The Plant Journal (2004) doi: 10.1111/j.1365-313X.2004.02139.x

LeSTIG1, an extracellular binding partner for the pollen receptor kinases LePRK1 and LePRK2, promotes pollen tube growth *in vitro*

Weihua Tang, Dior Kelley, Inés Ezcurra[†], Robyn Cotter[‡] and Sheila McCormick[*]

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, CA 94710, USA

Received 26 February 2004; revised 30 April 2004; accepted 7 May 2004.
[*]For correspondence (fax +1 (510) 559 5678; e-mail sheilamc@nature.berkeley.edu).
Current address: [†]Department of Plant Biology and Forest Genetics, SLU, Box 7080, SE-75007 Uppsala, Sweden.
[‡]Xoma (US) LLC, 2910 7th St., Berkeley, CA 94710, USA.

Summary

As pollen tubes grow through the pistil they are thought to perceive and respond to diverse signals. The tomato pollen-specific receptor kinases LePRK1 and LePRK2 might participate in signaling during pollen tube growth. We previously showed that the extracellular domain of LePRK2 interacts with a pollen protein, LAT52, before but not after pollen germination. To determine whether LePRK2 might have different binding partner(s) after pollen germination, we characterized two more proteins that, like LAT52, were identified in yeast two-hybrid screens using the extracellular domains of LePRK1 and LePRK2 as baits. We show that LeSHY, a leucine-rich repeat protein from pollen, and LeSTIG1, a small cysteine-rich protein from pistil, can bind the extracellular domains of both LePRK1 and LePRK2 *in vitro*. *In vitro* binding assays with the extracellular domain of LePRK2 suggested that LeSTIG1 could displace binding of LAT52, consistent with the idea that LePRK1 and LePRK2 might interact with different ligands at different stages of pollen tube growth. Exogenous LeSTIG1 promotes pollen tube growth *in vitro*. The interaction of these pollen kinases with LeSTIG1 supports the notion that LePRK1 and LePRK2 are involved in mediating pollen–pistil interactions.

Keywords: *in vitro* binding, pollen–pistil interaction, pollen tube growth promotion, signaling, stigma exudate.

Introduction

Plant receptor kinases play an important role in many signal transduction pathways (for review, see Torii, 2000) and are involved in diverse processes of plant development, including pollination (for review, see Tichtinsky *et al.*, 2003). When a mature pollen grain lands on the stigma of the pistil, it hydrates, extends a tube that grows rapidly through the transmitting tract of the style, and eventually reaches an ovule to deliver the sperm. Pollination is essential for the reproduction of flowering plants and is therefore tightly controlled *in vivo*. In tomato, the pollen-specific receptor kinases LePRK1, LePRK2, and LePRK3 are thought to function in pollination (Kim *et al.*, 2002; Muschietti *et al.*, 1998). All these LePRKs have extracellular domains with five or six leucine-rich repeat (LRR) motifs, a transmembrane domain and an intracellular serine/threonine kinase domain. A pollen-specific extracellular protein, LAT52 (Twell *et al.*, 1989), is required for pollen to germinate *in vitro* and to achieve fertilization *in vivo* (Muschietti *et al.*, 1994). LAT52 interacts with the extracellular domain of LePRK2 (ECD2) in pollen (Tang *et al.*, 2002) and this interaction is thought to activate a signaling cascade required to initiate pollen tube growth.

LAT52 binds LePRK2 before but not after pollen germination (Tang *et al.*, 2002). However, because LePRK2 increases expression after pollen germination and localizes to the pollen tube wall, LePRK2 might continue to play a role in signaling after pollen germination by binding additional partners. These additional partners could also be produced by pollen, like LAT52. We expected, however, that the pistil might also produce binding partners for pollen receptor

kinases, because many experiments suggest that pistil components facilitate pollen tube growth and contribute to pollen tube guidance (for review, see Lord and Russell, 2002). In the specific case of signaling mediated by LePRKs, it was intriguing that pollen expressing an antisense-LAT52 construct had a more severe mutant phenotype *in vitro* than *in vivo* (Muschietti *et al.*, 1994), suggesting that some stigma factor(s) could be compensating for the lack of LAT52 during *in vivo* pollen germination. Furthermore, LePRK2 was phosphorylated in pollen membranes and was specifically dephosphorylated when pollen membranes were treated with stigma/style extracts (Muschietti *et al.*, 1998); the stigma/style extracts also dissociated the LePRK1–LePRK2 complex in pollen membranes (Wengier *et al.*, 2003). These results indicate that the LePRKs might interact with factor(s) from stigma and style, either directly or indirectly.

In addition to LAT52, we isolated several other candidate interactors from yeast two-hybrid screens of cDNA libraries prepared from pollen (Tang *et al.*, 2002), and from stigma/style tissue. We show here that two of these proteins, one from the pollen library and one from the stigma/style library, can bind the extracellular domains of both LePRK1 and LePRK2 *in vitro*. These proteins, both predicted to be extracellular, are named LeSHY and LeSTIG1, because of their sequence similarity to previously reported proteins from petunia (Guyon *et al.*, 2000) and tobacco (Goldman *et al.*, 1994). Exogenous LeSTIG1 abolished the interaction of LAT52 and LePRK2 in extracts prepared from mature pollen, suggesting that *in vivo* it might replace LAT52 in binding LePRK2 after pollen germinates on the stigma. In addition, we demonstrate that LeSTIG1 promotes *in vitro* pollen tube growth when added to pollen germination medium.

Results

Yeast two-hybrid screens yield candidate interactors for LePRKs

The pollen tube wall-localized LePRKs are thought to interact with extracellular signaling molecules. We previously isolated several proteins that interact with the extracellular domains of LePRK1, LePRK2 and LePRK3 (ECD1, ECD2 and ECD3) from the pollen library by yeast two-hybrid screens (see Tang *et al.*, 2002, supplemental table), and confirmed by co-immunoprecipitation that one of them, LAT52, interacted with ECD2 before pollen germination (Tang *et al.*, 2002). Another interactor from the pollen library contained a predicted N-terminal signal peptide followed by 10 LRR motifs, which are thought to mediate protein–protein interactions (Kobe and Kajava, 2001). This candidate is similar (69% overall amino acid identity, Figure S1a) to a petunia pollen-specific protein, previously called PGPS/D4 (Guyon *et al.*, 2000), and now called SHY (Loverine Taylor, personal communication). In petunia, *SHY* expression is upregulated in the early stages of pollen tube growth and is induced by flavonols, which are required for petunia pollen germination (Guyon *et al.*, 2000). We named the homologous LRR tomato candidate LeSHY (GenBank accession number AY376852). *LeSHY* was highly expressed in mature pollen and after pollen germination *in vitro*, but no transcripts were detected in the root, leaf or seedling (Figure S1b).

As binding partners for LePRKs might reasonably be present in the extracellular matrix of stigma and style, we also screened a tomato stigma/style cDNA library, using ECD1, ECD2 or ECD3 as baits. From each individual screen, we obtained about a dozen positive cDNA clones encoding candidate interacting proteins that contained a predicted N-terminal signal peptide. Some of the candidates were obtained from a screen with only one of the three baits, while others were obtained from both the ECD1 and ECD2 screens or from both the ECD1 and ECD3 screens.

