



New pollen-specific receptor kinases identified in tomato, maize and *Arabidopsis*: the tomato kinases show overlapping but distinct localization patterns on pollen tubes

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Abstract

We previously characterized LePRK1 and LePRK2, pollen-specific receptor kinases from tomato (Muschiatti *et al.*, 1998). Here we identify a similar receptor kinase from maize, ZmPRK1, that is also specifically expressed late in pollen development, and a third pollen receptor kinase from tomato, LePRK3. LePRK3 is less similar to LePRK1 and LePRK2 than either is to each other. We used immunolocalization to show that all three LePRKs localize to the pollen tube wall, in partially overlapping but distinct patterns. We used RT-PCR and degenerate primers to clone homologues of the tomato kinases from other Solanaceae. We deduced features diagnostic of pollen receptor kinases and used these criteria to identify family members in the *Arabidopsis* database. RT-PCR confirmed pollen expression for five of these *Arabidopsis* candidates; two of these are clearly homologues of LePRK3. Our results reveal the existence of a distinct pollen-specific receptor kinase gene family whose members are likely to be involved in perceiving extracellular cues during pollen tube growth.

Abbreviations: EGF, epidermal growth factor; LRR, leucine-rich repeat; RLK, receptor-like kinase; TNF, tumor necrosis factor; TTS, transmitting-tract-specific protein; WAK, wall-associated kinase

Introduction

The first plant gene encoding a putative receptor kinase, referred to as a receptor-like kinase (RLK), was

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF243041 (ZmPRK1); AF243040 (LePRK3); PRK1: AF246966(Lp), AF246964(Nt), AF246965(St); PRK2: AF246970(Lpim), AF246969(Lp), AF246967(Nt), AF246968(St); PRK3: AF252412(Lp), AF252413(St), AF267178 (Lper); PRK4: AF252415 (LePRK4), AF252414 (NtPRK3*).

cloned less than 10 years ago (Zhang and Walker, 1993). Since then we have learned that plant RLKs play key roles in various biological processes, such as cell-cell recognition during development, defense against pathogens, and self-incompatibility (reviewed by Torii, 2000). The RLK proteins that have been isolated from various plant species all have an extracellular domain, a transmembrane domain and a kinase domain with Ser/Thr-type specificity. These RLKs can be classified into several major groups,

based on the structures of their extracellular domains. These include the leucine-rich repeat (LRR) group, the S-domain group (members of which have extracellular domains related to the S-locus glycoprotein), the lectin-like domain group, the EGF receptor-like group (represented in *Arabidopsis* by WAK1), and the TNF receptor-like group (represented by Crinkly4 in maize). More recently, an RLK with a chitinase-like extracellular domain (Kim *et al.*, 2000) and a group of salicylic acid-induced RLKs whose extracellular domains have four conserved cysteine residues (Du and Chen, 2000) were described.

The LRR subfamily is the largest group of RLKs in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000; Shiu and Bleeker, 2001). A characteristic leucine-rich repeat motif of about 25 amino acid residues occurs in numerous eukaryotic proteins. LRRs are thought to be involved in mediating protein-protein interactions (Kajava, 1998). The LRR receptor kinases have been found to regulate various developmental processes, phytohormone perception, and defense responses. For instance, *Arabidopsis* CLAVATA1 controls meristem cell fate (Waites and Simon, 2000), HAESA plays a role in floral abscission (Jinn *et al.*, 2000), and BRI1 perceives brassinosteroids via its extracellular domain (He *et al.*, 2000). Recently CLV3, a small secreted protein, has been identified as the ligand for CLAVATA1 (Waites and Simon, 2000). Together with some recent work, for example the demonstration that KAPP (kinase-associated protein phosphatase) associates with several different RLKs (Trotochaud *et al.*, 2000 and references therein), a paradigm of plant signaling via receptor kinases is nearly established. Even with the barrier of the cell wall, plant RLKs indeed function as receptor kinases, can bind extracellular ligand(s) and transduce signals to intracellular components.

Pollen tube growth serves as a model system for studying cell-cell recognition, communication and signaling. During pollination, a pollen tube emerges from the hydrated pollen grain on the stigma, then penetrates the surface, traverses the transmitting tract, and finally reaches the ovule for fertilization. This is a very complicated and tightly controlled process, presumably involving signaling between each pollen tube and the pistil, and perhaps signaling between pollen tubes. Pollen tubes may follow several different guidance cues as they traverse different type of tissues to target an ovule. Recent evidence suggests that lipid-transfer protein (Park *et al.*, 2000), stilar pectin (Mollet *et al.*, 2000), and TTS (Wu *et al.*, 1995, 2000;

but see also Sommer-Knudsen *et al.*, 1998) may serve as extracellular cues. Within the pollen tube cytoplasm, a pollen-specific Rho-GTPase (Rop) mediates cytoskeletal changes in response to oscillations in the calcium gradient (reviewed by Palanivelu and Preuss, 2000). However, it is still largely unknown how growing pollen tubes perceive various extracellular cues and transmit them to intracellular signal transduction pathway(s). Pollen-expressed receptor kinases are plausible candidates for this role.

To the best of our knowledge, only four pollen-expressed RLKs have been reported. The first pollen-specific receptor-like kinase, PRK1, was isolated from *Petunia inflata* (Mu *et al.*, 1994). In this paper, for clarity, we will use the name PiPRK1 for the *P. inflata* pollen receptor kinase. Although PiPRK1 is expressed in germinating pollen, experiments with antisense constructs only established that it is required during pollen maturation (Lee *et al.*, 1996). Whether PiPRK1 also plays a role in regulating pollen tube growth remains unknown. The first evidence indicating that receptor kinases were involved in pollen tube growth and pollen-pistil interactions came from the pollen tube localization of LePRK1 and LePRK2, two receptor-like kinases we previously isolated from *Lycopersicon esculentum* (Muschietti *et al.*, 1998). Both of the tomato proteins and PiPRK1 fall into the LRR-RLK family: each has 5–6 LRRs in the extracellular domain that is presumed to bind one or more extracellular ligands. Although LePRK1, LePRK2 and PiPRK1 have kinase activity, they have amino acid differences in several of the normally invariant residues within the kinase subdomains (Muschietti *et al.*, 1998), suggesting that they belong to a LRR-RLK subfamily that is distinct from other groups. Another LRR-receptor kinase, RKF1, is pollen-specific (Takahashi *et al.*, 1998), but it has 13 LRRs and retains the invariant amino acids in the kinase subdomains.

Because LePRK1 and LePRK2 are expressed late in pollen development, and especially because the level of LePRK2 dramatically increases after pollen germinates (Muschietti *et al.*, 1998), it is likely that they are involved in mediating pollen tube growth. In addition, LePRK2 is phosphorylated in pollen membranes, and is specifically dephosphorylated after the membranes are incubated with style extracts (Muschi-etti *et al.*, 1998). Here we characterize several new receptor kinases from a variety of plant species, and thereby define a subfamily of LePRK-like proteins, all pollen-expressed, that share structural similarity and characteristic amino acid variations. We demon-

strate distinct but overlapping localization patterns for three tomato representatives of this subfamily, using a modified immunolocalization method. Together this information will help in defining and understanding the roles these receptor kinases play during pollen tube growth.

Materials and methods

Plant materials

Lycopersicon esculentum (cvs. VF36 and VFNT Cherry), *Lycopersicon pennellii* (LA2963), *Lycopersicon peruvianum* (LA385), *Lycopersicon pimpinellifolium* (LA1645), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum* cv. Samsun), *Arabidopsis thaliana* (Col-0) and *Zea mays* (B73) were grown under standard greenhouse conditions. Pollen was collected and stored at -80°C until required, or used directly for germination and immunolocalization experiments.

