The Plant Cell, Vol. 10, 319–330, March 1998 © 1998 American Society of Plant Physiologists

RESEARCH ARTICLE

Pollen Tube Localization Implies a Role in Pollen–Pistil Interactions for the Tomato Receptor-like Protein Kinases LePRK1 and LePRK2

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We screened for pollen-specific kinase genes, which are potential signal transduction components of pollen–pistil interactions, and isolated two structurally related receptor-like kinases (RLKs) from tomato, LePRK1 and LePRK2. These kinases are similar to a pollen-expressed RLK from petunia, but they are expressed later during pollen development than is the petunia RLK. The abundance of LePRK2 increases when pollen germinates, but LePRK1 remains constant. Both LePRK1 and LePRK2 are localized to the plasma membrane/cell wall of growing pollen tubes. Both kinase domains have kinase activity when expressed in *Escherichia coli*. In phosphorylation assays with pollen membrane preparations, LePRK2, but not LePRK1, is phosphorylated, and the addition of tomato style, but not leaf, extracts to these membrane preparations results at least partially in specific dephosphorylation of LePRK2. Taken together, these results suggest that LePRK1 and LePRK2 play different roles in postpollination events and that at least LePRK2 may mediate some pistil response.

INTRODUCTION

Recognition between the pollen and pistil is a key step in the control of plant fertilization. Although many types of pollen may contact the stigma, only pollen of the same or closely related species will be compatible. A successful or compatible pollination begins when the pollen grain contacts the surface of the stigma and hydrates. After hydration, the pollen tube emerges, penetrates the transmitting tract matrix of the style, and elongates until it reaches the ovule. Many of the molecules thought to be involved in interactions of the pistil with the pollen reside within the extracellular matrix (ECM) of the transmitting tract, where they come into contact with the growing pollen tube (reviewed in Cheung, 1996). Arabinogalactan proteins, proline-rich glycoproteins, and extensin-like proteins constitute the major classes of proteins present in this ECM. One extensively studied arabinogalactan protein, the tobacco TTS (transmitting tissue–specific) protein, seems to play a triple role in pollen tube growth as a nutrient and chemical guide and as one of the style components involved in pollen tube adhesion (Cheung et al., 1995; Wu et al., 1995). Similarly, an extensin-like pollen protein, Pex1, may play a role in adhesion during pollen tube growth through the pistil (Rubinstein et al., 1995).

Although ECM proteins, such as TTS, and pollen proteins, such as Pex1, may be important for compatible pollen–pistil interactions, it is not known whether they are involved in the recognition process that determines the specificity of the interaction. In self-incompatibility (SI) systems, in which self-pollen is rejected to prevent inbreeding, the recognition components have been best characterized in the female partner (reviewed in Dodds et al., 1996; Kao and McCubbin, 1996). However, recently the style glycoproteins (S glycoproteins), which play a role in SI in poppy, were shown to bind specifically to a plasma membrane component of pollen (Hearn et al., 1996); in Brassica, a pollen coat protein was shown to bind to S glycoproteins (Hiscock et al., 1995b). In neither case was allelic specificity shown. Among potential recognition components, there is substantial evidence for protein kinase involvement in both the male and female partners. For example, a style-specific receptor-like kinase (SRK) has been implicated in the recognition process that occurs in the SI system in Brassica (Stein et al., 1991; Goring

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and Rothstein, 1992; Nasrallah et al., 1994). In poppy (Franklin et al., 1992; Rudd et al., 1996), Brassica (Hiscock et al., 1995a), and rye (Wehling et al., 1994), pollen proteins show changes in phosphorylation status during incompatible interactions. Kinases may also play a role during compatible pollinations in which different pollen proteins are phosphorylated (Wehling et al., 1994).

We sought to isolate receptor-like protein kinases from tomato pollen because they were potential signaling components in compatible, incompatible, and interspecific crosses. The cultivated tomato (*Lycopersicon esculentum*) and its wild relatives (e.g., *L. pennellii* and *L. peruvianum*) provide material for examining these various crossing relationships (Mutschler and Liedl, 1994). One pollen-specific receptor-like kinase (PRK1) has been described in petunia (Mu et al., 1994); however, it appears to play a role during earlier stages of pollen development (Lee et al., 1996). We reasoned that kinases expressed very late during pollen development would more likely be involved in postpollination events. Here, we describe two functional receptor-like kinases (RLKs) from tomato that are found exclusively in mature and germinating pollen and are associated with the pollen tube membrane/cell wall. One of these kinases is specifically dephosphorylated when incubated with style extracts, suggesting that it plays a role soon after pollination.

RESULTS

LePRK1 and *LePRK2* Encode RLKs

To isolate kinase genes that were expressed in mature pollen of *L. esculentum*, mixed oligonucleotide pools encoding the conserved kinase subdomains VI and IX (Hanks and Hunter, 1995) were used as primers for reverse transcriptase–polymerase chain reaction (RT-PCR) by using mature pollen poly(A)$^+$ RNA. The cDNAs encoding products of the expected length (~200 bp) were cloned, and the deduced sequences were compared for similarity to kinase subdomains VII and/or VIII. Of several candidate clones, two showed pollen specificity by RNA gel blot analysis and were therefore used to screen an amplified cDNA library prepared from mature tomato anthers (McCormick et al., 1987). We screened the positive plaques by PCR to select phage with the largest inserts and completely sequenced the inserts. One contained a complete open reading frame; the other was truncated at the 5′ end and was completed by 5′ rapid amplification of cDNA ends (see Methods).

The deduced amino acid sequences of both clones have characteristics of transmembrane receptor protein kinases, so the genes were named *LePRK1* and *LePRK2* (for *L. esculentum* pollen receptor kinase). A comparison of the polypeptide sequences is shown in Figure 1A, and a schematic of their predicted hydropathy plots is shown in Figure 1B. The N-terminal stretch of hydrophobic amino acids followed by

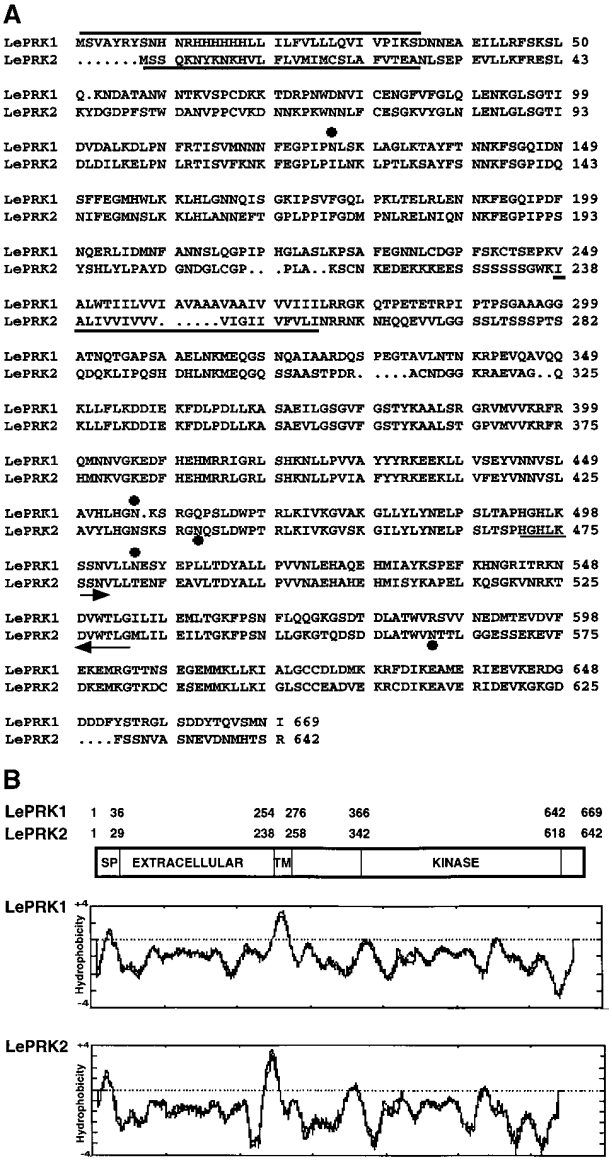

Figure 1. *LePRK1* and *LePRK2* Encode RLKs.

