germinates abnormally and cannot achieve fertilization. Plant J 6:321-338.

Muschietti JP, Eyal Y, McCormick, S (1998) Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK1 and LePRK2. Plant Cell 10:319-330.

Nishikawa S, Zinkl GM, Swanson RJ, Maruyama D, Preuss D (2005) Callose (beta-1,3 glucan) is essential for Arabidopsis pollen wall patterning, but not tube growth. BMC Plant Biol 5:22.

Potocký M, Jones MA, Bezvoda R, Smirnoff N, Zárský V (2007) Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. New Phytol 174:742-751.

Rasband, W.S. 1997-2007 ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/.

Rossetti S, Bonatti PM (2001) In situ histochemical monitoring of ozone- and TMV-induced reactive oxygen species in tobacco leaves. Plant Physiol Biochem 39:433–442.

Steer M, Steer J, (1989) Pollen tube tip growth. New Phytol 111:323–358.

Takeda S, Gapper C, Kaya H, Bell E, Kuchitsu K, Dolan L (2008) Local positive feedback regulation determines cell shape in root hair cells. Science 319:1241-1244.

Tang W, Ezcurra I, Muschietti J, McCormick S (2002) A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2. Plant Cell 14:2277-2287.

Tang W, Kelley D, Ezcurra I, Cotter R, McCormick S (2004) LeSTIG1, an extracellular binding partner for the pollen receptor kinases LePRK1 and LePRK2, promotes pollen tube growth in vitro. Plant J 39:343-353.

Tang W, Coughlan S, Crane E, Beatty M, Duvick J (2006) The application of laser microdissection to in planta gene expression profiling of the maize anthracnose stalk rot fungus *Colletotrichum graminicola*. Mol Plant Microbe Interact 19:1240-1250.

Taylor LP, Hepler P (1997) Pollen germination and tube growth Annual Review of Plant Physiology and Plant Molecular Biology 48:461-491.

Taylor WR, Jones DT, Segal AW (1993) A structural model for the nucleotide binding domains of the flavocytochrome b-$_{245}$ β-chain. Protein Sci. 2:1675-1685.

Toews MW, Warmbold J, Konzack S, Rischitor P, Veith D, Vienken K, Vinuesa C, Wei H, Fischer R (2004) Establishment of mRFP1 as a fluorescent marker in *Aspergillus nidulans* and construction of expression vectors for high-throughput protein tagging using recombination in vitro (GATEWAY). Curr Genet 45:383-389.

Twell D, Klein TM, Fromm ME, McCormick S (1989a) Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. Plant Physiol 91:1270-1274.

Twell D, Wing R, Yamaguchi J, McCormick S (1989b) Isolation and expression of an anther-specific gene from tomato. Mol Gen Genetics 217: 240-245.

Twell D, Yamaguchi J, Wing RA, Ushiba J, McCormick S (1991) Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. Genes Dev 5: 496-507

Wengier D, Valsecchi I, Cabanas ML, Tang W, McCormick S, Muschietti J (2003) The receptor kinases LePRK1 and LePRK2 associate in pollen and when expressed in yeast, but dissociate in the presence of style extract. Proc Natl Acad Sci USA 100:6860-6865.

Williams L, Fletcher JC (2005). Stem cell regulation in the Arabidopsis shoot apical meristem. Current Opinion in Plant Biology 8: 582-586.

Wong HL, Pinontoan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, Kojima C, Yoshioka H, Iba

K, Kawasaki T, Shimamoto K (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell 19:4022–4034.

Zhang Y, McCormick S (2007) A distinct mechanism regulating a pollen-specific guanine nucleotide exchange factor for the small GTPase Rop in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104:18830-18835.

Zonia L, Munnik T (2008) Vesicle trafficking dynamics and visualization of zones of exocytosis and endocytosis in tobacco pollen tubes. J Exp Bot 59:861-873.