RT-PCR

Mature pollen RNA was used for reverse transcription (RT) reactions. For maize, a ca. 200 bp fragment was obtained using the degenerate primers IX-2 and VI (Muschietti *et al.*, 1998). For tomato, a primer corresponding to kinase domain IV (P3: 5'-TC(CT)TC(CT)TT(CT)CT(AG)TA(AG)TA-3') was used for the RT-PCR. The resulting cDNA was amplified using P3 and a 5' primer corresponding to a portion the extracellular domain (P2: 5'-AA(CT)AA(CT)AA(AG)TT(CT)TCIGG-3'). PCR products of the sizes predicted for LePRK1 (ca. 882 bp) and LePRK2 (ca. 831 bp) were sequenced.

For *Arabidopsis*, primer sequences are as follows: AtPRK3 (GenBank CAB86675) forward primer 5'-GTTTTTCCTTCACCCCTTCTCT-3' and reverse primer 5'-CCCTAATCCTTTTCAACAC-3'; AtPRKa (GenBank AP000383) forward primer 5'-AACACCGCCTCAACCAAA-3' and reverse primer 5'-TATCAACCGAGAAGCAAAGAC-3'; AtPRKb (GenBank AC012561) forward primer 5'-CAGATTGTCTCTTGCGATTCAAA-3' and reverse primer 5'-TCTTCCCTTTTCATTTCTTGTC-3'; AtPRKc (GenBank AAC67207) forward primer 5'-CCAGGACCATCAAGTCTACAAA-3' and reverse primer 5'-TCTTCATCTTCTCAACAGCC-3'; AtPRKd (GenBank AB025636) forward primer 5'-CCTTTTCTTCTCCACA CCA-3' and reverse primer 5'-ACGACCTACC

ATCAGTTTCACTC-3'. AtPRKe (GenBank CAA16528.1) appears to be mis-annotated in the *Arabidopsis* database with an erroneous amino acid extension at the C-terminus of the protein; we believe that AtPRKe encodes a 684 amino acid protein.

A pollen-specific pectate lyase-like gene, At59 (Kulikauskas and McCormick, 1997), was used for the positive control. At59 primers are forward primers 5'-ATGGCAGCAGCTTTTCTTG-3' and reverse primer 5'-CGTTCCTATCTGCAACGAGG-3'.

cDNA and genomic library screening

For maize, an amplified cDNA library from immature starch-filled maize pollen (Ky21; Rubenstein *et al.*, 1995) was screened with the ca. 200 bp RT-PCR product. A ca. 1.4 kb cDNA (cDNA11) was used to screen a genomic library (B73; Zhang and Walker, 1993) to obtain the complete gene, ZmPRK1. The intron/exon boundaries were confirmed by RT-PCR and PCR on genomic DNA. For tomato, an amplified cDNA library from mature anther poly(A)⁺ RNA of *L. esculentum* cv. VF36 (McCormick *et al.*, 1987) was screened with a 480 bp *EcoRI/PvuII* DNA fragment of the LePRK3-VFNT PCR product to obtain a LePRK3 cDNA clone from *L. esculentum* cv. VF36. The 5' region of the LePRK3 cDNA was cloned (Marathon cDNA Amplification RACE Kit, Clontech, Palo Alto, CA) using API1(Clontech) and a primer specific for the extracellular region of LePRK3 (3RACE5-1, 5' AGAGAGATGCCATTTTGGAGAAGAAATCTGGAGG-3'). The 3' end of the cDNA was obtained by PCR, using the amplified cDNA library as template, with two primers specific to the LePRK3 kinase domain (3RACE3-2, 5'-CAGTCAGCAATTGAAGAGAATA GAGTATCAGAAT-3'; 3RACE3-3, 5'-AGAAACAGAAAAGGATTCCC TTGAAATGATGGAGA-3') and the λ gt10 reverse primer (Clontech). The sequence of the LePRK3 coding region was deduced by comparing the sequences of the cDNA and PCR products, and confirmed by RT-PCR with *L. esculentum* cv. VF36 pollen RNA. The intron/exon boundaries were confirmed by PCR on genomic DNA.

Cloning of PRK3 homologues

PRK3 homologues from *L. pennellii*, potato (*S. tuberosum*) and tobacco (*N. tabacum* cv. Samsun) were obtained by RT-PCR with primers PRK3E (5'-TTCATTGATGGAAACCAGTTTTCCGGA-3') and PRK3K (5'-CAACTTCTCTCTTTTCCGTAATGG TATGC-3') from the LePRK3 cDNA sequence.

The RT reaction used the PRK3K primer, and PCR on the resulting cDNA used the PRK3E and PRK3K primers. PCR products of the predicted size (ca. 850 bp) were subcloned. To confirm that tomato had an equivalent of *NtPRK3**, RT-PCR with primers PRK3A (5'-GCG(CT)AAGGA(AT)GATCA(AT)TT(TC)GA(CA)AAG-3') and PRK3B (5'-CCAAGGACTT CAGC(AT)GCTGCC-3') yielded two fragments, one corresponding to the expected size for *LePRK3* (ca. 220 bp) and one of ca. 250 bp.

DNA sequence and parsimony analysis

DNA sequence analysis was carried out with Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, MI). Database searches used the BLAST Network Service (Altschul *et al.*, 1997). Amino acid alignments were performed with DNASTAR software (DNASTAR, Madison, WI), ClustalW and Kyte/Doolittle analysis.

Putative pollen RLKs from *Arabidopsis* were evaluated using several web-based programs to get predictive information about protein topology. Accession numbers not shown in Table 1 are AtPRK3b (AF296832), AtPRKg (AAB95307), AtPRKh (AAC95351) and AtPRKi (AAB65497). An initial Fitch parsimony heuristic search was performed with 1000 random addition replicates with tree bisection-reconnection (TBR) branch swapping. The resulting Fitch trees were used as starting trees for a heuristic search with successive weighting (Farris, 1969; Carpenter, 1988) applied, TBR branch swapping, and MULPARS in effect. The heuristic search was continued until the tree length remained constant in two successive rounds. Confidence limits for clades were assessed by conducting a 'fast' bootstrap (Felsenstein, 1985) of 1000 replicates with no swapping and successive weights applied.

DNA and RNA analysis

Genomic DNA was extracted from leaves (Della-porta *et al.*, 1983). For *Arabidopsis*, total RNA was extracted according to Kulikauskas and McCormick (1997). Maize and tomato RNA was extracted (Logemann *et al.*, 1987) and samples separated on formaldehyde gels, transferred to nylon membranes and UV-cross-linked with a Stratalinker (Stratagene, La Jolla, CA). Blots were pre-hybridized, hybridized and washed according to Sambrook *et al.* (1989).

Expression of the extracellular domain and preparation of polyclonal antibody

A PCR product corresponding to the extracellular domain of *LePRK3* (amino acids 23–246) was subcloned into the *NheI*-*Bam*HI sites of the pRSETB vector (Invitrogen, San Diego, CA) and used to express the extracellular domain recombinant protein in *E. coli* BL21(DE3) pLysE (Novagen, Madison, WI) according to the manufacturer's instruction. The recombinant protein was affinity-purified on a Ni-NTA agarose column (Qiagen, Valencia, CA) and the expected size band (ca. 29 kDa) was eluted after SDS-PAGE. The protein was concentrated and dialyzed with Centriprep-10 (Amicon, Beverly, MA). Protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL). Mouse polyclonal antibodies were obtained as described in Muschietti *et al.* (1998).