(A) Alignment of the deduced amino acid sequences of the LePRK1 and LePRK2 polypeptides. The putative signal peptides and transmembrane domains are marked by solid overlines and underlines. The potential N-linked glycosylation sites for LePRK1 and LePRK2 are indicated by superior and inferior stars, respectively. Arrows represent regions corresponding to the degenerate primers used in RT-PCR. Gaps were introduced to optimize the alignment. The nucleotide sequences of the full-length clones *LePRK1* (GenBank accession number U58474) and *LePRK2* (GenBank accession number U58473) are 2387 and 2375 bp long, respectively.

(B) Structural features of the LePRK1 and LePRK2 polypeptides. The domains of LePRK1 and LePRK2 polypeptides are indicated. Hydropathy plots generated by TMpred software (Hofmann and Stoffel, 1993) are shown below and are drawn relative to the size of LePRK1. SP, signal peptide; TM, transmembrane domain.

three positively charged amino acids is indicative of a signal peptide (von Heijne, 1983). The proteins have 26 (LePRK1) or 20 (LePRK2) hydrophobic amino acids followed by a short tract of basic amino acids, which is typical of type Ia integral membrane proteins (Weinstein et al., 1982). There are two potential N-linked glycosylation sites (N*S/T; the asterisk represents any amino acid) in the cytoplasmic domains of each protein and an additional potential site in the extracellular domain of LePRK1. Overall, the extracellular domains of LePRK1 and LePRK2 are 50% identical to each other and 37 to 38% identical to the extracellular domain of PRK1 from petunia (Mu et al., 1994). The extracellular domains of LePRK1 and LePRK2 contain six and five consecutive copies, respectively, of a leucine-rich repeat (LRR) of 22 to 26 amino acids (Figure 2A). LRRs are thought to be involved in interactions with other proteins (Kobe and Deisenhofer, 1994). Figure 2B shows an alignment of the LRRs of LePRK1 and LePRK2 with those of several LRR-containing proteins. This alignment is restricted to the first 16 amino acids of the motif, which is the most conserved region of LRRs (Kobe and Deisenhofer, 1994).

An alignment of the kinase domains of LePRK1, LePRK2, the petunia PRK1 (Mu et al., 1994), and three other plant RLKs is shown in Figure 3. There are 12 characteristic subdomains in the catalytic kinase domain, based on the location of conserved amino acid residues (Hanks and Hunter, 1995). Despite their clear similarity to kinases, the kinases that are specifically expressed in pollen have amino acid substitutions in some of the invariant or highly conserved positions within the kinase subdomains. In the pollen kinases, the invariant A residue of subdomain II, involved in maintenance of the hydrophobic pocket that encloses the adenine ring of the ATP, is substituted with a V residue, another hydrophobic amino acid. In addition, the V residue of subdomain I is replaced by a T residue in LePRK1 and LePRK2 and by an S residue in PRK1. The highly conserved D residue in subdomain VIb, thought to be the catalytic base that would accept a proton, is replaced by an H residue, a positively charged amino acid. The almost invariant DFG triplet of subdomain VII is substituted in LePRK1 and LePRK2 by a DYA triplet and by a DYT triplet in PRK1. Similar substitutions at these positions are found in two maize pollen RLKs (H.-U. Kim, P. Dodds, R. Kulikauskas, and S. McCormick, unpublished data), suggesting that pollen-specific RLKs may represent a distinct subclass of plant RLKs. On the basis of the distribution of certain amino acids found in subdomains VIb and VIII (Hanks and Hunter, 1995), LePRK1 and LePRK2 are classified as serine/threonine or dual specificity kinases.

LePRK1 and *LePRK2* Are Expressed Late in Pollen Development and Are Single-Copy Genes

We predicted that pollen proteins involved in pollen–pistil interactions would be expressed during the later stages of pollen development. To determine the expression patterns of *LePRK1* and *LePRK2*, poly(A)$^+$ RNA was prepared from different tissues and developmental stages and analyzed by RNA gel blot hybridization (Figure 4). DNA probes corresponding to portions of the extracellular domains of *LePRK1* and *LePRK2* (amino acids 36 to 248 for LePRK1 and 29 to 238 for LePRK2) were used. A single transcript of the expected length (~2.2 kb) was detected for both genes only in mature pollen. No signal was detected in leaf, root, sepal, petal, pistil, or green fruit. Significantly, no signal was detected in immature anthers, defined here as anthers containing the tetrad through early binucleate pollen stages (Twell et al., 1990). Thus, the immature anther sample includes the stage that corresponds to the first detectable expression of the petunia *PRK1* mRNA (Mu et al., 1994).

DNA gel blots with digested genomic DNA from *L. esculentum* were hybridized with the same probes used for the

A

LePRK1	84	VFGLQLE**N**KGLS**G**T**I**DVDALK.DLPN 108
	109	FRTISVM**N**NNFE**G**P**I**PNLS.KLAG.. 131
	132	LKTAYFT**N**NKFS**G**QIDN.SFFEGMHW 156
	157	LKKLHLG**N**NQIS**G**K**I**P..SVFGQLPK 180
	181	LTELRLE**N**NKFE**G**QIP...DFNQER. 202
	203	LIDMNFA**N**NSLQ**G**P**I**PHGLASL.KPS 228
LePRK2	79	VYGLNLENLGLS**G**TIDLDIL.KELPN 103
	104	LRTISVFKNKFE**G**PLP..ILNK.LPT 126
	127	LKSAYFSNNKFS**G**PIDQNIFEGMNS. 151
	152	LKKLHLANNEFT**G**PLPP.IF.GDMPN 175
	176	LRELNIQNNKFE**G**PIPPSYSHLYLPA 201
LRR Consensus		L**L*L**N*a***a****a*****

B

Protein	Number of repeats	Consensus sequence	Reference
LePRK1	6	L**a*a*NN*a*G*IP	This work
LePRK2	5	L**L*a*NN*F*GPIP	This work
Xa21	23	L**a*L**N*a*G*IP	(Song et al., 1995)
TMK1	11	L**L*L**N*a*G*aP	(Chang et al., 1992)
DRT100	4	L**L*L**N*a*G*IP	(Pang et al., 1992)
Toll	15	L**L*L**N*a**.aP	(Hashimoto et al., 1988)
Pex 1	8	L**a*a**N*F*G*aP	(Rubinstein et al., 1995)
PRK1	5	L**L*a**N***G***	(Mu et al., 1994)
RLK5	21	L**L*L**N*aSG*IP	(Walker, 1993)
PGIP	10	L**L*L**N*L*G*IP	(Stotz et al., 1994)
GPIbα	7	L*SL*L**N*LSG*LP	(Titani et al., 1987)
Cf-9	28	L**L*LS*N*a*G*IP	(Jones et al., 1994)
Cf-2	38	L**L*L**N*a*G*IP	(Dixon et al., 1996)

Figure 2. LRRs of LePRK1 and LePRK2 and Alignment with Other LRRs.