Figure Legends

Figure 1. *LePRK2* expression is reduced in antisense *LePRK2* pollen. Quantitative RT-PCR of *LePRK2* and *LePRK1* mRNA levels, using total RNA of mature pollen (A) or in vitro germinated pollen (B) as templates. WT: wild-type; 1-6, antisense *LePRK2* lines 1-6. The tomato actin gene was used as a control. In vitro germinated pollen was harvested 6 hours after culturing in optimized germination medium. Experiments were performed in triplicate. Error bar = standard error.

Figure 2. Protein levels of LePRK2 are reduced in antisense *LePRK2* pollen. A and B. Immunoblots showing LePRK2 (A) and LePRK1 (B) protein levels in germinated pollen of wild-type (WT), GFP-expressing and antisense *LePRK2* lines 1-4; C. The LePRK2 protein level was normalized against the LePRK1 protein level. The mean intensity average of two experiments, as measured with Image J. Two bands are detected by anti-LePRK2 antibodies (Wengier et al., 2003).

Figure 3. Germination percentage and growth rate of antisense *LePRK2* pollen. A. Germination percentage of tomato pollen plotted against culture time. Wild-type VF36 (WT1 and WT2) and homozygous transgenic plants carrying *pLAT52::GFP* (GFP) are controls; 2, 3 and 4 are antisense *LePRK2* lines 2, 3 and 4. A combination of two independent experiments is shown. WT1 and antisense LePRK2 line 2 were measured in experiment 1. WT2, GFP and antisense LePRK2 lines 3 and 4 were measured in experiment 2. B. Pollen tube length measured after 3 hour in vitro

culture. 1-6 are antisense *LePRK2* lines 1-6. C. Tomato pollen tubes from wild-type (left) or from antisense *LePRK2* line 2 (right), after 10 hour in germination medium. In each plate, pollen was added at 1mg/ml. Arrows point to the "mats" of pollen tubes.

Figure 4. Pollen tubes of antisense *LePRK2* plants are delayed in reaching ovaries. Wild-type tomato pistils hand-pollinated with either wild-type pollen (WT), GFP-expressing pollen (GFP) or antisense *LePRK2* pollen (line 2 and line 4) were dissected and stained with decolorized aniline blue to reveal pollen tubes. A-D: Germination of indicated pollen tubes on stigmas 5 hours after pollination; E-L: Ovaries were dissected after pollination at the times indicated to reveal the presence or absence of pollen tubes. Scale bar = 200μm, arrows point to pollen tubes.

Figure 5. Antisense *LePRK2* pollen tubes have large vacuoles near the tip region and shorter intervals between callose plugs. A. The tip regions of a representative GFP-expressing pollen tube (control) and antisense *LePRK2* pollen tube of lines 2 and 4. The pollen tubes were imaged after 5 hours in vitro culture. Scale bar=10 μm. DIC: differential interference contrast. B. Representative pictures of pollen tubes with callose plugs stained by decolorized aniline blue. Wild-type and antisense *LePRK2* pollen tubes after 10 hr and 12 hour in vitro culturing respectively. Insets show enlargements of the callose plugs. Scale bar=200 μm. G: pollen grain. T: tip of pollen tube. C. The average interval lengths between callose plugs in pollen tubes of wild-type, GFP-expressing or antisense *LePRK2* lines after 12 hours in vitro culturing. Error bar=standard error (n=3).

Figure 6. Responses of antisense *LePRK2* pollen tubes to 1mM $CaCl_2$ treatment. A and B. Tip-localized ROS production of pollen tubes detected by nitroblue tetrazolium (NBT) staining. A. Average of minimum intensities of NBT-stained tubes of wild-type (WT), GFP-expressing pollen (GFP) and antisense *LePRK2* lines 1, 2 and 4 after 6 hour in vitro culturing with or without 1 mM $CaCl_2$. The results for individual experiments are shown in separate panels. For each experiment an average of 30 tubes per well and 3 wells per treatment were measured. Error bar = standard error. Asterisks indicate significant differences at the same data point ($P<0.05$; t test). B. Representative NBT-staining pictures for pollen tubes of wild-type and antisense *LePRK2* lines 2

and 4. Scale bar = 10 μm. C. Pollen tube lengths for wild-type (WT), GFP-expressing pollen (GFP) and antisense *LePRK2* lines 2 and 4 after 3 hour culture with or without 1 mM $CaCl_2$. Error bars = standard error (n=3).