The candidate interactors from stigma/style include several small cysteine-rich proteins (molecular mass ranging from 8 to 16 kDa), which are different from the small cysteine-rich proteins obtained from the pollen library screen (including LAT52). One of the stigma/style candidates (now termed LeSTIG1, GenBank accession number AY376851) is similar to a tobacco stigma-specific protein, STIG1 (Goldman *et al.*, 1994) (Figure S2a). These two proteins show 72% amino acid identity overall, and even higher similarity in the C-terminal cysteine-rich region (all 16 cysteine residues are conserved). STIG1 and LeSTIG1 are probably secreted proteins, not only because they have predicted N-terminal signal sequences and no predicted retention signal sequences, but also because *STIG1* is specifically expressed in the stigmatic secretory zone, which is thought to secrete many compounds that are required for penetration of the pistil and pollen tube growth (Goldman *et al.*, 1994). The *LeSTIG1* expression pattern is similar to that of *STIG1* (Figure S2b); the *LeSTIG1* transcript was not detected in any of the vegetative tissues examined (leaf, root, and seedling), or in pollen. In the pistil, the *LeSTIG1* expression was strong in the stigma, weak in the style, and not detectable in the ovary. We obtained *LeSTIG1* twice from both the ECD1 and ECD2 screens, but not from the screen with ECD3, although the ECD3 screen yielded several other candidate interactors. Retransformation of *LeSTIG1* into yeast cells harboring bait plasmids confirmed that LeSTIG1 specifically interacted with ECD1 and ECD2, but not with ECD3 (data not shown).

Binding specificity and competition among candidate interactors

To test the binding specificity of these candidate interactors with the LePRKs, we performed *in vitro* binding assays using GST-fusion proteins and His-tagged ECDs. Figure 1 shows

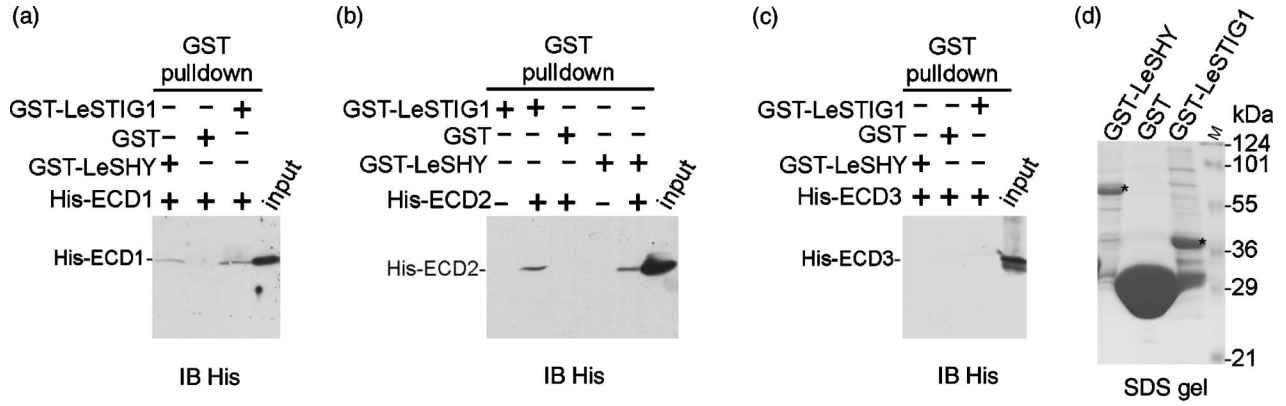

Figure 1. *In vitro* binding assays.
(a–c) *In vitro* binding assays with His-ECD1 (a), His-ECD2 (b) and His-ECD3 (c). GST-LeSTIG1 (~120 pmol), GST-LeSHY (~80 pmol), or GST (~300 pmol) was incubated with the indicated His-fusion protein (~100 pmol). Proteins bound to glutathione-sepharose were separated by SDS-PAGE and detected by anti-His antibodies. One-fifth of the corresponding His-fusion protein used in each assay was loaded onto the right lane of each gel as an input control. +, present; −, absent.
(d) SDS-PAGE analysis of GST-fusion proteins used in these assays. M: molecular mass marker. *Target GST-fusion protein with expected size.

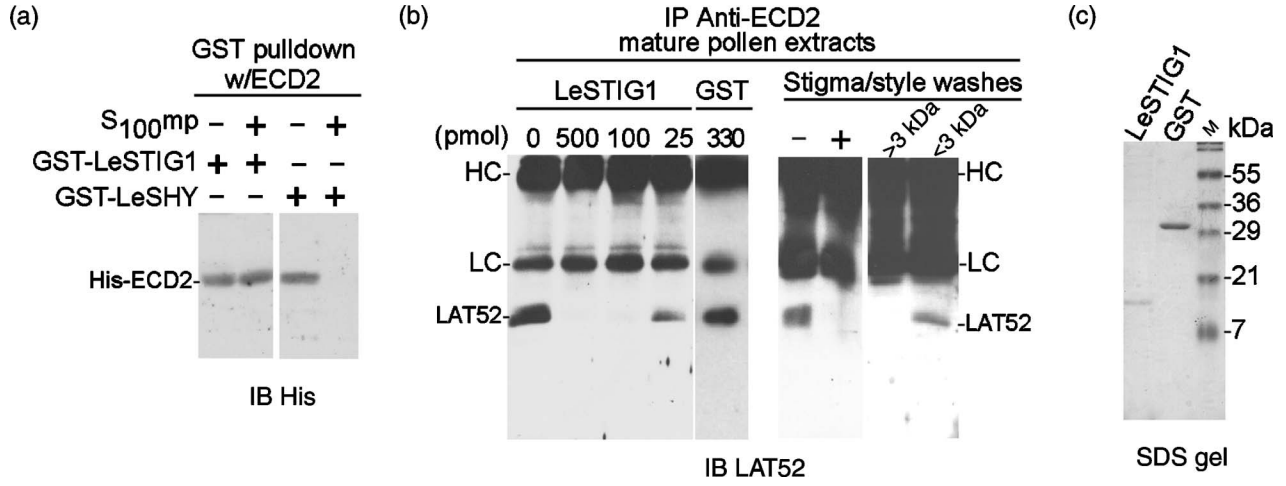

Figure 2. Competition among candidate ligands assessed by *in vitro* binding assays.
(a) The effect of LAT52 addition on *in vitro* binding of GST-LeSTIG1 or GST-LeSHY to His-ECD2. Left gel: GST-LeSTIG1 (~120 pmol) and His-ECD2 (~100 pmol) were incubated with or without S_{100}mp (estimated to contain ~100–250 pmol native LAT52). Right gel: GST-LeSHY (~80 pmol) and His-ECD2 (~120 pmol) were incubated with or without S_{100}mp. The sepharose-bound proteins in each lane were analyzed by immunoblotting with anti-His antibody to detect His-ECD2.
(b) The effect of LeSTIG1 protein and stigma/style washes on the LAT52–LePRK2 interaction. Left gel: Proteins (1 mg) extracted from mature pollen (estimated to contain ~100–250 pmol native LAT52) were incubated with LeSTIG1 proteins (purified from *E. coli* extracts, GST portion removed) or GST proteins. Samples were immunoprecipitated with anti-ECD2 antibody. The precipitated proteins were analyzed by immunoblotting with anti-LAT52 antibody. HC, LC: heavy chain and light chain of antibody. Right gel: Protein extracted from mature pollen (1 mg) incubated with or without stigma/style washes. Two fractions (40 μl each) of stigma/style washes were used. >3 kDa: greater than 3 kDa (protein concentration 1 μg μl^{-1}); <3 kDa: smaller than 3 kDa.
(c) SDS-PAGE analysis of the LeSTIG1 and GST proteins used in (b). M: molecular mass marker.

that both GST-LeSTIG1 and GST-LeSHY bound to His-ECD1 (Figure 1a) and His-ECD2 (Figure 1b), but not to His-ECD3 (Figure 1c). Figure 1(d) shows the partially purified GST-fusion protein used in the binding assays. Although the amount of GST protein used in the binding assays was at least equivalent to the amounts of GST-LeSTIG1 and GST-LeSHY proteins used in the same assay (Figure 1d), GST alone did not bind His-ECD1 or His-ECD2 (Figure 1a,b), and GST-LeSTIG1 or GST-LeSHY are not recognized by anti-His antibody (Figure 1b). These results show that LeSTIG1 and LeSHY interact specifically with ECD1 and ECD2.