(A) Alignment of the LRR motifs of LePRK1 and LePRK2 with the LRR consensus (Kobe and Deisenhofer, 1994). Gaps (denoted by dots) were included to facilitate the alignment. Amino acids that are conserved in all of the LRRs of LePRK1 or LePRK2 are in boldface. Asterisks indicate any amino acid; a, an aliphatic (A, V, L, I, F, Y, or M) amino acid.

(B) Alignment of a subregion (first 16 amino acids) of the LRRs of LePRK1 and LePRK2 with LRRs of other proteins. Letter designations are used if the amino acid is present at that position in more than half of the repeats in that protein.

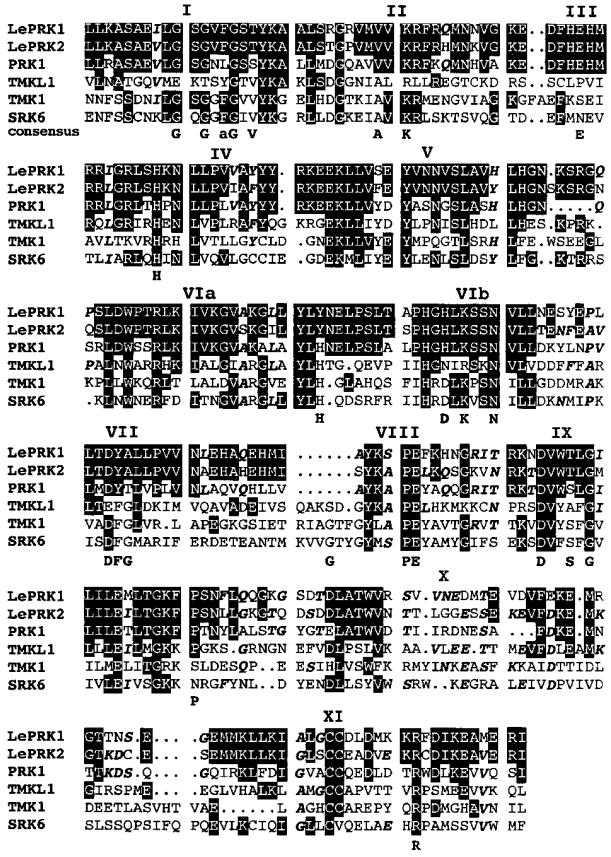

Figure 3. Alignment of the Kinase Domains of Pollen RLKs with Other Selected Plant RLKs.

An alignment of predicted sequences of LePRK1, LePRK2, petunia PRK1 (Mu et al., 1994), SRK6 (Stein and Nasrallah, 1993), TMK1 (Chang et al., 1992), and TMKL1 (Valon et al., 1993) is shown. Amino acid residues identical to those in both LePRK1 and LePRK2 are indicated in reverse type, and conserved amino acids are indicated in boldface italics. The locations of the 12 protein kinase subdomains are indicated by roman numerals, and the invariant or highly conserved amino acids in these subdomains (Hanks and Hunter, 1995) are in boldface capital letters (consensus). This alignment was generated using the Genetics Computer Group (Madison, WI) Pileup program (Higgins and Sharp, 1989) and optimized by eye. Dots indicate gaps included to facilitate the alignment.

RNA gel blot analyses. The blots showed only one band when the DNA was treated with several different restriction enzymes (Figure 5 and data not shown), indicating that each gene is present in the *L. esculentum* genome as a single copy. The *LePRK1* and *LePRK2* extracellular domain probes are only 40% identical at the DNA level and do not cross-hybridize under the high-stringency conditions used for the hybridization. An HaeIII enzyme polymorphism between *L. esculentum* and *L. pennellii* for *LePRK1* (data not shown) and an EcoRV enzyme polymorphism for *LePRK2* (data not shown) were used to map the genes by using a mapping population of 47 plants (Tanksley et al., 1992) and MapManager version 2.6 (Manly, 1993). The *LePRK1* gene was conclusively (lod score = 4.5) mapped to chromosome 5, between the restriction fragment length polymorphism markers TG503 and TG379. *LePRK2* is unlinked to *LePRK1* and was tentatively (lod score = 2.1) mapped to chromosome 7, near the marker TG166.

LePRK1 and LePRK2 Are Membrane-Associated Proteins

To obtain more direct information about the products of the *LePRK1* and *LePRK2* genes, DNA constructs corresponding to the extracellular domains of LePRK1 and LePRK2 were expressed in *Escherichia coli*, and the resulting proteins were purified and used to raise antibodies. The resulting polyclonal antibodies were used for immunodetection. Anti-LePRK1 detected a single protein of ~75 kD in crude extracts prepared from mature pollen, whereas anti-LePRK2 detected a single protein of ~70 kD. There was no cross-reaction between the two antibodies, and no proteins were detected in immature anther, leaf, or root extracts (data not shown). In addition, Figure 6 shows that the ~75 and ~70 kD proteins were immunodetected only in the microsomal fraction (P_{100}) of mature pollen and germinated pollen and not in the soluble fraction (S_{100}). An α-tubulin antibody was used to show equivalent protein loading among different samples; it was previously noted that tubulin partitions equally between soluble and membrane fractions (Nick et al., 1995). It is interesting that the amount of LePRK1 was equivalent in mature pollen and germinated pollen but that

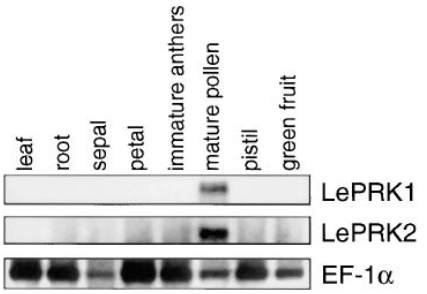

Figure 4. Expression of *LePRK1* and *LePRK2* Genes in Different Tomato Tissues.

Poly(A)$^+$ mRNA (1 μg) was loaded in each lane. The blot was hybridized first with a probe corresponding to a portion of the extracellular domain of LePRK1 and subsequently with a mixed probe corresponding to two sequences: one encoding a portion of the extracellular domain of LePRK2 and the second (loading control) encoding a portion of EF-1α. Unfortunately, the EF-1α hybridization did not give a sharp band; therefore, faint background is observed at the position of LePRK2.