Figure 7. The effects of STIL on tomato pollen tube growth. The graph shows average pollen tube length after 3 hour germination from antisense LePRK2, wild-type and GFP-expressing pollen tubes. The data are pooled from two experiments; each experiment contained 3 replicates, and at least thirty pollen tubes were measured in each replicate. Error bars = standard error.

Figure 8. Transient over-expression of LePRK2 and/or KPP fusion proteins in pollen tubes. A and B: Tomato pollen tubes over-expressing GFP (A) or LePRK2-GFP (B). C-F: Tobacco pollen tubes over-expressing RFP (C), LePRK2-RFP (D), GFP (E) or full-length KPP-GFP (F) individually. G: Tobacco pollen tubes co-expressing LePRK2-RFP and KPP-GFP. All constructs used the *LAT52* promoter. Pictures were taken using DIC, GFP or RFP channels, as indicated. Pollen tubes visible in the DIC channel but invisible in either the GFP or RFP channels are not transformed. Scale bar=50 μm.

Table1 Time for pollen tubes to reach ovaries

Hours post pollination	Number of pistils with pollen tubes at the ovary/total examined			
	Wide-type	GFP	Line 2	Line 4
3	0/2		0/1	0/1
5	0/2	0/1		
6	0/2	0/3	0/1	0/1
7	1/2	1/2	0/2	0/2
8	2/3	3/4	0/2	0/1
9	2/2	2/2	0/4	0/2
10	2/2	3/3	1/3	1/3
11	2/2	2/2	5/6	0/2
12		1/1	4/4	3/3
14	2/2	1/1	1/1	1/1

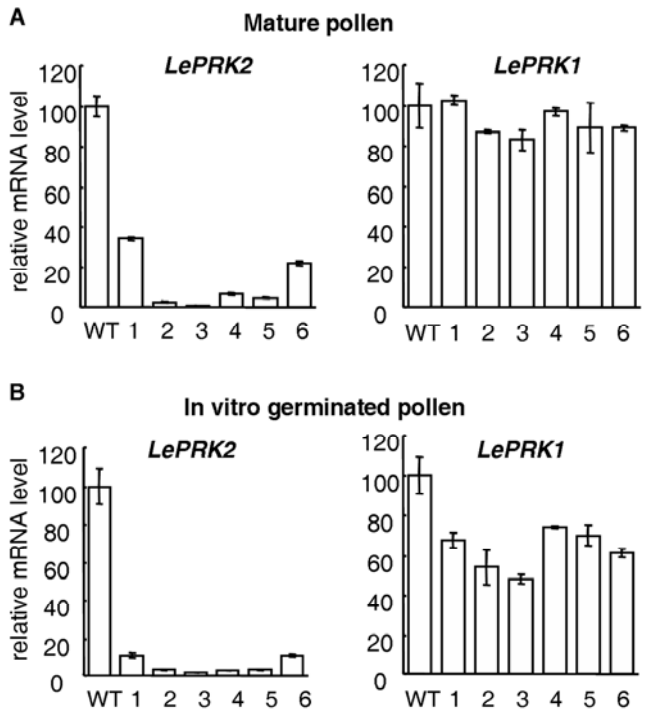

Figure 1. *LePRK2* expression is reduced in antisense *LePRK2* pollen. Quantitative RT-PCR of *LePRK2* and *LePRK1* mRNA levels, using total RNA of mature pollen (A) or in vitro germinated pollen (B) as templates. WT: wild-type; 1-6, antisense *LePRK2* lines 1-6. The tomato actin gene was used as a control. In vitro germinated pollen was harvested 6 hours after culturing in optimized germination medium. Experiments were performed in triplicate. Error bar = standard error.