Immunoblot analysis

In vitro pollen germination of *L. esculentum* cv. VF36 was according to Muschietti *et al.* (1998). Total protein was extracted from mature pollen and ca. 16 h germinated pollen as described by Muschietti *et al.* (1998) except that the extraction buffer contained Complete (Boehringer Mannheim, Indianapolis, IN) as the only protease inhibitor. Crude microsomal and cytoplasmic fractions were obtained essentially as described by Muschietti *et al.* (1998); note that the pellet was incubated with 0.1% TritonX-100 in extraction buffer for 30 min on ice before the centrifugation step, yielding the P100 fraction. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham, Piscataway, NJ) using the semi Dry Blotter (E & K Scientific Products, Saratoga, CA). Membranes were blocked in 5% milk, 2% glycine in 1 × TBS and incubated overnight at 4 °C with *LePRK3* antibody (diluted 1:1000 in 5% milk, 2% glycine, 0.2% Triton X-100, in 1 × TBS). Blots were washed (1 × TBS and 0.2% Triton X-100, 1 h, room temperature) then incubated with anti-mouse secondary antibody conjugated with horseradish peroxidase (diluted 1:1000 in 5% milk, 2% glycine, 0.2% Triton X-100, in 1 × TBS) for 2 h at room temperature. The blots were washed once (1 h) then developed with the Enhanced chemiluminescence (ECL) western blotting detection system (Amersham). For the deglycosylation experiment, the P100 fraction was treated with Endo H according to manufacturer's instructions (Boehringer Mannheim).

Table 1. Selected pollen-expressed receptor kinases from *Arabidopsis*, tomato, maize and petunia.

PRK	GenBank accession number	Amino acids	Kinase domain diagnostic amino acids				LRRs
			I	II	VIb	VII	
LePRK1	AAC12254	669	T	V	H	DYA	6
LePRK2	AAC11253	642	T	V	H	DYA	5
LePRK3	AF243040	612	A	V	N	DYA	5
ZmPRK1	AF243041	747	C	V	H	DYA	6
PiPRK1	AAA33715	720	S	V	H	DYT	5
AtPRK3	CAB86675	633	A	V	N	DYA	5
AtPRKa	AP000383	671	S	V	H	DYA	6
AtPRKb	AC012561	686	S	V	H	DYA	5
AtPRKc	AAC67207	629	S	V	H	DYG	5
AtPRKd	AB025636	709	S	V	H	DYG	6
Generic kinase			V	A	D	DFG	

Immunolocalization

Immunolocalization essentially followed Lin *et al.* (1996), but omitting cell wall digestion enzymes. Germinated pollen was allowed to settle (room temperature, 20 min) to the bottom of the petri dish before further processing. Most of the medium was carefully removed, and the remaining medium and germinated pollen transferred to a 15 ml Falcon tube. A 3× volume of fixative solution (4% paraformaldehyde, 50 mM Hepes pH 7, 2 mM MgCl₂, 10% sucrose) was added and the germinated pollen was allowed to settle. After ca. 2.5 h as much as possible of the fixative/germination medium was removed and replaced with fresh fixative solution. After 1 h the fixative solution was removed and replaced with 1× PBS. After 30 min the PBS was removed and the germinated pollen suspension was transferred onto slides (Probe On Plus, Fisher Scientific, Pittsburgh, PA). The germinated pollen grains were allowed to settle onto the slide for no longer than 12–15 min so that the slides did not dry. Then the slides were placed in a petri dish adjacent to rolled moist Kimwipes (Kimberly-Clark, Roswell, GA), blocked with 3% milk in PBS (room temperature, 1 h) then incubated with 1% milk in PBS with either LePRK1 (1:50), LePRK2 (1:50), LePRK3 (1:20) or α -tubulin (1:50)(Amersham) antibodies (4 °C, overnight), washed with 1× PBS (3×, 20 min) then with FITC conjugated goat F(ab')₂ anti-mouse IgG (H+L) antibody (1:50) (Caltag Laboratories, San Francisco, CA) (27 °C, 2 h). Control slides were incubated with FITC alone. Slides were mounted with Vectashield (Vector, Burlingame, CA),

after additional PBS washes, sealed with nail polish and stored at –20 °C. Slides were examined with an Axiophot epifluorescence photomicroscope (Zeiss, Thornwood, NY). Some images were acquired with a deconvolution microscope from Applied Precision.

Results

Isolation of *ZmPRK1*

We have previously isolated two pollen-specific receptor-like kinase genes from *L. esculentum* by RT-PCR, using mixed primers encoding the conserved kinase subdomains VI and IX (Muschiatti *et al.*, 1998). To isolate kinase genes that were expressed in mature pollen of *Zea mays*, the same degenerate primers were used for RT-PCR, with maize mature pollen poly(A)⁺ RNA. An amplified product cDNA was cloned and its deduced sequence was similar to that region of LePRK1 and LePRK2. We then screened a pollen cDNA library and subsequently a genomic library of maize, to obtain the complete sequence of *ZmPRK1* (for *Zea mays* pollen-expressed receptor-like kinase1; GenBank accession number AF243041). *ZmPRK1* has two small introns (111 and 115 bp) and encodes a protein of ca. 79 kDa. RNA blot analysis showed that *ZmPRK1* is specifically expressed in mature pollen and is barely detectable in tri-nucleate stage immature pollen (Figure 1). According to the deduced amino acid sequence, *ZmPRK1* has an extracellular domain containing 5 LRRs, a single transmembrane domain, and a cytoplasmic kinase domain; these domains show

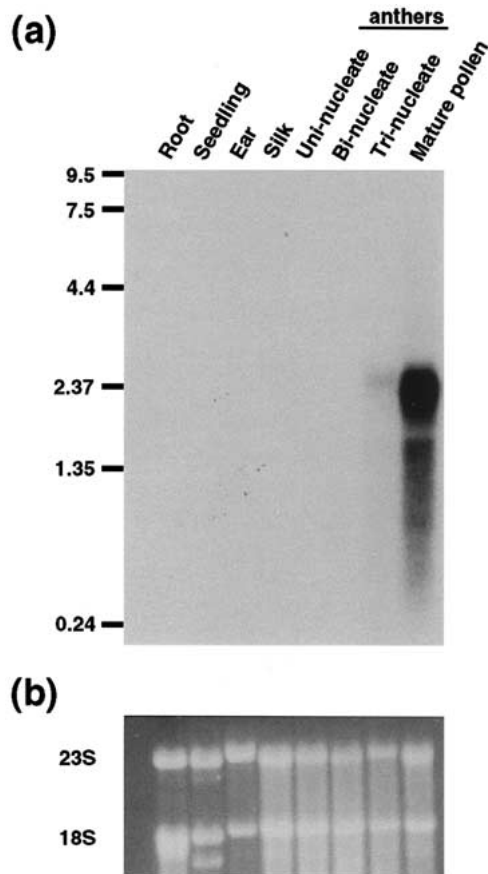


Figure 1. *ZmPRK1* expression is pollen-specific. Total RNA (5 μ g) was separated by gel electrophoresis, transferred to a nylon membrane and hybridized with a cDNA fragment containing the entire *ZmPRK1* kinase domain. RNA size markers (in kb) are shown on the left.

43%, 31% and 55% amino acid identity, respectively, to the corresponding domains of LePRK1. Furthermore, *ZmPRK1* has the same amino acid substitutions in some of the normally invariant positions within the kinase subdomains (Table 1), lending more support to the idea that these pollen-specific RLKs represent a distinct subclass of plant RLKs. *ZmPRK1* is so far the first pollen-specific LRR-RLK identified in maize. DNA blot analysis showed that *ZmPRK1* corresponds to a single-copy gene (data not shown) and maps to chromosome 1, bin 1.06.

Isolation of LePRK3

We reasoned that a RT-PCR survey for pollen-expressed kinases in other Solanaceae would allow us to quickly pinpoint highly conserved amino acids in LePRK1 and LePRK2. For this purpose, we aligned

the sequences of the four available pollen-receptor kinases (*PiPRK1*, *LePRK1*, *LePRK2* and *ZmPRK1*) and designed primers based on two highly conserved domains, one in the extracellular domain and one corresponding to kinase domain IV. With these primers, RT-PCR on pollen RNA of other Solanaceae yielded products that corresponded to the sizes expected for *LePRK1* and *LePRK2*.

Upon cloning and sequencing some of the *LePRK2*-sized products we found that one clone, derived from RT-PCR with RNA prepared from a different cultivar of *L. esculentum* (cv. VFNT Cherry), was quite different in sequence. Because different cultivars of *L. esculentum* were expected to have nearly identical sequences for a given gene, this sequence was unlikely to represent a *bona fide* *LePRK2* sequence. We therefore named this gene *LePRK3*.