We have confirmed three binding partners for ECD2: LAT52 (Tang *et al.*, 2002), LeSTIG1, and LeSHY. Multiple binding partners might bind to a receptor synergistically, competitively or independently. To determine the binding relationships for LAT52, LeSTIG1, and LeSHY, we tested the effect of LAT52 on the *in vitro* interactions of LeSTIG1 with ECD2 and of LeSHY with ECD2. Native LAT52 protein is enriched in the soluble protein fraction of mature pollen extracts (S_{100}mp) (Tang *et al.*, 2002), and thus we added S_{100}mp into the *in vitro* binding assays. Figure 2(a) shows that the addition of S_{100}mp did not affect the binding of

GST-LeSTIG1 to His-ECD2, but abolished the interaction between GST-LeSHY and His-ECD2. These results indicate that LAT52 or other soluble components in mature pollen cannot compete with LeSTIG1 for binding to ECD2, but might displace LeSHY for binding to ECD2.

We also tested the effect of LeSTIG1 on the interaction between LAT52 and LePRK2. In mature pollen extracts, LAT52 can be precipitated with antibodies that specifically recognize ECD2 (Tang *et al.*, 2002 and Figure 2b). *Escherichia coli*-expressed LeSTIG1 was purified (Figure 2c) and incubated with mature pollen extracts before immunoprecipitation with anti-ECD2. Figure 2(b) (left gel) shows that the addition of 25 pmol LeSTIG1 reduced the amount of LAT52 that could be precipitated, and that the addition of 100 pmol or more of LeSTIG1 completely abolished the precipitation of LAT52 with anti-ECD2 antibody. These results suggest that LeSTIG1 can outcompete LAT52 for binding to ECD2. As a control, *E. coli*-expressed GST was also purified (Figure 2c) and incubated with mature pollen extracts, and it did not affect co-immunoprecipitation of LAT52 with LePRK2 (Figure 2b). We did not use *E. coli*-expressed LAT52 as a control, because GST-LAT52 alone was not able to bind ECD2 *in vitro* (Tang *et al.*, 2002 and data not shown). LeSTIG1 can outcompete LAT52 in binding with LePRK2, but LAT52 cannot outcompete LeSTIG1, suggesting that LeSTIG1 has a higher binding affinity than does LAT52.

Tobacco STIG1 is thought to be present in the exudate of stigma and style (Goldman *et al.*, 1994). As LeSTIG1 also has a predicted N-terminal signal peptide and no predicted retention sequence, and *LeSTIG1* expression is restricted to the stigma and style, we presume that, like STIG1, LeSTIG1 is present in the exudate of stigma and style. If the *in vitro* competition results (Figure 2b, left gel) reflect the situation *in vivo*, then the exudate of stigma and style might also dissociate the LePRK2-LAT52 complex. To test this, a stigma/style exudate was prepared and incubated with mature pollen extracts before immunoprecipitation. Figure 2(b) (right gel) shows that LAT52 was not precipitated with anti-ECD2 antibody when the stigma/style exudate was added. We previously reported that a metal ion (Mg^{2+} and/or Ca^{2+}) is required for the LAT52–LePRK2 interaction (Tang *et al.*, 2002). To exclude the possibility that a change in ion concentration (as a side effect of the addition of stigma/style exudate) was responsible, the stigma/style exudate was size-fractionated using centrifugal filters with a 3 kDa cutoff. Figure 2(b) (right gel) shows that the addition of the retained fraction (>3 kDa) abolished the LAT52–LePRK2 interaction, but the addition of the filtrate (<3 kDa) did not affect the LAT52–LePRK2 interaction. These results show that the dissociation of the LAT52–LePRK2 complex was not caused by a change in ion concentration.

We did not similarly test LePRK1 competition for two reasons. First, only a small amount of LAT52 was co-immunoprecipitated with anti-ECD1 (Tang *et al.*, 2002). As LePRK1 and LePRK2 can form a complex in pollen (Wengier *et al.*, 2003), we cannot conclude whether LAT52 directly interacts with LePRK1, or whether it is co-immunoprecipitated as a result of its association with LePRK2 (Tang *et al.*, 2002, Figure 2b). Secondly, we do not have *in vivo* evidence for the LePRK1 and LeSTIG1 or LeSHY interaction.

Exogenous LeSTIG1 promotes pollen tube growth in vitro

Ligands are thought to elicit specific cellular responses upon binding to receptors. As LeSTIG1 and LeSHY are ligand candidates for the pollen receptors, we wanted to test whether LeSTIG1 and LeSHY have any effects on pollen tube growth. We slightly modified an *in vitro* pollen tube growth assay (Lush *et al.*, 1997) that had been used to assess the effects of sucrose, RNase A (Lush *et al.*, 1997), and a galactose-rich style glycoprotein (Sommer-Knudsen *et al.*, 1998) on the growth of *Nicotiana alata* pollen tubes. Clusters of tomato pollen grains were placed on microscope slides and covered with gelled medium that had been supplemented with GST-LeSTIG1, GST-LeSHY, GST, or with no supplemental protein. The clusters were then cultured at 26°C. We began to see pollen tubes growing outward from the clusters after 30 min in all the tested media (data not shown), but after further incubation the tube lengths differed in the different media. The pollen tubes extending from individual clusters were photographed after approximately 20 h of culture. Figure 3(a) shows representative photographs of the results: one each for the no protein and 100 nM GST controls, and two replicates each for the 100 nM GST-LeSTIG1 and 100 nM GST-LeSHY tests. The pollen tubes (the thin curved lines extending from the center cluster of pollen grains) cultured in 100 nM GST-LeSTIG1 (the center panel) were generally longer than those from other tested conditions (left and right panels).

To quantify pollen tube growth, tube growth from each cluster was measured in two ways. One measure, referred to as maximum radius, represented the longest pollen tube from each cluster. The second measure, the average length of the 20 longest tubes from each cluster, was designed to correct for the possibility of one unusually long pollen tube affecting the first measure. As there is a population effect on pollen tube growth (Brewbaker and Majumder, 1961; Chen *et al.*, 2000), a large cluster of pollen grains can produce longer pollen tubes than can form a small cluster (Lush *et al.*, 1997). We therefore measured the average diameters of all the clusters, and selected clusters whose average diameters were within the range of 0.08–0.28 mm for quantification.