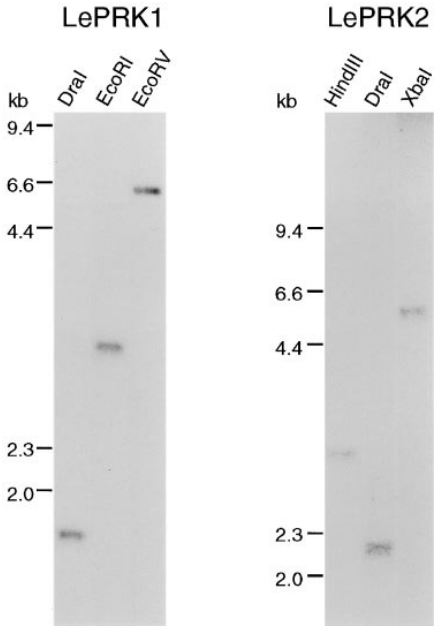

Figure 5. Genomic DNA Gel Blot Analysis of *LePRK1* and *LePRK2.*

Genomic DNA (2 μg) of *L. esculentum* was digested with the indicated restriction enzymes, blotted, and hybridized with DNA probes corresponding to a part of the extracellular domains of LePRK1 and LePRK2. DNA markers are indicated at left in kilobases.

LePRK2 increased after pollen germination. Because the antibodies detecting LePRK1 and LePRK2 are monospecific and different, we cannot conclude anything regarding the difference in intensity of the signals for these proteins in mature pollen.

We used immunolocalization to confirm the apparent membrane localization of LePRK1 and LePRK2. Essentially, we followed the protocol of Lin et al. (1996) and used a similar antibody dilution. In vitro–germinated pollen was incubated with the antibodies and subsequently with Texas red–conjugated secondary antibody. Fluorescent images were detected using epifluorescence microscopy. Figure 7 shows that the signal was most strongly detected at the margins of the pollen tubes (Figures 7A and 7B) and that incubation with the secondary antibody alone yielded no fluorescent signal in pollen tubes (Figure 7C). The out-of-focus signal in Figure 7B corresponds to signal associated with the pores. Tomato pollen has three pores, and the plasma membrane/intine partially extrudes from all of them; then one pore takes precedence and the tube grows out. The results shown in Figure 7 are representative of the results obtained in seven experiments, with 25 to 50 pollen tubes observed in each experiment. We sometimes saw enhanced signal at the tip (Figure 7A); however, as discussed by He and Wetzstein (1995), this is likely due to the fixation protocol. The exine (outer pollen wall) shows some autofluorescence that is most notable near the pores on the grain; this fluorescence was present to the same extent in all preparations and was independent of the presence or absence of the primary antibody (Figure 7C).

LePRK1 and LePRK2 Have Kinase Activity

Because the pollen kinases have several variant amino acids in the conserved kinase subdomains, it was important to test the proteins for kinase activity. DNA constructs corresponding to the kinase domains of LePRK1 and LePRK2 were expressed

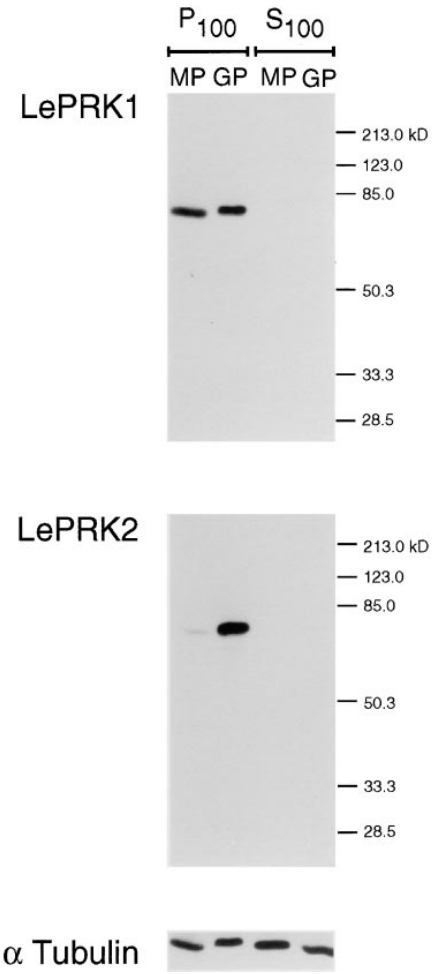

Figure 6. Immunodetection of the LePRK1 and LePRK2 Polypeptides in Pollen Extracts.

Protein extracts (30 μg) from *L. esculentum* pollen were separated by SDS-PAGE, blotted to nitrocellulose, and incubated with antibodies raised against LePRK1 and LePRK2 extracellular domains or with an α-tubulin antibody. Molecular mass markers are indicated at right in kilodaltons. P_{100}, crude microsomal fraction; S_{100}, cytoplasmic fraction; MP, mature pollen; GP, germinated pollen.

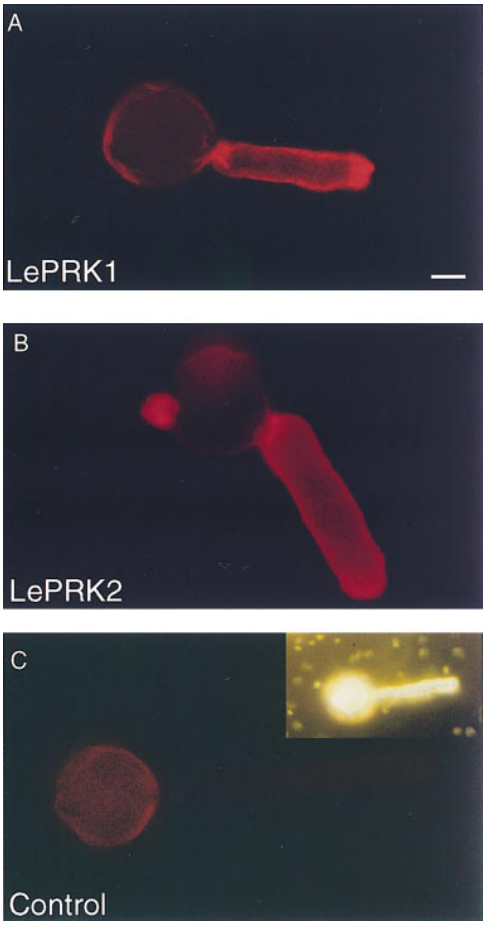

Figure 7. Immunolocalization of LePRK1 and LePRK2.

In vitro–germinated pollen was fixed, incubated with the primary antibody indicated, and visualized with a Texas red–conjugated secondary antibody. Fluorescent images were observed with an epifluorescence microscope.
(A) Anti-LePRK1 antibody.
(B) Anti-LePRK2 antibody.
(C) Secondary antibody alone. The pollen tube extends to the right of the grain; for reference, the inset shows a dark-field image of the same pollen grain.
Bar in **(A)** = 10 μm for **(A)** to **(C)**.

in *E. coli*, and the resulting proteins were purified and assayed. As negative controls, mutant versions were constructed by changing the K residue in subdomain II to R (see Figure 3). In all previously tested protein kinases, this lysine is essential for kinase activity (Hanks and Hunter, 1995). Figure 8A shows that the wild-type constructs could autophosphorylate in the presence of γ-^{32}P-ATP, whereas the mutant proteins had no detectable kinase activity. Figure 8B shows that similar amounts of the proteins were loaded in each lane. These results provide biochemical evidence that LePRK1 and LePRK2 are functional protein kinases.