In order to obtain the complete sequence of *LePRK3*, we screened a mature anther cDNA library of tomato with a portion of the *LePRK3* RT-PCR product. Two cDNAs, of 1.6 kb and 0.85 kb, were obtained. Sequencing results confirmed that the two clones were identical in the region of overlap, but neither was full-length. 5' RACE with a gene-specific primer yielded a sequence that encoded the N-terminus of the *LePRK3* protein. We used phage DNA from the pollen cDNA library as template, with the λ gt10 reverse primer and a 3'-gene-specific primer, and isolated the 3' region of the gene. Because of the composite nature of this deduced sequence, we confirmed the existence of the full-length cDNA using primers within the deduced 5' and 3' UTRs. The sequenced region is 2090 bp long and includes 144 bp of 5' UTR and 110 bp of 3' UTR. *LePRK3* has one intron, of 289 bp. The predicted protein has 612 amino acids and, as found in the other pollen receptor kinases, has an extracellular domain with six leucine-rich repeats (Figure 2). *LePRK1* and *LePRK2* show amino acid identities of 50% in the extracellular domain, 47% identity in the cytoplasmic juxtamembrane domain (termed variable domain in Figure 2) and 75% identity in the kinase domain (Muschiatti *et al.*, 1998). By contrast, *LePRK3* is more divergent, showing only 36% or 39% amino acid identity to *LePRK1* or *LePRK2* in the extracellular domain, only 14% or 19% identity in the variable domain, and only 51% or 53% identity in the kinase domain.

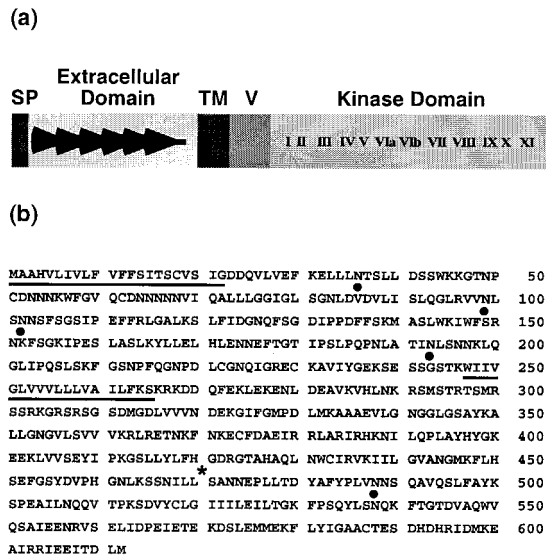


Figure 2. Schematic of *LePRK3* gene and encoded protein. a. Gene structure. SP indicates signal peptide, arrows indicate LRRs, TM indicates transmembrane domain, V indicates variable domain and roman numerals indicate conserved domains within kinase. b. The deduced amino acid sequence. The hydrophobic signal peptide and transmembrane domain are marked by solid underlines. The 5 potential glycosylation sites are indicated with dots, and the position of the 289 bp intron is indicated with an asterisk. The GenBank accession number for *LePRK3* is AF243040.

LePRK3 is expressed late in pollen development

We initially identified *LePRK3* via RT-PCR, so we knew it was expressed in pollen. To determine the expression pattern of *LePRK3*, an RNA blot was prepared, using total RNA from different tissues and developmental stages (Figure 3). Using a probe corresponding to part of *LePRK3* (amino acids 177–336), we detected a transcript of ca. 2.9 kb only in mature anther and pollen RNA samples. The coding region corresponds to a mRNA size of ca. 2 kb; we presume that additional sequences in the 5' and 3' UTRs account for the larger transcript size. Thus, like *LePRK1* and *LePRK2* (Muschiatti *et al.*, 1998), *LePRK3* is also specifically expressed in pollen. A DNA blot probed with the extracellular domain indicates that *LePRK3* is also a single-copy gene (data not shown).

LePRK3 is glycosylated

Antibodies raised against the extracellular domains of *LePRK1* and *LePRK2* recognized proteins in pollen membrane fractions (Muschiatti *et al.*, 1998). The predicted size for *LePRK3* is 68 kDa. Figure 4 shows that antibody raised against the extracellular domain

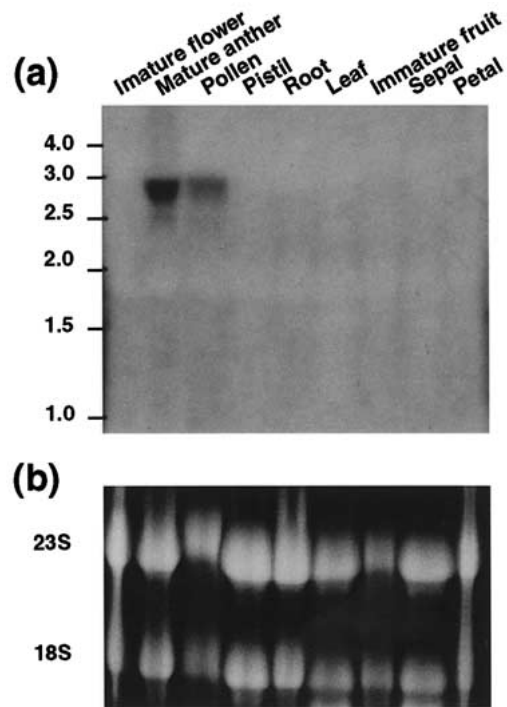


Figure 3. *LePRK3* expression is pollen-specific. a. Total RNA (15 μ g) was separated by gel electrophoresis, transferred onto a nylon membrane and hybridized with a 480 bp *EcoRI/PvuII* DNA fragment from the *L. esculentum* (cv. VFNT) PCR product. RNA size markers (in kb) are shown on the left. B. EtBr-stained gel to show loading.

of *LePRK3* variably recognized a 68 kDa protein, a 78 kDa protein, or both. In the course of our experiments, protein extracts were prepared on 15 different days, so perhaps the pollen used for these different extracts varied in some way. *LePRK3* has 4 predicted glycosylation sites in the extracellular domain. It seemed possible that the 78 kDa protein represented a glycosylated version of *LePRK3*, and indeed, endo H treatment of a membrane preparation containing the 78 kDa version of the protein yielded a 68 kDa protein (Figure 4). *LePRK3* can be immunolocalized to pollen tubes (see below), but it is notable that neither version of the *LePRK3* protein was detectable by immunoblot when membrane preparations were prepared from germinated pollen (data not shown). However, these same membrane preparations showed the expected (Muschiatti *et al.*, 1998) distinct signal for *LePRK1*. It is possible that the *LePRK3* epitopes are less accessible in membrane preparations from germinated pollen.

Tomato PRKs immunolocalize to the pollen tube wall

One way to address whether LePRK1, LePRK2 and LePRK3 have similar or different roles during pollen development and germination is to determine their cellular or sub-cellular localization patterns. Immunolocalization with antibodies raised against the extracellular domains of LePRK1 and LePRK2 showed uniform labeling on the pollen tube margins (Muschiatti *et al.*, 1998). However, Muschiatti *et al.* (1998) used a fixation/permeabilization method designed for the immunodetection of Rop1 (Lin *et al.*, 1996), which is a cytoplasmic protein. In this protocol, the pollen tube walls are partially digested with cellulase and macerated in order to allow the anti-Rop antibodies' access to the epitopes. In retrospect, this method could have somewhat disturbed the *in vivo* location of the receptor-like kinases, whose extracellular domains are predicted to be in the pollen tube cell walls.