Figure 3(b) shows that both measurements of pollen tube length (the maximum radius in left graph and the average tube length in center graph) were two- to threefold greater with 100 nM GST-LeSTIG1 than those with no protein or with GST at the same concentration. *T*-tests (Table 1) show that

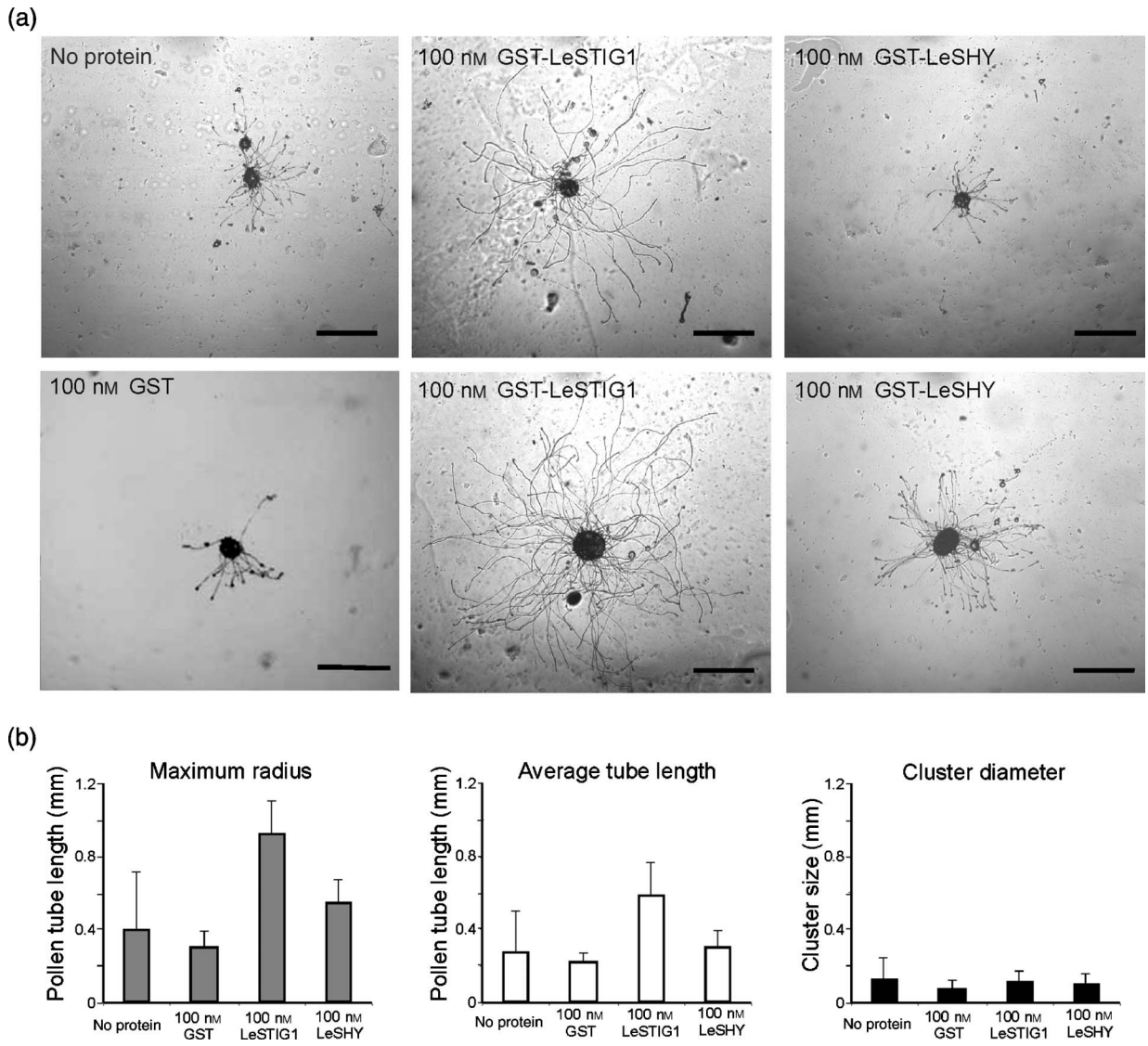

Figure 3. *In vitro* pollen tube growth assays (experiment I).
Numerous clusters of tomato pollen grains were separately cultured under gelled pollen germination medium supplemented with GST, GST-LeSTIG1, or GST-LeSHY, or with no protein. Photographs were taken after 20 h culturing.
(a) Representative photographs from experiment I. Pollen tubes (the thin curved lines in the photographs) grew outward from the cluster of pollen grains. The protein added to the pollen germination medium is noted. Scale bars are 0.5 mm.
(b) Quantitative analyses of the pollen tube growth assay, experiment I. Average measurements from at least eight replicates for each treatment are presented on three separate graphs. The left and center graphs show two measures of pollen tube lengths from the same grain cluster. The left graph (gray bars) represents maximum radius from cluster. The center graph (white bars) represents the average length of the longest 20 tubes from the cluster. The right graph (black bars) represents the average diameters of the grain clusters. Error bars represent the standard deviation.

Table 1 *T*-test results for pollen tube lengths and cluster size measurements in Figure 3b

Probability	100 nM LeSTIG1 versus 100 nM GST	100 nM LeSTIG1 versus no protein	100 nM LeSHY versus 100 nM GST	100 nM LeSHY versus no protein
Maximum radius	0.0000032[a]	0.00044[a]	0.029	0.48
Average tube length	0.00058[a]	0.0080[a]	0.014	0.84
Cluster diameter	0.30	0.91	0.37	0.87

[a]The difference is significant, $P < 0.01$.

the differences in pollen tube lengths between 100 nM GST-LeSTIG1 and 100 nM GST and between 100 nM GST-LeSTIG1 and no protein were significant at the probability level of 0.01. Figure 3(b) (right graph) and the *t*-test results (Table 1) show that the average diameter of the clusters with 100 nM GST-LeSTIG1 was not significantly larger than for those with no protein or 100 nM GST. Thus, we believe that the difference in the length of pollen tubes between different treatments was not caused by a population effect.

Figure 3(b) also shows that both measurements of pollen tube length were slightly greater (1.1–1.7-fold) with 100 nM GST-LeSHY than those with no protein or with 100 nM GST, but the *t*-test results (Table 1) indicate that these differences in pollen tube length were not significant at the probability level of 0.01. Altogether, the results from the pollen tube growth assays performed on that experimental day (referred to as experiment I) indicate that exogenous GST-LeSTIG1, but not GST or GST-LeSHY, significantly increased pollen tube lengths within 20 h of *in vitro* culturing.

As the growth of pollen tubes can vary from day to day, even when the *in vitro* germination conditions are very well controlled, we repeated the growth assay. Figure 4 shows quantitative analyses of the results of experiment II (a) and experiment III (b). The average diameters of all the clusters were within the range of 0.2–0.4 mm in experiment II (Figure 4a, right), and within the range of 0.11–0.31 mm in experiment III (Figure 4b, right). Although the absolute values of pollen tube length in the same treatment were different among the three experiments, both measurements of pollen tube length (left and center graphs) in the medium with 100 nM GST-LeSTIG1 were greater than those in the medium with 100 nM GST or with no protein (Figure 4, left and center graphs). Therefore, we conclude that 100 nM GST-LeSTIG1 can reproducibly promote pollen tube growth *in vitro*.