LePRK2 Is Phosphorylated in Pollen Membranes

Many receptor kinases undergo autophosphorylation. After we confirmed that the kinase domains of LePRK1 and LePRK2 have kinase activity in vitro, we tested endogenous phosphorylation activity in mature pollen membranes. Microsomal fractions were prepared from mature pollen of *L. esculentum* and labeled with γ-^{32}P-ATP to reveal the profile of auto- and trans-phosphorylated proteins in mature pollen membranes (Figures 9A to 9C, lanes 1). The phosphorylated protein pattern did not resemble the Coomassie Brilliant Blue R 250 staining pattern (data not shown), which argues that the labeled proteins are indeed phosphorylated and not labeled due to nonspecific association with γ-^{32}P-ATP. The phosphorylated protein pattern was similar when the labeling time was varied from 10 sec to 10 min (data not shown). One of the major phosphorylated proteins was immunoprecipitated by the LePRK2-specific antibody (Figure 9A, lane 2), thus establishing that this 70-kD phosphoprotein was indeed LePRK2. LePRK1 can be immunoprecipitated by LePRK1-specific antibodies (data not shown); however, we were not able to correlate any of the phosphorylated proteins with LePRK1 because none of them comigrates with LePRK1 or was immunoprecipitated by LePRK1-specific antibodies (data not shown).

LePRK2 Is at Least Partially Dephosphorylated by Tomato Style Extracts

A role for LePRK2 in pollen–pistil communication would presumably involve direct interaction with a style component. Therefore, we examined the phosphorylation pattern of the pollen microsomal fractions in the presence or absence of style extract (Figure 9B). Incubation of the pollen microsomal fraction with *L. esculentum* style extract for 5 min either before or after adding γ-^{32}P-ATP led to the disappearance of the 70-kD phosphorylated band (Figure 9B, lanes 2 and 3;

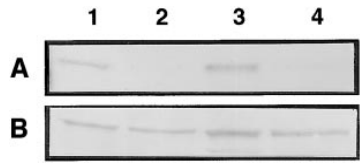

Figure 8. Analysis of Kinase Activity of LePRK1 and LePRK2.

Maltose binding protein (MBP) fusion proteins were expressed in *E. coli*, affinity purified using an amylose resin, and incubated with γ-^{32}P-ATP in phosphorylation buffer (see Methods). The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and then exposed to x-ray film. Lanes 1 contain MBP–LePRK1; lanes 2, MBP mutant version of LePRK1; lanes 3, MBP–LePRK2; and lanes 4, MBP mutant version of LePRK2.
(A) Autoradiography of the phosphorylated fusion proteins.
(B) Ponceau-S–stained nitrocellulose membrane.

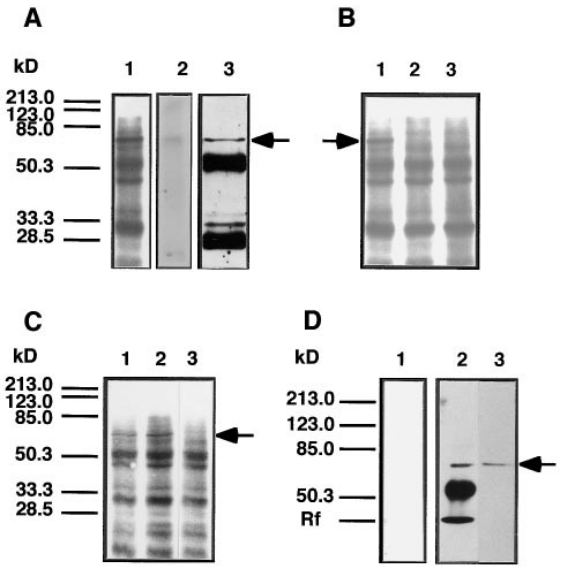

Figure 9. LePRK2 Is Phosphorylated in Pollen Microsomal Fractions and Is at Least Partially Dephosphorylated in the Presence of Tomato Style Extracts.

Pollen microsomal fractions (40 μg) were incubated for 5 min with γ-^{32}P-ATP in buffer (untreated), in buffer with 30 μg of style extract proteins (treated), or in buffer with 30 μg of leaf extract proteins, as described in Methods. Total pollen proteins or proteins immunoprecipitated by using the LePRK2 antibody were separated by SDS-PAGE, blotted to nitrocellulose, and then subjected to autoradiography. After autoradiography, when indicated, the membrane was used for immunodetection with the LePRK2 antibody. All lanes show autoradiograms, except for lane 3 in **(A)** and lanes 2 and 3 in **(D)**, which show immunoblots. The position of LePRK2 is indicated in **(A)** to **(D)** by arrows. SDS–acrylamide gels are 12%, except for **(D)**, which is 8%. Molecular mass markers are indicated at left in kilodaltons.

(A) LePRK2 is phosphorylated in pollen membranes. Lane 1, profile of phosphorus-labeled proteins in untreated pollen microsomal fraction; lane 2, immunoprecipitation with LePRK2 antibody of phosphorus-labeled proteins in untreated pollen microsomal fraction; and lane 3, immunodetection of LePRK2 immunoprecipitated from untreated pollen microsomal fraction. The intense bands at ~52 and ~28 kD correspond to antibody heavy and light chains.

(B) Phosphorylated LePRK2 disappears in the presence of tomato style extract. Lane 1, profile of phosphorus-labeled proteins in untreated pollen microsomal fraction; lane 2, profile of phosphorus-labeled proteins in pollen microsomal fraction treated with style extract before labeling; and lane 3, profile of phosphorus-labeled proteins in pollen microsomal fraction treated with style extract after labeling.

(C) LePRK2 phosphorylation status is not affected by tomato leaf extract. Lane 1, profile of phosphorus-labeled proteins in untreated pollen microsomal fraction; lane 2, profile of phosphorus-labeled proteins in pollen microsomal fraction incubated with tomato leaf extract; and lane 3, profile of phosphorus-labeled proteins in pollen microsomal fraction treated with style extract.

(D) LePRK2 is at least partially dephosphorylated by tomato style extract. Lane 1, immunoprecipitation with LePRK2 antibody of phosphorus-labeled proteins in pollen microsomal fraction treated with style extract; lane 2, immunodetection of LePRK2 immunoprecipitated from pollen microsomal fraction treated with style extract; the intense bands at ~52 and ~28 kD correspond to antibody heavy and light chains; and lane 3, immunodetection of LePRK2 in pollen microsomal fraction treated with style extract.