In this study, we modified the method and used only a mild fixation but no enzymatic digestion of pollen tubes, for immunolocalization with the antibodies raised against the extracellular domains of LePRK1, LePRK2 and LePRK3 separately. Figure 5 shows that under these fixation and incubation conditions, the three anti-LePRK antibodies have overlapping but distinct patterns of labeling, while no signal could be seen on pollen tubes when an antibody raised against a cytoplasmic protein (α -tubulin) or the secondary antibody alone is used (Figure 5j, k). The images shown in Figure 5 illustrate the different labeling patterns observed, in each of 4 separate experiments. For each antibody at least 200 intact pollen tubes were scored. Table 2 shows the frequency for one of the patterns, from over 700 pollen tubes (intact and burst), from 2 additional experiments. The anti-LePRK1 antibody showed rather uniform labeling along the margins of the pollen tube (Figure 5a–c), although some also showed a hoop-like striping along the tube (Figure 5b). The anti-LePRK2 antibody labeled the pollen tube margins (Figure 5d–f) and in addition most (see Table 2) showed a collar-like labeling near the grain-tube interface (Figure 5d and f). Anti-LePRK3 antibodies labeled the margins of the pollen tube (Figure 5g–i) and also showed hoop-like striping along the tube (Figure 5g); a minority (Table 2) showed a collar-like labeling near the grain-tube interface (Figure 5i). The number of tubes with hoop-like striping was not tabulated; although many had distinctive striping, others appeared to have none, and others were intermediate, perhaps depending on the

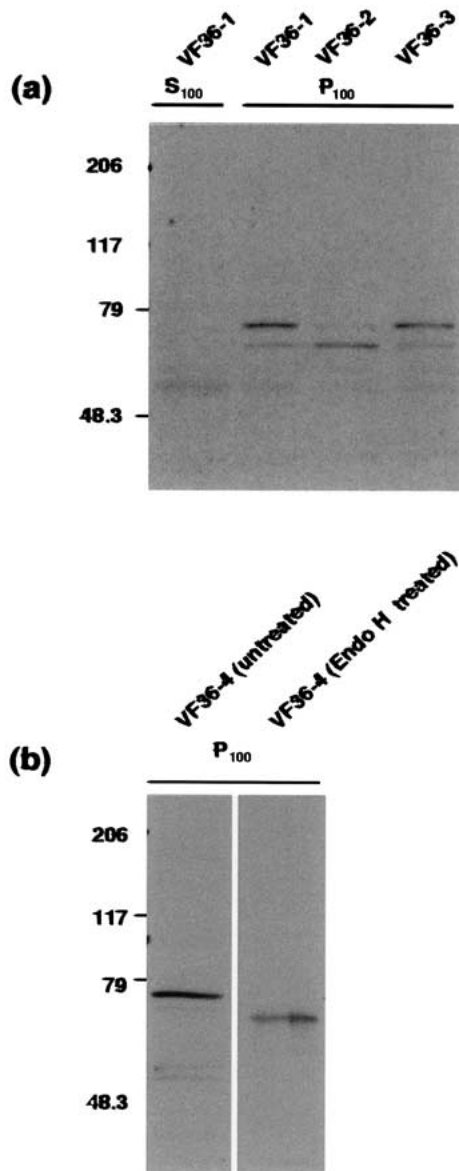


Figure 4. Immunoblot analysis of LePRK3 in pollen extracts. a. Pollen protein extracts (100 μ g) from 3 separate plants were separated on a 7.5% SDS-PAGE gel, blotted onto nitrocellulose membrane and incubated with antibody raised against the extracellular domain of LePRK3 (1:1000). P100, crude microsomal fraction; S100, crude cytoplasmic fraction. Molecular mass markers (in kDa) shown on the left. b. Pollen protein extract (80 μ g) from a plant whose pollen only had the 78 kDa version of LePRK3 was either not treated or endo H-treated, and immunoblotted as described in a.

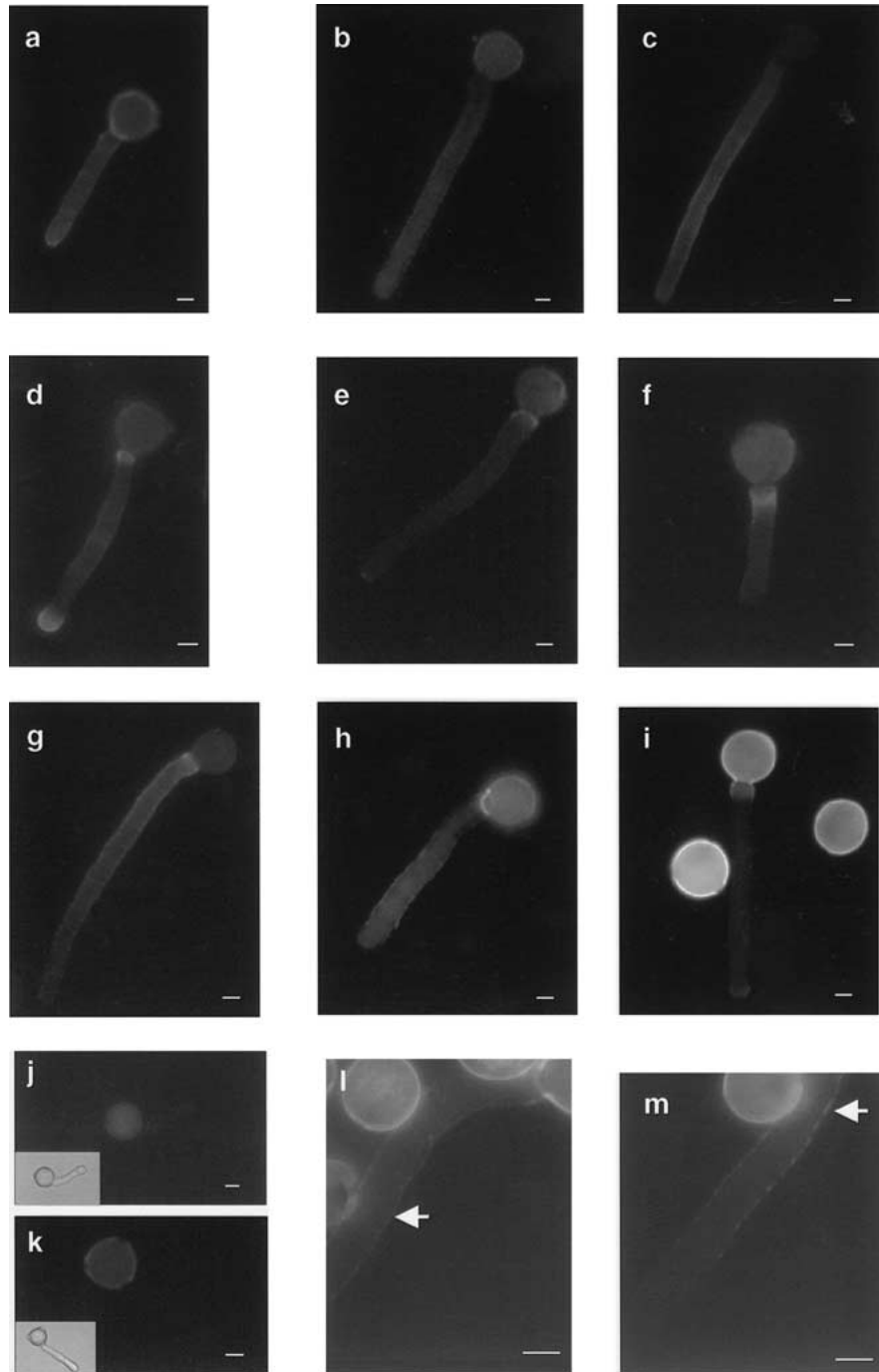


Figure 5. Immunolocalization of LePRK proteins. Pollen was germinated *in vitro*, fixed and incubated with primary antibodies, and detected with a fluorescein isothiocyanate-conjugated secondary antibody. Scale bar is 10 μm . a–c, anti-LePRK1 antibody; d–f, anti-LePRK2 antibody; g–i, anti-LePRK3 antibody; j, secondary antibody alone, the inset shows a bright-field image of the same pollen grain; k, anti- α -tubulin antibody, the inset shows a bright-field image of the same pollen grain; l and m, anti-LePRK3 antibody, with 16 h germinated tubes. A montage of one pollen tube, to show the segmented pattern of labeling at the margin (membrane/wall) in more detail. The arrows indicate the position of overlap.