Figure 4(a) also shows, in experiment II, with similarly sized clusters, that the pollen tube lengths in the medium with 100 nM GST-LeSHY were slightly less (0.7–0.8-fold) than with 100 nM GST, but this difference was not significant. Considering the results from experiment I (Figure 3b), we conclude that the addition of GST-LeSHY at the concentration of 100 nM does not cause any significant difference in pollen tube length. To test whether a higher or lower concentration of GST-LeSHY had an effect, we tested 2 mM (Figure 3b), 50 and 25 nM (data not shown), but no difference in the lengths of pollen tubes was observed between

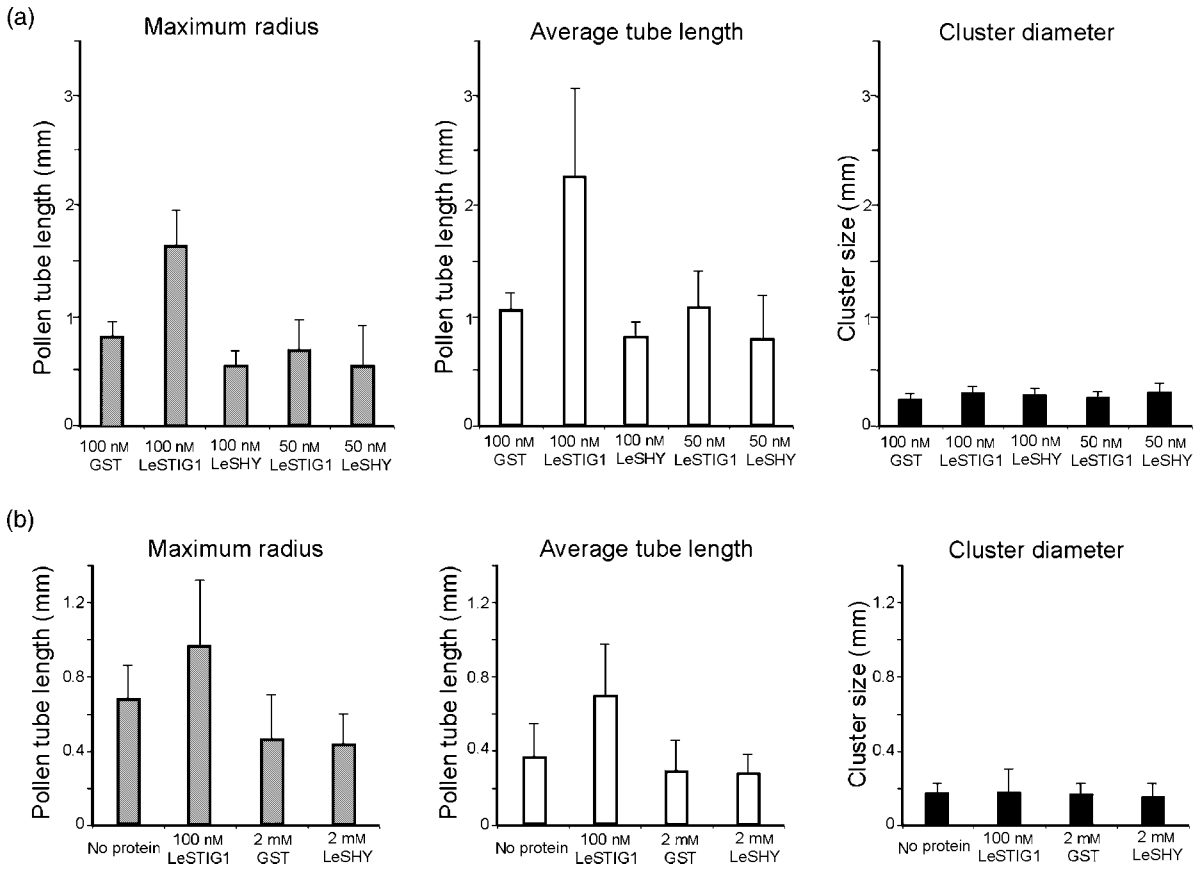

Figure 4. *In vitro* pollen tube growth assays (experiments II and III).
(a, b) Quantitative analyses of the results of experiments II and III, respectively. The measurements were plotted as in Figure 3(b).

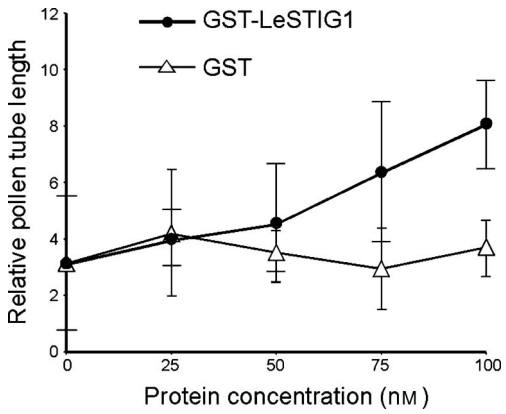

Figure 5. Concentration dependence of GST-LeSTIG1 protein on pollen tube growth *in vitro* (experiment I).
Pollen germination medium supplemented with different concentrations of GST-LeSTIG1 or GST were tested for their effects on *in vitro* pollen tube growth. The relative pollen tube lengths represent the average pollen tube length divided by the average cluster diameter, to correct for population effect. Results are plotted as relative pollen tube length, with standard errors at each concentration of supplemental protein.

GST-LeSHY and GST at the same concentration. We thus conclude that exogenous GST-LeSHY within the range of 25–2 mM does not significantly affect pollen tube length in our system. However, we cannot exclude that LeSHY has an effect on pollen tube growth; because LeSHY is produced by pollen, the endogenous supply might have saturated any response.

In contrast to GST-LeSHY, exogenous GST-LeSTIG1 can enhance pollen tube growth *in vitro*. If the pollen tube growth enhancement is specifically caused by GST-LeSTIG1, we should expect dilution to reduce and eventually abolish the response. Thus we prepared pollen germination medium supplemented with lower concentrations of GST-LeSTIG1 and GST, and performed the growth assays simultaneously with experiment I. As the length of pollen tubes increased with the cluster diameter (as mentioned earlier), we divided the average pollen tube length by the cluster diameter, referred to as the relative pollen tube length, then plotted the data. Figure 5 shows that the difference in pollen tube lengths was reduced when the concentration of supplemental protein was 75 nM, further reduced when LeSTIG1 was 50 nM, and completely abolished if diluted to 25 nM. This result indicates that the effective concentration of GST-LeSTIG1 for promoting pollen tube growth is around 100 nM (4 μg ml^{-1}), comparable with the effective concentration, 2 μg ml^{-1}, for a tobacco style transmitting tissue-specific protein called TTS, which caused a twofold increase in the growth rate of tobacco pollen tubes (Wu *et al.*, 2000). LeSTIG1 contains a cysteine-rich region, but not all cysteine-rich proteins have a promotive effect on pollen tube growth, because GST-LAT52 did not increase pollen tube growth in our assays (data not shown).

Some pollen tube growth factors such as TTS (Cheung *et al.*, 1995) also function as directional cues for pollen tube growth *in vitro*, although others, such as GABA (Palanivelu *et al.*, 2003) appear not to. We tested whether LeSTIG1 could attract pollen tubes by providing a directional source of GST-LeSTIG1. In two independent experiments in which we tested source concentrations of up to 2 mM, the pollen tubes did not turn toward or away from the GST-LeSTIG1 source (data not shown). Thus LeSTIG1 probably does not guide pollen tube growth but rather plays a role in positive regulation of growth.