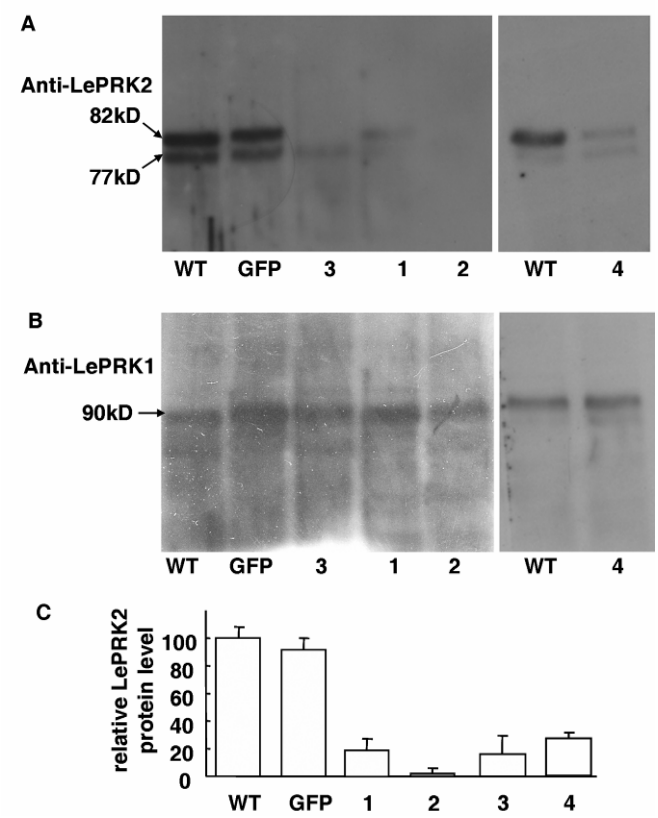

Figure 2. Protein levels of LePRK2 are reduced in antisense *LePRK2* pollen. A and B. Immunoblots showing LePRK2 (A) and LePRK1 (B) protein levels in germinated pollen of wild-type (WT), GFP-expressing and antisense *LePRK2* Lines 1-4; C. The LePRK2 protein level was normalized against the LePRK1 protein level. The mean intensity average of two experiments, as measured with Image J. Two bands are detected by anti-LePRK2 antibodies (Wengier et al., 2003).

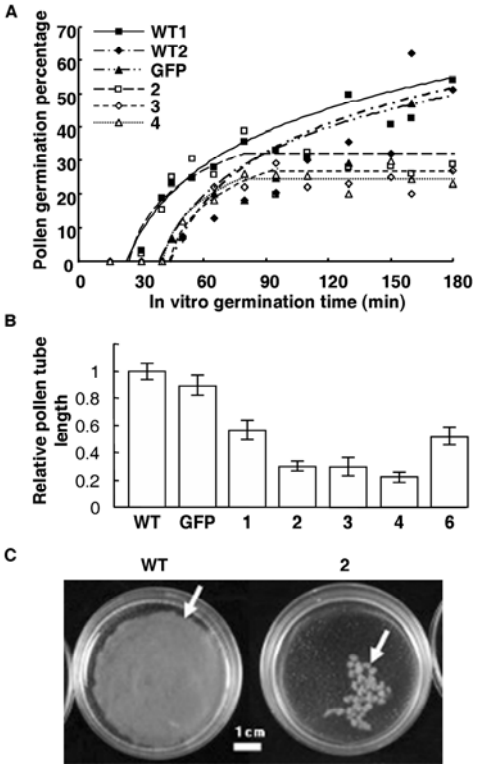

Figure 3. Germination percentage and growth rate of antisense *LePRK2* pollen. A. Germination percentage of tomato pollen plotted against culture time. Wild-type VF36 (WT1 and WT2) and homozygous transgenic plants carrying *pLAT52::GFP* (GFP) are controls; 2, 3 and 4 are antisense *LePRK2* lines 2, 3 and 4. A combination of two independent experiments is shown. WT1 and antisense LePRK2 line 2 were measured in experiment 1. WT2, GFP and antisense LePRK2 lines 3 and 4 were measured in experiment 2. B. Pollen tube length measured after 3 hour in vitro culture. 1-6 are antisense *LePRK2* lines 1-6. C. Tomato pollen tubes from wild-type (left) or from antisense *LePRK2* line 2 (right), after 10 hour in germination medium. In each plate, pollen was added at 1mg/ml. Arrows point to the "mats" of pollen tubes.