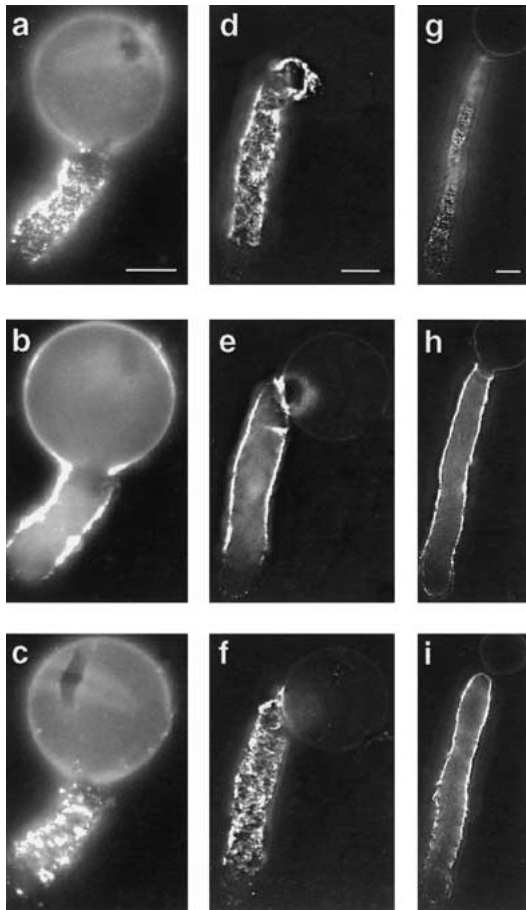


Figure 6. Immunolocalization images with a deconvolution microscope. Three Z-section images for each antibody are shown. Anti-LePRK1 antibody: a, top; b, middle; c, bottom. Anti-LePRK2 antibody: d, top; e, middle; f, bottom. Anti-LePRK3 antibody: g, top; h, middle; i, near bottom. Scale bar is 10 μ m.

plane of focus. On tubes with hoop-like striping, the pattern seen with both anti-LePRK1 and LePRK3 was very regular along the length of the tube (Figure 5b, g) and at further 2.5-fold magnification there were clear gaps between labeled regions (Figure 5l and m). Note that the images in Figure 5l and m are from LePRK3 immunolocalization at 16 h germination (see immunoblot results, above). No obvious hoop-like striping was seen with anti-LePRK2. Labeling at the pollen tube tips was sometimes seen (Figure 5d, i); as discussed by He and Wetzstein (1995), apical regions are thought to be more sensitive to fixation than other regions of the pollen tube. Indeed, in some experiments there were numerous burst tubes with intense labeling near the tip.

Table 2. Frequency (%) of collar fluorescence at the grain-tube interface.

	Collar	No collar
Anti-LePRK1	0	100
	0	100
Anti-LePRK2	98	2
	89	11
Anti-LePRK3	14	86
	17	83

To obtain more detailed images, we used a deconvolution microscope to remove out of focus light, using the same preparations shown in Figure 5. Figure 6 shows 3 different Z-sections of single pollen grains. Figure 6a, d and g are focused near the top surface of the pollen tube, Figure 6b, e and h are focused at the mid-plane of the pollen tube, and Figure 6c, f and i are focused near the bottom surface of the pollen tube. LePRK1 shows a patchy signal on the surface of the grain and reduced labeling at the tip. LePRK2 shows a pattern similar to that seen with LePRK1, but the collar seen in Figure 5 is more distinctive when the out-of-focus light is removed. The hoop-like striping pattern for the LePRK3 immunolocalization is particularly striking on the top surface image (Figure 6g); there are clearly horizontal gaps along the pollen tube. The striping pattern seen with LePRK3 and LePRK1 is similar to the patterns seen with anti-AGP antibody (Li *et al.*, 1992) and with antibodies that detect pectins (Stepka *et al.*, 2000). The collar-like labeling is similar to the pattern seen with an anti-callose antibody (Li *et al.*, 1999).

Homologues of LePRK1, LePRK2, and LePRK3 in the Solanaceae

As discussed above, we designed degenerate primers corresponding to conserved regions of the pollen kinases in order to obtain partial sequences of homologues from other Solanaceae. Once we had discovered the existence of LePRK3, we designed two other primers, PRK3-1 and PRK3-2, to isolate homologues of LePRK3. In Figure 7, the deduced amino acid sequences of these PCR products for each of the genes are aligned with the appropriate portion of the *L. esculentum* sequences of LePRK1, LePRK2 and LePRK3. Within the *Lycopersicon* genus, *L. pimpinellifolium* is the most closely related to *L. esculentum*, followed by *L. pennellii* and then *L. peruvianum* (Miller and