Figure 9C, lane 3; and Figure 9D, lane 1) previously identified as LePRK2. The same result was obtained after incubating pollen microsomal fractions with *L. pennellii* style extract (data not shown). In contrast, incubation of the pollen microsomal fraction in the presence of *L. esculentum* leaf extract did not affect the phosphorylation of the 70-kD band (Figure 9C, lane 2). A comparison of Figure 9A, lane 2, with Figure 9D, lane 1, shows that the LePRK2 antibody did not immunoprecipitate a phosphoprotein if the membranes have been incubated with style extract. This suggests that a component(s) of the style extract caused either degradation or dephosphorylation of LePRK2. Although we cannot rule out the possibility that some degradation occurred, Figure 9D, lane 2 (an immunoblot of Figure 9D, lane 1), shows that detectable levels of intact LePRK2 protein were still present after incubation in the presence of style extract, followed by immunoprecipitation with the LePRK2 antibody. Similarly, the immunoblot in Figure 9D, lane 3, shows that LePRK2 protein is detectable after incubation of pollen microsomal membranes with style extracts. We therefore concluded that at least some LePRK2 is dephosphorylated after incubation with style extracts. Because none of the other phosphoproteins is affected by the presence of style extract (Figures 9B and 9C), we can conclude that this dephosphorylation is specific to LePRK2. We can only speculate at this stage about the biochemical nature of this dephosphorylation. Boiling of the style extract (10 min) before incubation or addition of the phosphatase inhibitors microcystin (10 μM) or okadaic acid (1 μM) to the labeling reaction did not prevent LePRK2 dephosphorylation (data not shown). Although additional experiments are required to determine whether phosphorylation and dephosphorylation of LePRK2 occur in vivo, our results suggest that LePRK2 dephosphorylation may mediate communication between tomato pollen and pistil.

DISCUSSION

Although many plant protein kinase genes have been isolated during the past few years, the roles for most remain unknown (reviewed in Braun and Walker, 1996). Protein kinases in somatic tissue might be involved in any of several potential steps in cell development or response to the environment; therefore, their specific role may be difficult to predict using a reverse genetics approach. However, pollen interacts only with the sporophytic tissue of the anther during its development and with the pistil after pollination. Our

data for LePRK1 and LePRK2 support their involvement in postpollination events. First, RNA gel blot analysis clearly shows that *LePRK1* and *LePRK2* expression is restricted to very late stages in pollen development. This timing of expression is different from the *PRK1* gene from petunia that is expressed as early as the binucleate pollen stage (Mu et al., 1994). This difference in the timing of expression could reflect a difference in the transduced signal or in the pathway where these proteins function, despite the similarity in the structure of the proteins. Furthermore, the fact that disruption of the *PRK1* gene by antisense strategy yielded aborted pollen might suggest that the PRK1 protein plays a role in interacting with sporophytic tissues during pollen development (Lee et al., 1996). Second, immunoblot analysis and immunolocalization of the LePRK1 and LePRK2 proteins show that they are most likely localized in the plasma membrane of the pollen tube. Third, the level of LePRK2 protein appears to increase dramatically after pollen germination. The time of expression and immunolocalization of LePRK1 and LePRK2 is consistent with a role for these proteins during pollen–pistil interactions, such as pollen hydration and/or pollen tube germination or growth.

The receptor kinases that have been isolated from animals, microorganisms, and plants vary mostly in the structure of the extracellular domain. The extracellular domains of LePRK1 and LePRK2 have LRRs, which may mediate interaction with a proteinaceous ligand. As shown in Figure 2B, in addition to the conserved first three leucines, there is a highly conserved amino acid subregion (N*a*G*I/aP; a represents any aliphatic amino acid) present in all the otherwise dissimilar proteins of the list. The conserved glycine is characteristic of extracellular LRRs (Jones et al., 1994). Thus, from a structural point of view, all of these proteins could be classified together as a subgroup of LRR proteins. Functionally, however, they comprise two groups: RLKs involved in the initiation and transduction of different signals through their kinase activity (Xa21, TMK1, PRK1, RLK5, and Cf-9) and proteins involved in adhesive processes (Toll and GP Ibα). Human platelet glycoprotein Ibα is a transmembrane protein involved in platelet adhesion (Lopez et al., 1987; Titani et al., 1987). Toll has an adhesive role in dorsoventral patterning in Drosophila embryogenesis (Hashimoto et al., 1988) as well as a signal transducing role through the interaction of its extracellular domain with a soluble extracellular ligand (Morisato and Anderson, 1995). It is interesting that the (N*a*G*I/aP) subregion is also present in the globular domain of Pex1, the maize pollen protein with an extensin domain and a proposed adhesive role (Rubinstein et al., 1995). Considering the LRR structure of LePRK1 and LePRK2 and their kinase activity, it is plausible that LePRK1 and LePRK2 play both adhesive and signaling roles in pollen tubes.

Adhesive signal transduction pathways in mammals involve phosphorylation cascades, intracellular calcium regulation, and formation of cytoskeletal complexes (Schaller and Parsons, 1994) as linked processes that precede the formation of focal adhesion sites (Clark and Brugge, 1995). Various lines of evidence suggest that similar processes may be occurring during pollen–pistil interactions. First, phosphorylation of pollen proteins has been shown to occur in compatible and incompatible interactions (Franklin et al., 1992; Wehling et al., 1994; Rudd et al., 1996). Second, an intracellular gradient of calcium in the pollen tube is essential for pollen tube growth (Pierson et al., 1994). Last, pollen tubes contain a localized arrangement of microfilaments (actin and kinesin-like proteins) in the tip of the pollen tube, and when grown in vivo, pollen tubes also show regions of association of actin microfilaments (Pierson et al., 1986). Lord and Sanders (1992) suggested that these regions are focal adhesion sites with the style extracellular matrix.

The existence of a functional cytoplasmic kinase domain in LePRK1 and LePRK2 clearly suggests that an extracellular signal is being transmitted into the pollen grain or tube. In most known receptor kinases, interaction of the ligand with the receptor activates the intracellular kinase activity, resulting in autophosphorylation and/or phosphorylation of target proteins. However, binding of a ligand can also result in the shutdown of intracellular kinase activity, as shown for the EGF receptor of Drosophila (Schweitzer et al., 1995). Similarly, a shutdown signal may be responsible for the dephosphorylation of LePRK2 that is observed upon exposure of pollen membranes to style extract (Figure 9). In other words, the putative style ligand may be transducing its signal by shutting down a previously activated kinase. The lack of extract specificity (*L. esculentum* versus *L. pennellii*) suggests that this signal may be a component of compatible pollen–pistil interactions and not part of the unilateral pollination barrier that exists between these species (Mutschler and Liedl, 1994). In any case, this result strongly suggests a role for LePRK2 in pollen–pistil interactions. The nature of this role needs to be studied further at the biochemical and physiological levels.

METHODS

Plant Material

Lycopersicon esculentum cv VF36 and *L. pennellii* LA2963 plants were grown under standard greenhouse conditions. Pollen was obtained by vibrating the anthers and was stored at −80°C until required or used directly for immunolocalization studies and germination experiments. *L. esculentum* cv VF36 and *L. pennellii* LA2963 stigmas and styles used for preparation of the extracts were collected and directly processed.