Discussion

We previously showed that LAT52 interacts with the extracellular domain of the pollen receptor LePRK2 (Tang *et al.*, 2002). Here, we characterized two more extracellular binding partners for LePRK1 and LePRK2, LeSTIG1 and LeSHY. Their interactions with the extracellular domains of LePRK1 and LePRK2 were confirmed by *in vitro* binding assays. However, in the binding assays, neither LeSTIG nor LeSHY interacted with the extracellular domain of LePRK3, although it is also composed of LRRs (Kim *et al.*, 2002). These results indicate that LeSTIG1 and LeSHY interact with LePRK1 and LePRK2 specifically and do not bind indiscriminately to proteins with LRRs.

The interaction of LePRK2 with LAT52, a pollen protein, indicated a role for the pollen receptor LePRK2 in autocrine signaling (Tang *et al.*, 2002). As the expression of LeSTIG1 is restricted to the stigma and style, the interactions with LeSTIG1 indicate a different signaling role for LePRK1 and LePRK2: mediating pollen–pistil interactions. When we originally studied LePRK1 and LePRK2, we predicted that at least LePRK2 would play a role in pollen–pistil interaction, based on the observation that the style extract caused a specific dephosphorylation of LePRK2 (Muschietti *et al.*, 1998). Later, we obtained convincing evidence that LePRK1 and LePRK2 were involved in pollen–pistil interactions, because a 3–10 kDa fraction of stigma/style extracts (called SE for convenience) could dissociate the LePRK1–LePRK2 complex and dephosphorylate LePRK2 in pollen membranes (Wengier *et al.*, 2003). Although it is still not clear whether the active component of SE is LeSTIG1 or another yet uncharacterized binding partner for LePRK2, the identification of LeSTIG1 as a long sought-after pistil partner for LePRK1 and LePRK2 provides convincing evidence that these two pollen receptors directly interact with pistil components.

We have characterized three structurally unrelated proteins as extracellular binding partners for the pollen receptors. Could all three of these proteins be ligands? LeSHY is an LRR protein. Although both LAT52 and LeSTIG1 harbor C-terminal cysteine-rich regions, the pattern of cysteine positions is not conserved. In animal cells, different types of

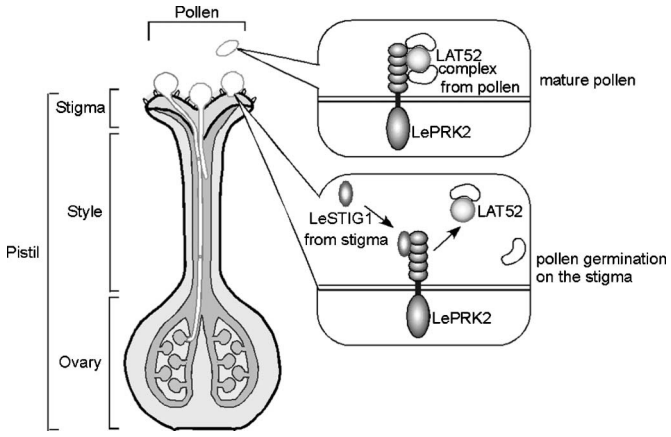

Figure 6. Model for LePRK2 signaling during pollen germination and tube growth.
In mature pollen membranes, a large protein complex including LAT52 interacts with the extracellular domain of LePRK2 (top). When a pollen lands on the stigma, a stigma protein, LeSTIG1 replaces LAT52 and binds to LePRK2 (bottom).

ligands have been reported to bind the epidermal growth factor receptor (EGFR) and initiate different signaling pathways. These ligands include the epidermal growth factor (EGF) and several EGF-like growth factors (for review, see Carpenter, 1987), an envelope glycoprotein of human cytomegalovirus called gB (Wang *et al.*, 2003), and decorin, an extracellular matrix proteoglycan, whose protein core is composed of 10 LRR motifs (Iozzo *et al.*, 1999; Santra *et al.*, 2002). Competition between EGF and gB (Wang *et al.*, 2003) and between EGF and decorin (Iozzo *et al.*, 1999) have been reported. In plants, one LRR receptor kinase, tomato BRI1/SR160, is thought to have two completely different ligands: a peptide hormone, systemin, and a steroid hormone, BR, and therefore this receptor kinase is thought to be involved in both defensive and developmental signaling (for review, see Wang and He, 2004). In the case of the LePRKs, one of the extracellular binding partners, LeSHY, is an LRR protein from pollen. This finding is in some way reminiscent of CLAVATA2, which is an LRR protein that interacts as a co-receptor with the LRR receptor kinase CLAVATA1 to regulate *Arabidopsis* meristem size (Jeong *et al.*, 1999). However, unlike CLAVATA2, LeSHY has no transmembrane domain. LeSHY also shares some structural similarity with decorin, which acts as an antagonistic ligand of the EGFR tyrosine kinase (Santra *et al.*, 2002). It will be interesting to determine whether LeSHY causes a response contrary to those of LAT52 or LeSTIG1 during pollen tube growth. For the other two extracellular binding partners, we previously reported LAT52 as a candidate ligand from pollen (Tang *et al.*, 2002), and here we propose that LeSTIG1 is a candidate ligand from stigma/style. Our results indicate that a plant receptor kinase might specifically interact with multiple ligands.

We hypothesize that LePRK2 has different signaling partners during the process of pollen tube growth (Figure 6). This idea is biologically reasonable because pollen tubes grow through different tissues of the pistil before reaching the embryo sac, and they need to respond to multiple signals along the way. Our competition binding assays were designed to mimic the *in vivo* biological process of pollen germination and tube growth. Before landing on the stigma, a complex composed of LePRK2 and LAT52 is present on the plasma membrane of mature pollen (Tang *et al.*, 2002). When a mature pollen lands on the stigma, it contacts the stigma exudate, and the LAT52–LePRK2 complex would then meet LeSTIG1. We propose that on the stigma, LePRK2 switches partners from LAT52 to LeSTIG1. Consistent with these ideas, adding LeSTIG1 or stigma/style washes to the mature pollen extracts abolished the interaction of LAT52 with LePRK2 (Figure 2b), and native LAT52 in mature pollen extracts did not outcompete LeSTIG1 (Figure 2a). As pollen tubes extend by tip growth, LePRKs would not be able to carry LeSTIG1 with them through the entire journey. Eventually, when the tip of the pollen tube grows into the ovary, where LeSTIG1 is not present, the LePRK2 receptor complex might change binding partners again. We do not yet know at which stage(s) the pollen partner LeSHY might bind to LePRKs *in vivo*. The only clue is that the *LeSHY* transcript is present in both mature and germinated pollen.

The canonical concept of a ligand includes a specific interaction with a receptor and the elicitation of a cellular response. Several extracellular molecules of a diverse nature have been shown to promote or guide pollen tube growth in particular plant species, including lipids (Lush *et al.*, 1998; Wolters-Arts *et al.*, 1998) and an arabinogalactan protein TTS (Cheung *et al.*, 1995; Wu *et al.*, 1995, 2000) in tobacco, a small cysteine-rich adhesion protein SCA and a small basic protein Chemocyanin (Kim *et al.*, 2003; Park *et al.*, 2000) in lily, and γ-amino butyric acid (GABA) in *Arabidopsis* (Palanivelu *et al.*, 2003). Receptors involved in mediating these effects have not yet been identified. However, it is intriguing that a 5-amino acid peptide growth factor, phytosulfokine-α (PSK), mediates the pollen population effect in tobacco (Chen *et al.*, 2000), because an LRR receptor kinase binds PSK in carrot callus cells (Matsubayashi *et al.*, 2002). Whether a similar receptor–ligand interaction can explain PSK-mediated enhancement of pollen tube growth (Chen *et al.*, 2000) is untested. Here, we showed that LeSTIG1 can bind the extracellular domains of LePRK1 and

LePRK2, and that LeSTIG1 can promote pollen tube growth *in vitro*. This data is consistent with the idea that LeSTIG1 is a ligand that acts through LePRK2 and/or LePRK1 to regulate pollen tube growth. Goldman *et al.* (1994) reported that stigma exudates promote pollen tube growth in pistils. Although we still do not know which factor in the stigma exudate is responsible for the promotion effect, our results suggest that LeSTIG1 could be a candidate.