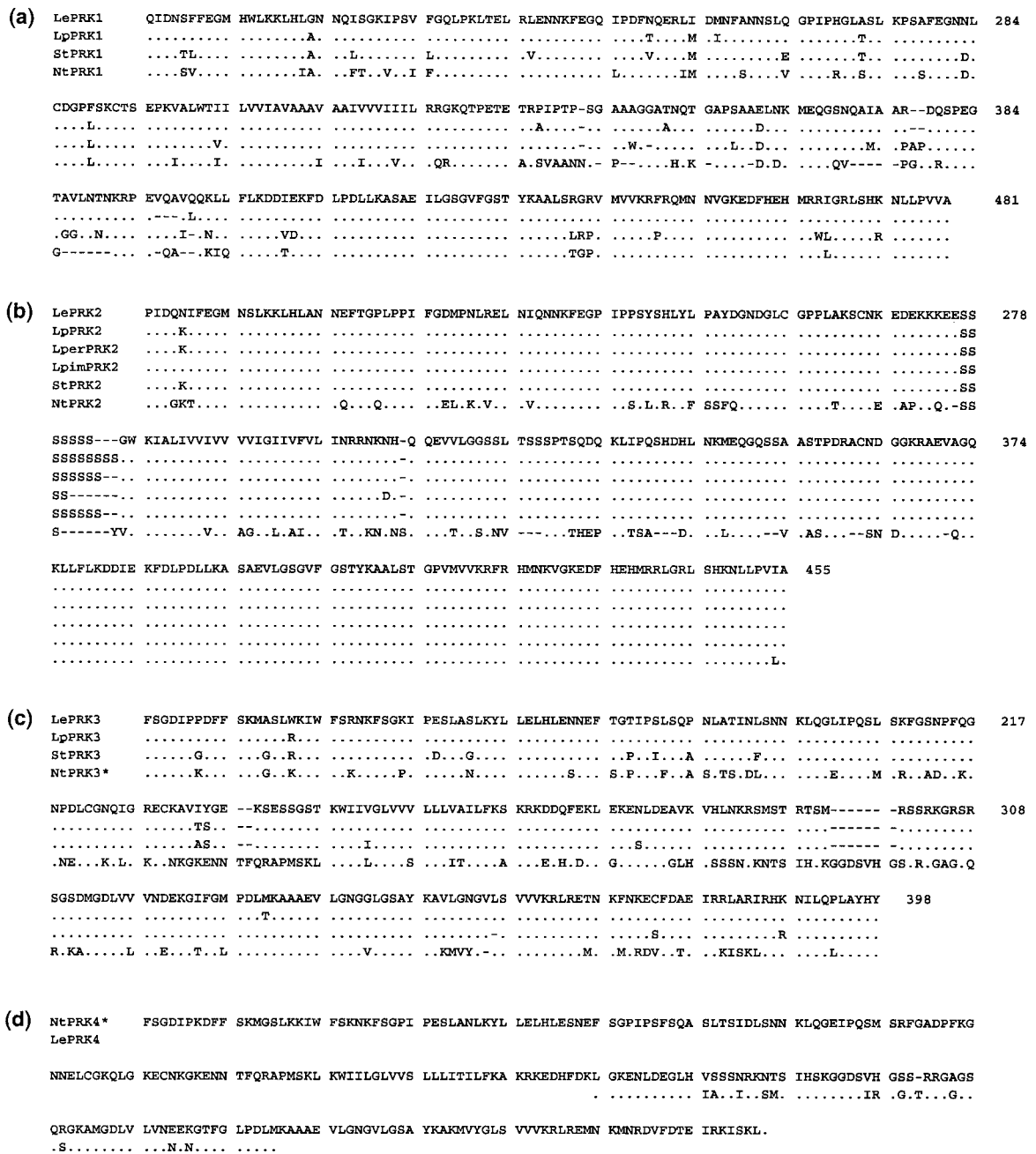


Figure 7. Alignment of putative homologues of pollen receptor-like kinases. a. Comparison of PRK1 homologues. b. Comparison of PRK2 homologues. c. Comparison of PRK3 homologues. Le, *L. esculentum*; Lpim, *L. pimpinellifolium*; Lp, *L. pennellii*; Lper, *L. peruvianum*; St, *S. tuberosum*; Nt, *Nicotiana tabacum*. d. Comparison of NtPRK3* and deduced amino acid sequence of RT-PCR product from *L. esculentum*. GenBank accession numbers for PRK1: AF246966 (Lp), AF246964 (Nt), AF246965 (St); for PRK2: AF246970 (Lpim), AF246969 (Lp), AF246967 (Nt), AF246968 (St); for PRK3: AF252412 (Lp), AF252413 (St), AF267178 (Lper); and for PRK4: AF252415 (LePRK4), AF252414 (NtPRK3*).

Tanksley, 1990). The evolutionary distance between tomato and potato has been estimated to be 5 million years and between tomato and tobacco 15 million years (Kawagoe and Kikuta, 1991). Thus we anticipated that the *Lycopersicon* sequences would be most similar to each other, followed by potato and then tobacco. For PRK1, this appears to be true. There are sporadic amino acid substitutions throughout the sequenced region. For the PRK2 sequences, there is exceptionally high conservation among the *Lycopersicon* and potato sequences; only the number of serine residues just N-terminal to the transmembrane domain vary. In the tobacco PRK2 sequence, although the kinase domain is nearly identical, there are numerous amino acid substitutions in the extracellular domain and in the variable domain. For PRK3, the *Lycopersicon* species and potato show sporadic amino acid substitutions, but the tobacco sequence, denoted NtPRK3*, is strikingly different. In order to test whether this tobacco sequence was the true PRK3 homologue, or if it might correspond to the sequence of another PRK gene, primers that amplify across this region were used for RT-PCR with RNA prepared from *L. esculentum* pollen. Sequencing confirmed that a sequence more similar to the NtPRK3* sequence exists in *L. esculentum* (Figure 7D). Thus the NtPRK3* sequence shown in Figure 7C probably corresponds to a portion of the tobacco homologue of another pollen-expressed RLK, which we named LePRK4, although the full-length sequence is not yet available. We predict that a true homologue of LePRK3 will exist in tobacco. It is similarly possible that the tobacco PRK2 sequence shown in Figure 2B corresponds to another PRK in tomato; we did not do PCR experiments to test this possibility.

Identifying Arabidopsis pollen receptor kinases

We were able to identify additional pollen receptor kinases in tomato after only limited efforts with degenerate primers and RT-PCR. It is difficult to predict whether more extensive efforts of this sort will yield all the pollen receptor kinases that exist in one species. We therefore decided to take advantage of the information from the *Arabidopsis* genome sequence, in order to identify candidate pollen receptor kinases. We performed BLAST searches of the *Arabidopsis* database using LePRK1. Numerous hits were obtained. Potential pollen receptor kinases were identified among these BLAST hits by checking for the presence of variant amino acid residues typical of pollen recep-

tor kinases, as discussed in Muschietti *et al.* (1998) and shown in Table 1. AtPRK candidates with these diagnostic amino acids and with topologies that were similar to the LePRKs (i.e. 5–7 LRRs) were tested by RT-PCR, using RNA prepared from pollen, flower and leaf. Each of the *Arabidopsis* candidates shown in Table 1 was indeed expressed in pollen and/or in flower, but not in leaf (data not shown).

To determine the relationships among the pollen receptor kinases, parsimony analysis was performed using PAUP 3.1.1 (Swofford, 1993) on a matrix of 347 characters, corresponding to part of the extracellular domain through kinase domain IV, as shown in Figure 7. Amino acid sequences were aligned using ClustalW (<http://www.clustalw.genome.ad.jp>) and optimized by eye. Twenty-five PRKs from eight species were used in the analysis: ten *Arabidopsis thaliana* (At) PRKs, i.e. AtPRK3a, AtPRK3b, AtPRKa, AtPRKb, AtPRKc, AtPRKd, AtPRKe, AtPRKg, AtPRKh, AtPRKi; three *Lycopersicon esculentum* (Le) PRKs, i.e. LePRK1, LePRK2, LePRK3; three *L. pennellii* (Lp) PRKs, i.e. LpPRK1, LpPRK2, LpPRK3; *L. pimpinellifolium* (Lpim) PRK2; *L. peruvianum* (Lper) PRK2; three *Solanum tuberosum* (St) PRKs, i.e. StPRK1, StPRK2, and StPRK3; three *Nicotiana tabacum* (Nt) PRKs, i.e. NtPRK1, NtPRK2, NtPRK3; and *Petunia inflata* (Pi) PRK1. The pollen kinase (PiPRK1) from *Petunia*, a member of the Solanaceae, was used as the outgroup, because PiPRK1 is expressed earlier during pollen development than are LePRK1, LePRK2 and LePRK3, and is therefore thought to play a functionally different role (Mu *et al.*, 1994; Muschietti *et al.*, 1998).

Figure 8 shows that there is strong bootstrap support for the close relationship of PRK1 and PRK2. Within the PRK1 and PRK2 clades, the relationship is consistent with the crossing relationships (Miller and Tanksley, 1990) and evolutionary distances (Kawagoe and Kikuta, 1991) of these species. The PRK3 clade is clearly distinct. Although we believe that NtPRK3 represents a portion of a fourth PRK (Figure 7d), it is clear that the NtPRK3 sequence is most closely related to *bona fide* PRK3 sequences. In addition, there is strong bootstrap support for inclusion of two of the AtPRK genes in the PRK3 clade, and we therefore named them AtPRK3a and AtPRK3b (Figure 8). The intron position of LePRK3 is conserved in both AtPRK3a and AtPRK3b, although the AtPRK3 introns are smaller (89 bp and 119 bp). Among the other *Arabidopsis* genes, AtPRKg, h, and i are sister to the PRK3 clade while AtPRKa, b, c, d, and e genes are

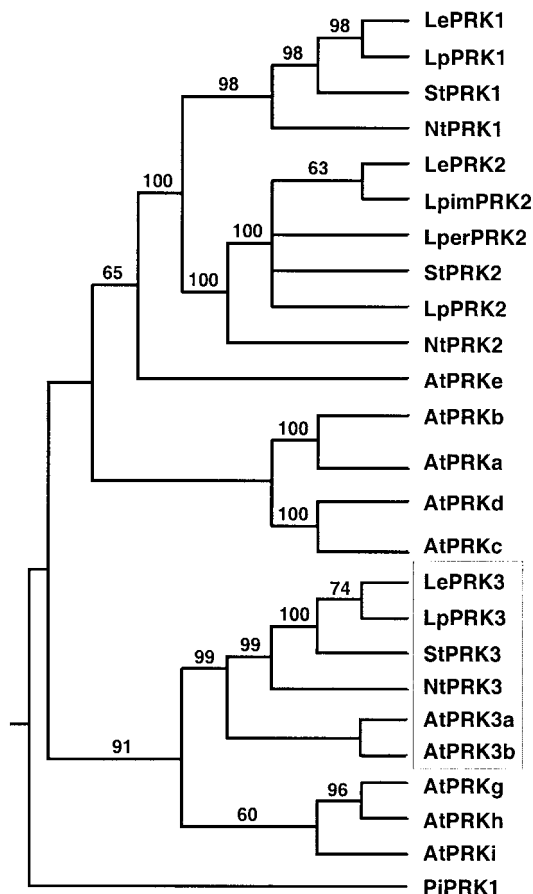


Figure 8. Phylogenetic relationship among PRKs. One of 13 most parsimonious successively weighted trees is shown. Bootstrap values > 60% are indicated. See Materials and methods for details. At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Lp, *L. pennellii*; Lpim, *L. pimpinellifolium*; Lper, *L. peruvianum*; St, *Solanum tuberosum*; Nt, *Nicotiana tabacum*; Pi, *Petunia inflata*.

more closely related to the PRK1 and PRK2 clades. In this group, AtPRKa and AtPRKb are likely duplicate genes. AtPRKe is sister to the PRK1/PRK2 clade. The *Arabidopsis* kinases reported here all fall into subsection III of the LRR cluster defined by Shiu and Bleeker (2001).