cDNA Cloning and DNA Sequencing

Poly(A)$^+$ RNA from *L. esculentum* mature pollen was prepared with an Oligotex Direct mRNA kit (Qiagen, Inc., Chatsworth, CA), following the manufacturer's procedures. Two reverse transcription (RT) reactions contained 250 ng of poly(A)$^+$ RNA, 10 units of avian myeloblastosis virus–RT, and 10 μM degenerate primers corresponding to

the conserved kinase subdomain IX: IX-1, CC(A/G)TAIGACCAIAC-(A/G)TC; IX-2, CCIA(G/C/A)IGACCAIAC(A/G)TC; IX-3, CC(A/G)TAIGA-(A/G)(A/T)AIAC(A/G)TC; and IX-4, CCIA(G/C/A)IGA(A/G)(A/T)AIAC-(A/G)TC. One RT reaction used a mixture of primers IX-1 (complexity of 4) and IX-4 (complexity of 24), maintaining equimolar concentrations of each of the 28 different oligonucleotides present in the reaction, whereas the other RT reaction used the same criteria and primers IX-2 (complexity of 6) and IX-3 (complexity of 16). The RT reactions were conducted at 42°C for 90 min in a final volume of 20 μL. Polymerase chain reactions (PCRs) were conducted on 5 μL of the resulting cDNA in a 50-μL volume containing Taq polymerase buffer, 100 μM each deoxynucleotide triphosphate, 0.2 units of Taq polymerase (Fisher Scientific, Pittsburgh, PA), and 4 μM primers designed from conserved kinase subdomains VI (A[T/C]GGICA[T/C]CTIAA[A/G]TCI TCIAA) and IX (the ones previously used for the RT reaction). The mixture was heated to 94°C for 3 min and amplified for 40 cycles under the following conditions: 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min, with a final extension cycle at 72°C for 10 min. PCR products of the predicted length (~200 bp) that were obtained using the primer IX-1 and IX-4 mixture were subcloned using the pCR-Script SK(+) cloning kit (Stratagene, La Jolla, CA) and sequenced with the Sequenase version 2.0 DNA sequencing kit, as described by the manufacturer (U.S. Biochemical). An amplified cDNA library from mature anther poly(A)$^+$ RNA of *L. esculentum* cv VF36 (McCormick et al., 1987) was plated (450,000 recombinant phage) and probed separately with each of the two PCR clones that showed pollen specificity. The 5′-AmpliFINDER RACE Kit (Clontech, Palo Alto, CA) was used to obtain the full-length clone corresponding to LePRK2.

DNA and peptide sequence analysis was performed using various programs of GCG (Genetics Computer Group) and BLAST programs (Altschul et al., 1990). The TMpred program was used to predict the membrane spanning domains of the two proteins (Hofmann and Stoffel, 1993).

RNA Gel Blot Analysis

Total RNA was extracted from mature pollen, leaf, root, sepal, petal, immature anthers (a mixture of the stages defined in Twell et al. [1990] as immature anthers [IA] and green petal anthers [GPA]), mature pistil, and green fruit, using a guanidinium thiocyanate method (Logemann et al., 1987). Poly(A)$^+$ RNA was isolated using the Poly-A-Tract system, as described by the manufacturer (Promega). Poly(A)$^+$ RNA (1 μg) was fractionated on a 1.3% formaldehyde gel and transferred to a nylon membrane (MSI, Westborough, MA). Prehybridization was for 4 hr at 45°C in 50% formamide, 4 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM sodium phosphate, pH 7.1, and 1 mM EDTA), 400 μg/mL salmon sperm DNA, 5 × Denhardt's solution (1 × Denhardt's solution is 0.1 g of Ficoll [type 400], 0.1 g of PVP, and 0.1 g of BSA [fraction V]), 1% SDS, and 50 mM potassium phosphate, pH 6.5. The probes were labeled with phosphorus-32 by random priming (Feinberg and Vogelstein, 1984). The probes were generated by PCR. The *LePRK1* probe was DNA corresponding to the extracellular domain (amino acids 37 to 249), the *LePRK2* probe corresponded to the extracellular domain (amino acids 30 to 237), and the *EF1-α* probe (Pokalsky et al., 1989) corresponded to amino acids 198 to 425. Hybridization was overnight at 45°C in prehybridization solution with the addition of 100 μg/mL dextran sulfate. Washes were performed in 0.1% SSPE, 0.1% SDS at 65°C for 1 hr, with two changes of the solution. After autoradiography, the membrane was boiled in 0.1% SDS, 0.1 mM EDTA, and 10 mM Tris, pH 7.5, for 20 sec to 1 min to remove the probe before rehybridization. The membrane was first probed with *LePRK1* and then with a mixture of *LePRK2* and *EF1-α*.

DNA Gel Blot Analysis

Genomic DNA was isolated from liquid nitrogen–frozen leaves, as described previously (Muschietti et al., 1994). Genomic DNA (2 μg) of *L. esculentum* and *L. pennellii* was digested for 5 hr with DraI, EcoRI, and EcoRV for *LePRK1* and with HindIII, DraI, and XbaI for *LePRK2*, fractionated on a 1% agarose gel, and transferred to maximum-strength Nytran (Schleicher & Schuell). The membranes were prehybridized and hybridized with the same *LePRK1* and *LePRK2* ^{32}P-labeled DNA probes used for the RNA gel blot analysis, as described by Church and Gilbert (1984). DNA blots of an F_2 population of *L. esculentum* (VF36) and *L. pennellii* (LA716) (Tanksley et al., 1992) were gifts of S. Gorman and C. Mainini (Plant Gene Expression Center, Albany, CA) and were prepared as described previously (Gorman et al., 1996). Map position was determined using MapManager version 2.6 software (Manly, 1993).

Expression of Extracellular Domains and Preparation of Polyclonal Antibodies

PCR products corresponding to the extracellular domains (amino acids 37 to 249 for LePRK1 and 30 to 237 for LePRK2) were obtained using Pfu DNA polymerase (Stratagene) and were subcloned into the NheI-BamHI sites of the pRSET vector (Invitrogen, San Diego, CA). These constructs were used to express the extracellular domain recombinant proteins in *Escherichia coli* BL21(DE3) pLysE (Novagen Inc., Madison, WI), as indicated by the manufacturer. The recombinant proteins were purified by affinity on Ni-NTA agarose (Qiagen), and the purified fractions were concentrated and dialyzed with Centriprep-10, according to the manufacturer's instructions (Amicon, Beverly, MA). Protein concentration was determined with the BCA Protein Assay (Pierce, Rockford, IL).

Polyclonal antibodies were prepared according to Karu (1993). Approximately 100 μg of purified protein in MPL plus TDM emulsion adjuvant (RIBI Immunochem Research, Hamilton, MT) was used per immunization per mouse for four boosts. The immune response was monitored by immunoblot on tail bleeds 2 weeks after immunization. Ascites fluids were induced by the sarcoma line T180 (ATTC accession number TIB 66) and collected 5 to 10 days later. Ascites fluids were stored at 80°C and used for immunodetection at a dilution of 1:1000.