Experimental procedure

Plant growth and tissue preparation

Tomato (*Lycopersicon esculentum* cv. VF36) plants were grown under standard greenhouse conditions. Mature pollen and *in vitro*-germinated pollen were obtained as described (Tang *et al.*, 2002). Tomato pistils were harvested from mature flowers, separated into component parts: stigma, style and ovary, and stored at −80°C.

Yeast two-hybrid screens and cDNA insert analysis

Yeast two-hybrid screens were performed as described (Tang *et al.*, 2002). The stigma/style prey library was prepared from mRNA extracted from the stigma/style of tomato pistils. The cDNA inserts in the positive colonies were sequenced. Database searches were conducted with the BLAST program at the Arabidopsis Information Resource (http://www.arabidopsis.org), at the National Center for Biotechnology Information (http://www.ncbi.nih.gov) and at the Institute for Genomic Research (http://www.tigr.org). Cellular locations were predicted using PSORT (http://psort.nibb.ac.jp) and SignalP (http://www.cbs.dtu.uk/services/signalP). Homologous proteins were aligned using the ClustalX 1.81 program developed by the National Center for Biotechnology Information.

RNA isolation and RT-PCR

Total RNA was extracted from tomato pollen, pistil (separated into stigma, style, and ovary), root, seedling, and leaf as described (Logemann *et al.*, 1987). Random-primed cDNA was made from 1 μg of total RNA, using Superscript II Reverse Transcriptase (Invitrogen). Gene-specific primers (Operon) are:

actin-F (5′-TTTCCTAGCATTGTTGGTC-3′),
actin-R (5′-GTGACTCACACCATCACCAG-3′);
LeSHY-F (5′-AATATTAGTGCAAACAATGCA-3′),
LeSHY-R (5′-TCACTGTTTTTGTACTCCCAT-3′);
LeSTIG1-F (5′CTCTTTCTAGCACACCAATTAC-3′),
LeSTIG1-R (5′-GATGCTTTTTATCCACTGAGATG-3′).

All PCR amplifications used these conditions: 94°C for 3 min, followed by 25 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec, and a final extension for 6 min at 72°C.

Fusion protein purification and in vitro *binding assays*

The extracellular domains of LePRK1, LePRK2, and LePRK3 (termed ECD1, ECD2, and ECD3) were fused with a 6-histidine (His) tag and expressed as described by Muschietti *et al.* (1998) and Kim *et al.* (2002). His-fusion proteins were further purified under native conditions as described by Tang *et al.* (2002). LeSTIG1, LeSHY, and LAT52 (with the signal peptides removed) were fused with GST in the pGEX-4T3 vector. The resulting constructs were transformed into *E. coli* strain BL21(DE3) (Stratagene, La Jolla, CA, USA), and fusion protein production was induced with 0.8 mM isopropyl-β-D-thiogalactoside (IPTG). The GST-fusion proteins were purified by Glutathione Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ, USA) following the manufacturer's protocol. In the experiment shown in Figure 2(b), the GST was removed from GST-LeSTIG1 by thrombin treatment on a Glutathione Sepharose 4B column (Amersham Pharmacia Biotech). Purified protein samples were dialyzed against a PGM buffer (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) at 4°C, and then quickly frozen in liquid nitrogen and stored at −80°C. For *in vitro* binding assays, GST (∼300 pmol), GST-LeSTIG1 (∼120 pmol) or GST-LeSHY (∼80 pmol) protein was purified as above except for the last elution step. While the proteins were still bound on 20 μl Glutathione Sepharose 4B, ∼100 pmol His-fusion protein was added, and incubated with 500 μl co-ip buffer (50 mM Tris–Cl, pH7.6, 100 mM NaCl, 0.5% Nonidet P-40) for 2 h at 4°C with constant rotation. The beads were washed three times with 1 ml wash buffer (50 mM Tris–Cl, pH 7.6, 100 mM NaCl, 0.2% Nonidet P-40) and then eluted twice with 20 μl of glutathione elution buffer. The supernatants were pooled, then analyzed by 15% SDS-PAGE followed by immunoblotting, using a primary antibody anti-RGSHis (Qiagen, Valencia, CA, USA) and a sheep anti-mouse secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). The proteins were detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Preparation of stigma/style washes and co-immunoprecipitation assays

Stigma/style pieces were separated from the ovaries of 60 tomato pistils by cutting with a razor blade, then incubated in 1 ml 100 mM NaCl solution for 4 h at 4°C with constant rotation (10 rpm). The tissue was pelleted and the supernatant (stigma/style wash) was considered to contain the exudate of stigma and style. The stigma/style washes were further partitioned using an Amicon YM3 centrifugal filter device (cutoff molecular mass of 3 kDa; Millipore, Billerica, MA, USA). The retentate contained molecules larger than 3 kDa, and the flow-through contained molecules less than 3 kDa. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Pollen protein extractions using PGM buffer and co-immunoprecipitation assays were performed as described (Tang *et al.*, 2002) except that stigma/style washes were added where indicated.

Pollen tube growth assays in gelled medium

In vitro assays for assessing the effects of LeSTIG1 and LeSHY on tomato pollen tube growth were performed as described by Lush *et al.* (1997) with minor modifications. The culture medium (25 mM Mes pH 6.0, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.8 mM $MgSO_4$, 1.6 mM boric acid, 183 mM sucrose, 4% PEG 4000, 0.8% low-melting agarose) was melted and maintained at 37°C. Supplemental proteins were added and thoroughly mixed before using the medium. Freshly collected tomato pollen was mixed with mineral oil (∼20 μl pollen per 20 μl oil) to make a paste, and was placed, using a needle, in clusters in the middle of a glass slide. The pollen clusters were immediately covered by a thin layer of the culture medium (∼20 μl per cluster), and the slides were placed in a humid chamber at 26°C. The clusters with extending pollen tubes were photographed after 20 h. Pollen tube lengths were measured using NIH image software (v1.62). Two types of measurements were used to assess growth. The shortest distance from the center of the cluster to the furthermost part of the

most distal pollen tube was measured. This distance was called 'maximum radius'. For the second measurement, individual pollen tubes were traced and the lengths of the 20 longest tubes from each cluster were recorded. The average was calculated and called 'average tube length'. The diameter of each cluster of pollen grains was also measured and designated cluster size. For each treatment and in each experiment, 12 replicates were prepared; the measurements of pollen tube growth for an average of eight clusters (those whose diameter fell in the range of the average ± 1 mm) were plotted. Two-tailed *t*-tests were performed using Excel (Microsoft). If by chance additional small clusters of pollen grains were placed close to the main cluster, only pollen tubes extending from the main cluster were measured.