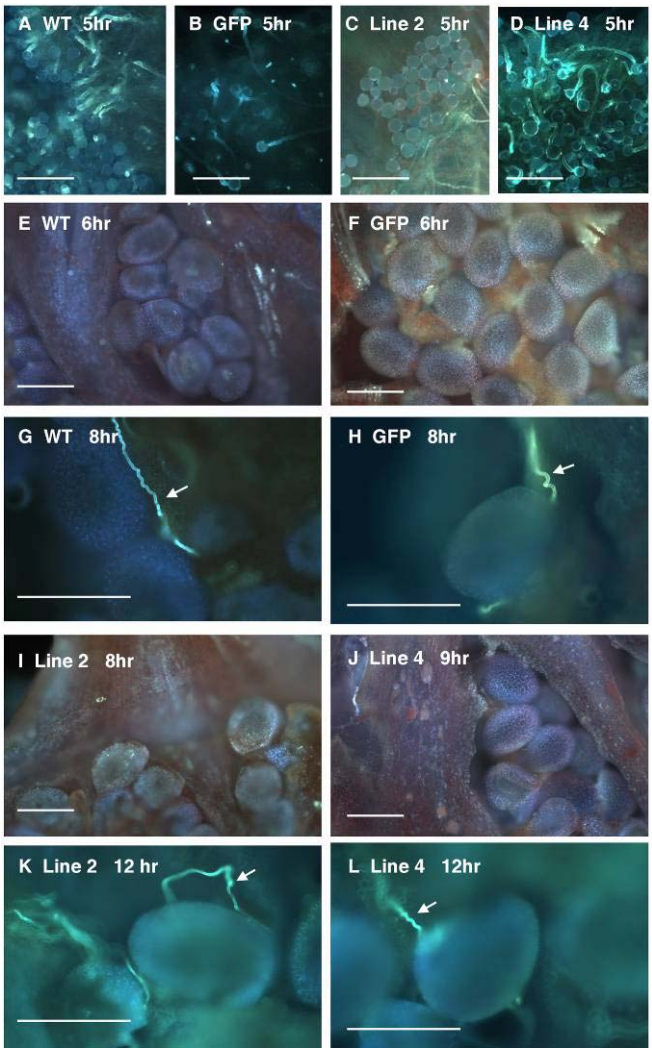

Figure 4. Pollen tubes of antisense *LePRK2* plants are delayed in reaching ovaries. Wild-type tomato pistils hand-pollinated with either wild-type pollen (WT), GFP-expressing pollen (GFP) or antisense *LePRK2* pollen (line 2 and line 4) were dissected and stained with decolorized aniline blue to reveal pollen tubes. A-D: Germination of indicated pollen tubes on stigmas 5 hours after pollination; E-L: Dissected ovaries at indicated hours after pollinated with pollen as labeled. Scale bar = 200μm, arrows point to pollen tubes.