SDS-PAGE and Immunoblotting

L. esculentum mature pollen, in vitro–germinated pollen, immature anthers containing binucleate microspores (Twell et al., 1990), and leaf samples were prepared by disrupting the tissue in a glass-glass homogenizer on ice, using four volumes of protein extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.5 μg/mL leupeptin, 10 μg/mL chymostatin, 2 μg/mL aprotinin, 1 μM Pefabloc [Boehringer Mannheim], and 10 μg/mL pepstatin). The homogenates were centrifuged at 10,000*g* for 10 min at 4°C, and the supernatants were reserved. The samples from mature pollen and from in vitro–germinated pollen were further fractionated into crude microsomal (P_{100}) and cytoplasmic fractions (S_{100}) by centrifugation at 100,000*g* for 3 hr at 4°C. The pellet (crude microsomal, P_{100}) was washed with extraction

buffer, centrifuged again for 10 min, and stored at −80°C. Approximately 30 μg of protein of each sample was separated on 10% SDS–polyacrylamide gels and blotted to nitrocellulose (Amersham Life Science Inc., Arlington Heights, IL) (Harlow and Lane, 1988). The membranes were blocked with 2% gelatin in 1 × TBS (1 × TBS is 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) for 30 min and incubated with antibodies raised against either LePRK1 or LePRK2 extracellular domains or α-tubulin (Amersham). The secondary antibody was sheep anti–mouse polyclonal antibody conjugated with horseradish peroxidase. All of the antibodies were diluted to 1:1000 in 1 × TBS, 0.2% Triton X-100, 4% nonfat dry milk, and 2% glycine. Membranes were incubated with each antibody for 1 hr and washed for 1 hr with 1 × TBS and 0.2% Triton X-100. Membranes were developed using the enhanced chemiluminescence kit (Amersham) and stripped following the manufacturer's protocol. Standards from Bio-Rad were used to estimate protein sizes.

Immunolocalization

Immunolocalization was essentially as described by Lin et al. (1996) except that the fixative solution was 4% paraformaldehyde, 50 mM Hepes buffer, pH 7.0, 2 mM $MgCl_2$, and 10% sucrose. Slides were incubated with primary antibody (1:50 dilution) at 28°C for 2 hr and with Texas red–conjugated goat anti–mouse IgG secondary antibody (Caltag Laboratories, San Francisco, CA) for 1 hr. After washes, slides were mounted with 1:1 Vectashield (Vector Lab, Burlingame, CA)–glycerol solution and observed with an epifluorescence microscope (Axiophot photomicroscope; Zeiss, Thornwood, NY) equipped with filter No. 15. Kodak Ektachrome (400 ASA) film was used for photography.

In Vitro Pollen Germination

In vitro pollen germination was as described by Muschietti et al. (1994), except that the polyethylene glycol concentration was 24% instead of 14%. For protein analysis, pollen was germinated (~50 mg of pollen in 5 mL of media) overnight at 28°C in a plastic Petri dish (60 × 15 mm) rotating at 50 rpm; the efficiency of germination was ~100%. The pollen tube mat was filtered and immediately used for microsomal protein fractionation. For immunolocalization, pollen was germinated for 1 hr when ~90% of the pollen grains had ~100- to 150-μm-long tubes.

Expression of Kinase Domains and Kinase Assays

Pfu-generated PCR products corresponding to the kinase domains (amino acids 348 to 669 for LePRK1 and 325 to 642 for LePRK2) were subcloned into the SalI-BamHI sites of the pMALc2 vector (New England BioLabs, Beverly, MA). As negative controls, mutant versions of LePRK1 and LePRK2 were constructed by site-directed mutagenesis of the conserved lysine of subdomain II (amino acid 396 for LePRK1 and 372 for LePRK2) to arginine (Kunkel et al., 1987). Wild-type and mutant constructs of LePRK1 and LePRK2 were used to express the recombinant proteins in *E. coli* BL21(DE3) pLysE (Novagen), as indicated by the manufacturer. The recombinant soluble proteins were purified by affinity chromatography on amylose resin, as indicated by the manufacturer (New England BioLabs), dialyzed overnight at 4°C against 20 mM Hepes, pH 7.0, 1 mM DTT, and 10% glycerol, and analyzed by SDS-PAGE.

For the in vitro kinase assay, the purified recombinant proteins (5 μg) were incubated in a 50-μL solution of phosphorylation buffer (50 mM Hepes, pH 7.0, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM Pefabloc, and 1 mM DTT) and 10 μM γ-^{32}P-ATP (2×10^4 cpm/pmol ATP) for 20 min at room temperature. The reaction was stopped by adding 10 μL of 5 × Laemmli SDS-PAGE sample buffer (1 × Laemmli SDS-PAGE sample buffer is 25 mM Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromphenol blue) and incubating for 30 min at 70°C. The labeled proteins were separated on 10% SDS–polyacrylamide gels and blotted to nitrocellulose. The membrane was stained with Ponceau S, according to the manufacturer's protocol (Sigma), and then exposed to film.

In Vitro Phosphorylation

Microsomal fractions were resuspended in PBC (phosphorylation buffer supplemented with 1 mM $CaCl_2$) to ~2 mg/mL of protein. Freshly collected stigmas and styles from 20 to 30 flowers or leaves were homogenized in PBC in an Eppendorf tube by using a plastic homogenizer. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C, and the supernatant (termed style or leaf extract) was used immediately for the experiment. Pollen microsomal proteins (40 μg) were incubated for 5 min at room temperature with either 30 μg of style extract or leaf extract proteins or with an equivalent volume of PBC. The phosphorylation reaction was started by adding 10 μM γ-^{32}P-ATP (10^4 cpm/pmol ATP) at room temperature for the indicated times. The reaction was stopped with EDTA (final concentration of 10 mM) and centrifuged at 10,000*g* for 10 min at 4°C; the pellet (pollen microsomal fraction) was resuspended in 30 μL of 2 × Laemmli SDS-PAGE sample buffer and boiled for 3 min. The labeled pollen proteins were separated on SDS–polyacrylamide gels, blotted to nitrocellulose, and then subjected to autoradiography.

For immunoprecipitation, the pellet (pollen microsomal fraction) was resuspended in 100 μL of RIPA buffer (1 × RIPA buffer is 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, and 20 mM Tris-HCl, pH 7.2) and incubated with 2 μL of the corresponding antibody for 2 hr at 4°C. The mixture was centrifuged at 10,000*g* for 10 min at 4°C to remove microsomes not dissolved by the RIPA, and then 100 μL of 10% protein A–Sepharose was added to the supernatant and incubated for 1 hr. Immunoprecipitates were washed twice with RIPA buffer and then resuspended in 40 μL of 2 × Laemmli SDS-PAGE sample buffer and boiled for 3 min. After brief centrifugation to precipitate the beads, the immunoprecipitated pollen proteins were separated by SDS-PAGE, blotted to nitrocellulose, and then subjected to autoradiography. After exposure to x-ray film, the membranes were subjected to immunodetection, as described above.

ACKNOWLEDGMENTS

We thank Yakang Lin for helpful suggestions in pollen immunolocalization and Julie Stone and John Walker for providing advice and a positive control for the kinase assay. We thank Elise Grenier for help with the DNA gel blots, Susan Gorman and Cathy Mainini for help with mapping, and Cathy Curie for providing her optimized pollen germination protocol. We thank David Hantz and his staff for excellent greenhouse assistance and Sothy Yi, Joy Liang, and Margaret Liu for collecting pollen. We thank Rima Kulikauskas and Robyn Cotter for help with formatting the figures. We thank all of the mem-

bers of our laboratory, especially Cathy Curie and Susan Gorman, for helpful discussions during the course of this work, and Peter Dodds, Paul Herzmark, Rima Kulikauskas, and Anabella Srebrow for their comments on the manuscript. This work was supported by U.S. Department of Agriculture–Current Research Information System Grant No. 5335-21000-008-00D. Y.E. was partially supported by a United States–Israel Binational Agricultural Research and Development Fund Postdoctoral Fellowship.

Received July 29, 1997; accepted December 23, 1997.

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