Acknowledgements

We thank Michele Engel, Rachel Holmes-Davis, Sheila Johnson-Brousseau, Pulla Kaothien, Heping Yang, and especially Jennifer Fletcher and Jorge Muschietti for insightful comments during the course of this work. We thank Vivian Ng, Lilian Chiang, Lauren Bartlow, and Kevin Ramirez, participants in the UC Berkeley Undergraduate Research Apprentice Program, for technical assistance. This work was supported by a CRADA from Syngenta and by the US Department of Agriculture CRIS number 5335-21000-020-00D.

Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2139/TPJ2139sm

Figure S1. LeSHY sequence and expression analysis.
(a) Alignment of deduced SHY protein sequences from tomato (LeSHY, GenBank accession number AY376852) and petunia (PhSHY, GenBank accession number AF049920). The predicted cleavage site of the N-terminal signal sequence is designated with an arrow. The first seven residues of each LRR are underlined and numbered. Identical residues are boxed in black. Similar residues are shaded in gray.
(b) Expression of *LeSHY*, as assessed by RT-PCR. MP: mature pollen, GP: *in vitro* germinated pollen, L: leaf, R: root, S: seedling, Sm: stigma, Sy: style, O: ovary. Actin was used as a control. Note: The small amount of *LeSHY* transcript detected in pistil tissues (stigma, style, and ovary) might be the result of pollen contamination, because the pistils were not collected from emasculated flowers.
Figure S2. LeSTIG1 sequence and expression analysis.
(a) Alignment of deduced STIG1 protein sequences from tomato (LeSTIG1, GenBank accession number AY376851), tobacco (NtSTIG1, GenBank accession number X77823), and petunia (PhSTIG1, GenBank accession number AF130352). The predicted cleavage site of the N-terminal signal sequence is designated with an arrow. The conserved cysteine residues are marked below with a dot. Identical residues are boxed in black. Similar residues are shaded in gray.
(b) Expression of *LeSTIG1*, as assessed by RT-PCR. Sm: stigma, Sy: style, O: ovary, Po: pollen, L: leaf, R: root, S: seedling. Actin was used as a control.

References

Brewbaker, J.L. and Majumder, S.K. (1961) Cultural studies of the pollen population effect and the self-incompatibility inhibition. *Am. J. Bot.* **8**, 457–464.

Carpenter, G. (1987) Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem.* **56**, 881–914.

Chen, Y.F., Matsubayashi, Y. and Sakagami, Y. (2000) Peptide growth factor phytosulfokine-alpha contributes to the pollen population effect. *Planta*, **211**, 752–755.

Cheung, A.Y., Wang, H. and Wu, H.M. (1995) A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell*, **82**, 383–393.

Goldman, M.H., Goldberg, R.B. and Mariani, C. (1994) Female sterile tobacco plants are produced by stigma-specific cell ablation. *EMBO J.* **13**, 2976–2984.

Guyon, V.N., Astwood, J.D., Garner, E.C., Dunker, A.K. and Taylor, L.P. (2000) Isolation and characterization of cDNAs expressed in the early stages of flavonol-induced pollen germination in petunia. *Plant Physiol.* **123**, 699–710.

Iozzo, R.V., Moscatello, D.K., McQuillan, D.J. and Eichstetter, I. (1999) Decorin is a biological ligand for the epidermal growth factor receptor. *J. Biol. Chem.* **274**, 4489–4492.

Jeong, S., Trotochaud, A.E. and Clark, S.E. (1999) The *Arabidopsis CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell*, **11**, 1925–1933.

Kim, H.U., Cotter, R., Johnson, S., Senda, M., Dodds, P., Kulikauska, R., Tang, W., Ezcurra, I., Herzmark, P. and 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.

Kim, S., Mollet, J.C., Dong, J., Zhang, K., Park, S.Y. and Lord, E.M. (2003) Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. *Proc. Natl Acad. Sci. USA*, **100**, 16125–16130.

Kobe, B. and Kajava, A.V. (2001) The leucine-rich repeat as a protein recognition motif. *Curr. Opin. Struct. Biol.* **11**, 725–732.

Logemann, J., Schell, J. and Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16–20.

Lord, E.M. and Russell, S.D. (2002) The mechanisms of pollination and fertilization in plants. *Annu. Rev. Cell Dev. Biol.* **18**, 81–105.

Lush, W.M., Opat, A.S., Nie, F. and Clarke, A.E. (1997) An in vitro assay for assessing the effects of growth factors on *Nicotiana alata* pollen tubes. *Sex. Plant Reprod.* **10**, 351–357.

Lush, W.M., Grieser, F. and Wolters-Arts, M. (1998) Directional guidance of *Nicotiana alata* pollen tubes *in vitro* and on the stigma. *Plant Physiol.* **118**, 733–741.

Matsubayashi, Y., Ogawa, M., Morita, A. and Sakagami, Y. (2002) An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine. *Science*, **296**, 1470–1472.

Muschietti, J., Dircks, L., Vancanneyt, G. and 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, J., Eyal, Y. and 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.

Palanivelu, R., Brass, L., Edlund, A.F. and Preuss, D. (2003) Pollen tube growth and guidance is regulated by POP2, an *Arabidopsis* gene that controls GABA levels. *Cell*, **114**, 47–59.

Park, S.Y., Jauh, G.Y., Mollet, J.C., Eckard, K.J., Nothnagel, E.A., Walling, L.L. and Lord, E.M. (2000) A lipid transfer-like protein is necessary for lily pollen tube adhesion to an in vitro stylar matrix. *Plant Cell*, **12**, 151–164.

Santra, M., Reed, C.C. and Iozzo, R.V. (2002) Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor,

partially overlapping but distinct from the EGF-binding epitope. *J. Biol. Chem.* **277**, 35671–35681.

Sommer-Knudsen, J., Lush, W.M., Bacic, A. and Clarke, A.E. (1998) Re-evaluation of the role of a transmitting tract-specific glycoprotein on pollen tube growth. *Plant J.* **13**, 529–535.

Tang, W., Ezcurra, I., Muschietti, J. and 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.

Tichtinsky, G., Vanoosthuyse, V., Cock, J.M. and Gaude, T. (2003) Making inroads into plant receptor kinase signalling pathways. *Trends Plant Sci.* **8**, 231–237.

Torii, K.U. (2000) Receptor kinase activation and signal transduction in plants: an emerging picture. *Curr. Opin. Plant Biol.* **3**, 361–367.

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

Wang, Z. and He, J. (2004) Brassinosteroid signal transduction-choices of signals and receptors. *Trends Plant Sci.* **9**, 91–96.

Wang, X., Huong, S.M., Chiu, M.L., Raab-Traub, N. and Huang, E.S. (2003) Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. *Nature*, **424**, 456–461.

Wengier, D., Valsecchi, I., Cabanas, M.L., Tang, W.H., McCormick, S. and 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.

Wolters-Arts, M., Lush, W.M. and Mariani, C. (1998) Lipids are required for directional pollen-tube growth. *Nature*, **392**, 818–821.

Wu, H.M., Wang, H. and Cheung, A.Y. (1995) A pollen tube growth stimulatory glycoprotein is deglycosylated by pollen tubes and displays a glycosylation gradient in the flower. *Cell*, **82**, 395–403.

Wu, H.M., Wong, E., Ogdahl, J. and Cheung, A.Y. (2000) A pollen tube growth-promoting arabinogalactan protein from *Nicotiana alata* is similar to the tobacco TTS protein. *Plant J.* **22**, 165–176.

LeSHY, GenBank accession number AY376852; LeSTIG1, GenBank accession number AY376851.