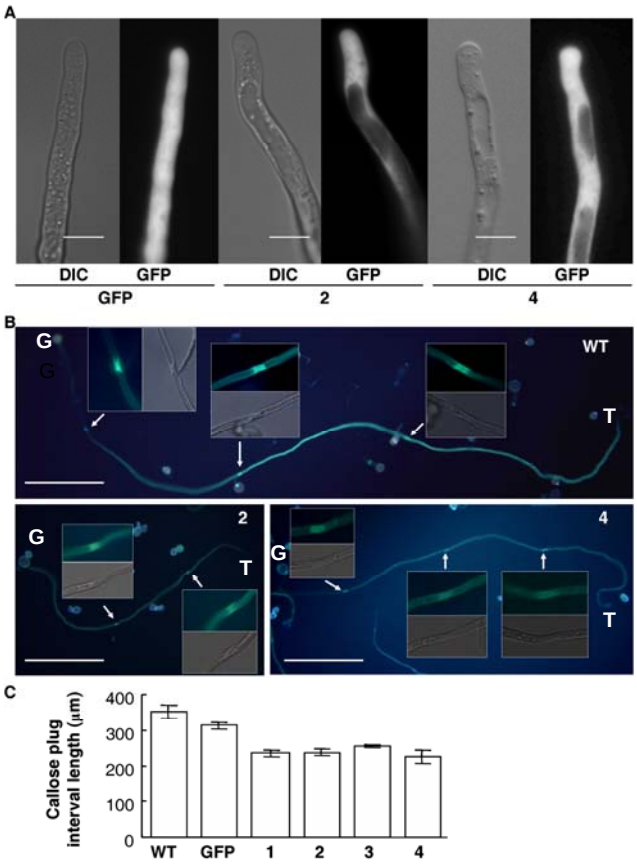

Figure 5. Antisense *LePRK2* pollen tubes have large vacuoles near the tip region and shorter intervals between callose plugs. A. The tip regions of a representative GFP-expressing pollen tube (control) and antisense *LePRK2* pollen tube of lines 2 and 4. The pollen tubes were imaged after 5 hours in vitro culture. Scale bar=10μm. DIC: differential interference contrast. B. Representative pictures of pollen tubes with callose plugs stained by decolorized aniline blue. Wild-type and antisense *LePRK2* pollen tubes after 10 hr and 12 hour in vitro culturing respectively. Insets show enlargements of the callose plugs. Scale bar=200 μm. G: pollen grain. T: tip of pollen tube. C. The average interval lengths between callose plugs in pollen tubes of wild-type, GFP-expressing or antisense *LePRK2* lines after 12 hours in vitro culturing. Error bar=standard error (n=3).

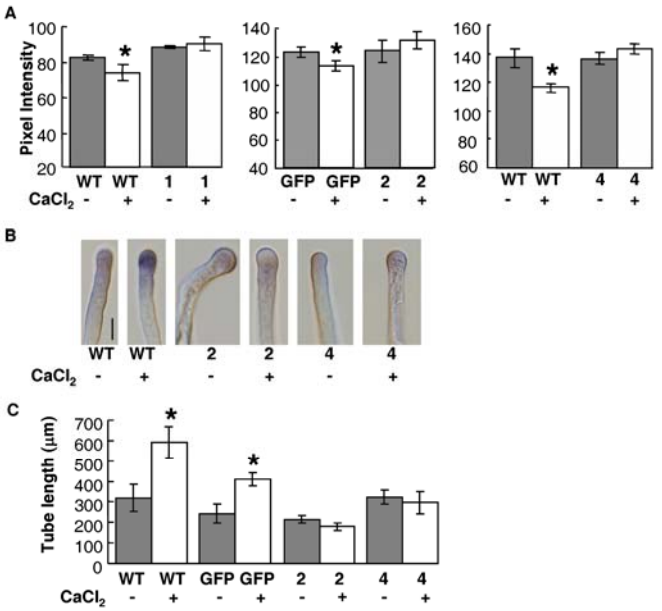

Figure 6. Responses of antisense *LePRK2* pollen tubes to 1mM $CaCl_2$ treatment. A and B. Tip-localized ROS production of pollen tubes detected by nitroblue tetrazolium (NBT) staining. A. Average of minimum intensities of NBT-stained tubes of wild-type (WT), GFP-expressing pollen (GFP) and antisense *LePRK2* Lines 1, 2 and 4 after 6 hour in vitro culturing with or without 1 mM $CaCl_2$. The results for individual experiments are shown in separated panel. For each experiment an average of 30 tubes per well and 3 wells per treatment were measured. Error bar = standard error. Asterisks indicate significant differences at the same data point ($P<0.05$; t test). B. Representative NBT-staining pictures for pollen tubes of wild-type and antisense *LePRK2* lines 2 and 4. Scale bar = 10 µm. C. Pollen tube lengths for wild-type (WT), GFP-expressing pollen (GFP) and antisense *LePRK2* lines 2 and 4 after 3 hour culture with or without 1 mM $CaCl_2$. Error bars = standard error (n=3).

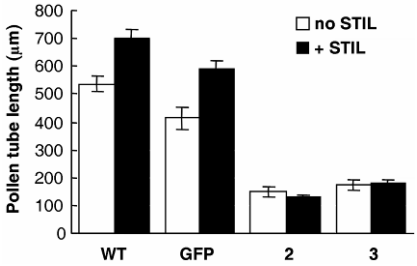

Figure 7. The effects of STIL on tomato pollen tube growth. The graph shows average pollen tube length after 3 hour germination from antisense LePRK2, wild-type and GFP-expressing pollen tubes. The data are pooled from two experiments; each experiment contained 3 replicates, and at least thirty pollen tubes were measured in each replicate. Error bars = standard error.

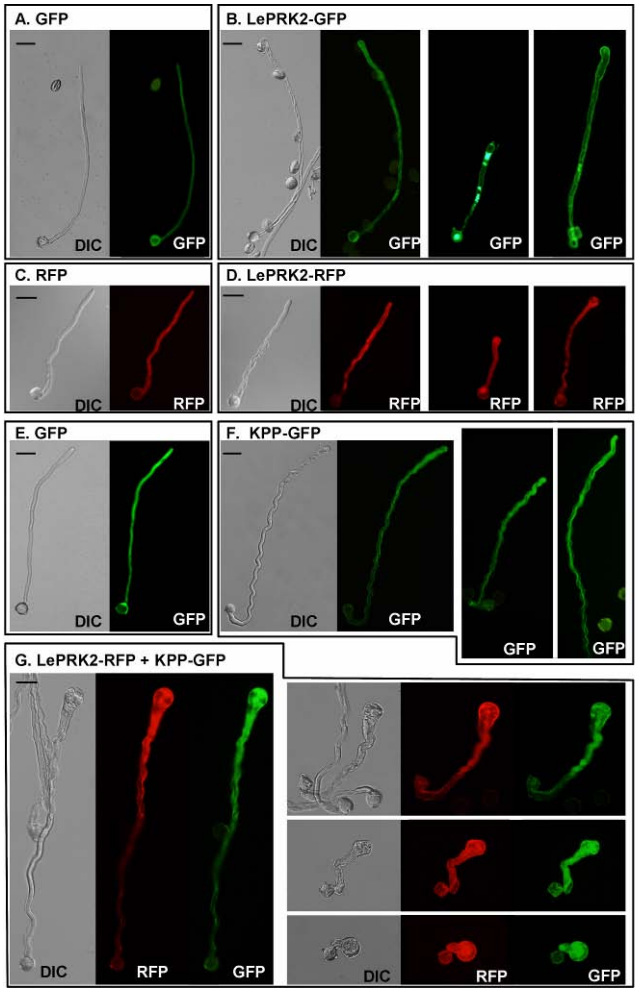

Figure 8. Transiently over-expression of LePRK2 and/or KPP fusion proteins in pollen tubes. A and B: Tomato pollen tubes over-expressing GFP (A) or LePRK2-GFP (B). C-F: Tobacco pollen tubes over-expressing RFP (C), LePRK2-RFP (D), GFP (E) or full-length KPP-GFP (F) individually. G: Tobacco pollen tubes co-expressing LePRK2-RFP and KPP-GFP. All the constructs used the *LAT52* promoter. Pictures were taken under DIC, GFP or RFP channel as indicated. Pollen tubes visible in the DIC channel but invisible in either the GFP or RFP channels are not transformed. Scale bar=50μm.