Discussion

We characterized 22 genes (full-length or partial) that encode LePRK-like receptor kinases, from tomato and tomato relatives, potato, tobacco, maize and *Arabidopsis*.

For the maize receptor kinase and for all three tomato kinases, expression is first seen in mature

pollen. All of these kinases have similar amino acid substitutions in the normally invariant residues in kinase subdomains (Table 1). Furthermore, although the size of the variable domains of these pollen receptor kinases is similar to that of BRI1 (66 amino acids), the variable domains are larger than those of many other LRR-RLKs, such as CLV1 (40), HAESA (26), SERK (35), RKF 1 (42). There is evidence (He *et al.*, 2000) that the variable domain is necessary for BRI1 to perceive brassinosteroids and transduce signals. Thus, from our work and that of others (Mu *et al.*, 1994; Takahashi *et al.*, 1998) we can conclude that there are at least 3 distinct subclasses of pollen receptor kinases.

Even though the LePRKs are a distinct sub-group, they do differ. The poly-serine motif at the C-terminus of the extracellular domain of the LePRK2-like proteins (Figure 7) is not found in LePRK1 and LePRK3. LePRK1 has two potential N-linked glycosylation sites in the extracellular domain and LePRK3 has four potential sites, but LePRK2 has none. The immunoblots reflect these differences. Only a single protein of the predicted size was detected for LePRK2. LePRK1 sometimes shows a doublet (not shown) that might represent glycosylation differences. We show here that the striking variation in protein size seen for LePRK3 reflects glycosylation differences (Figure 4). Either a single protein (the predicted 68 kDa or a larger 78 kDa) or two proteins (68 kDa and 78 kDa) were detected in a given pollen sample, and the pattern varied between pollen extracts prepared from different pollen collections. We have no evidence that LePRK3 can exist in different states of glycosylation (i.e. one site used, two sites used, etc.) because we saw no gradation in protein sizes. Although we cannot tell whether the glycosylated form is indicative of an earlier or of a later developmental stage, it might be possible to assess the maturity stage of pollen by probing protein extracts with LePRK3 antibody.

The three tomato pollen receptor kinases localize to the plasma membrane/cell wall of the pollen tube, in distinct but overlapping patterns. These distributions are most likely determined by the architectural structure of the pollen cell wall as it grows within the transmitting tract, incorporating material secreted from the pollen tube and surrounding transmitting tract tissue. In previous immunolocalization studies with LePRK1 and LePRK2 (Muschiatti *et al.*, 1998), both showed uniform labeling of the plasma membrane after cell wall digestion. It is interesting that the omission of the digestion step (Figures 5 and 6)

gave a more striking and distinctive fluorescent pattern along the pollen tubes, perhaps more indicative of how the extracellular domains of these proteins are positioned in the cell wall. Other cell wall components, such as arabinogalactans (Li *et al.*, 1992) and pectins (Stepka *et al.*, 2000) have also shown distinctive fluorescent hoop-like patterning, with and without the digestion step respectively. Obviously, accessibility of the antibody to the epitope is important and since the outer cell wall layer is a pectin coat, digestion was not required by Stepka *et al.* (2000) but was for localization of the arabinogalactans localized to the inner callose cell wall (Li *et al.*, 1992). Our results suggest that the extracellular domains of the tomato receptor kinases extend to the outer cell wall. The hoop-like pattern roughly correlates with oscillatory changes in the growth rate, a characteristic feature of pollen tube growth in the Solanaceae (Feijo *et al.*, 2001).

Stepka *et al.* (2000) described pollen from a hollow-style plant, *Ornithogalum*, that exhibited different fluorescence patterns of pectin distribution when the pollen tubes were germinated in different media. In solid medium, pectin showed the periodic hoop-like distribution; while in liquid medium, non-esterified pectin showed uniform labeling, and esterified pectins were only labeled at the tube tip. Our immunolocalization studies were with *in vitro* grown pollen tubes; the presence of 24% PEG (Jahnen *et al.*, 1989) in our germination medium might have provided enough resistance to better mimic that found in the transmitting tract. Feijo *et al.* (2001) find the hoop-like pattern particularly odd for arabinogalactans, because growth occurs at the tip, not at the shank of the pollen tube, where the arabinogalactans are deposited. Indeed, we expect that the kinases are continuously incorporated into new plasma membrane as tip growth proceeds, and we interpret the immunolocalization patterns we see along the shank of the tube (Figures 5 and 6) as a history of past growth oscillations at the tip. That the hoop-like pattern correlates with AGPs is particularly interesting, because at least some arabinogalactan proteins play crucial roles in cell-cell signaling and cell recognition during pollination (Wu *et al.*, 1995, 2000; but see also Sommer-Knudsen *et al.*, 1998). Double antibody staining would be required to determine if the immunolocalization patterns for the AGPs and for LePRK1 and LePRK3 are coincident.

The individual fluorescent patterns seen among the three tomato receptor-like kinases could indicate that each has a specific biological function in the ab-

sence of other kinase partners, while the overlapping patterns make heterodimers feasible. The different LePRKs may bind the same ligands, albeit perhaps with different affinities, or may bind different ligands, based on variability within the LRR motif (Figure 7), or on the composition of the heterodimer. In support of these notions, we have evidence that LePRK1 and LePRK2 can be co-immunoprecipitated from pollen membranes, suggesting that they may exist in heterodimers (Valsecchi, McCormick and Muschietti, in preparation). We are using yeast two-hybrid screens to isolate potential ligands for the tomato kinases. We have evidence that some putative ligands interact with the extracellular domains of LePRK1 and LePRK2, others with LePRK2 and LePRK3, and others with LePRK1 and LePRK3 (Tang, Ezcurra, Muschietti and McCormick, in preparation; Ezcurra, Cotter and McCormick, in preparation). Similarly, we have evidence from yeast two-hybrid screens that there is some overlap between downstream partners, i.e. some proteins interact with the cytoplasmic domains of LePRK1 and LePRK2, others with those of LePRK1 and LePRK3 (Ok, Cotter and McCormick, in preparation). Knowing which amino acids can vary within, for example, the LePRK1 clade in the Solanaceae (Figure 7), will, in the future, help us to determine the precise residues that are important for protein-protein interactions.

Why would the pollen tube need so many receptors? What different kinds of signals might they mediate? An early hypothesis predicted that a single chemical cue, in a continuous gradient emanating from the ovule, would be responsible for pollen tube guidance (Mascarenhas, 1993). Depending on the length of the pistil, a growing pollen tube will traverse between a few millimeters to several centimeters on its way to the ovary. Recent models predict that the maximum distance over which a pollen tube can be guided by diffusible chemical cues is 1–9 mm, implying that multiple chemical cues from intermediate targets in the pistil guide pollen tubes to the ovules (Lush, 1999). The growth of the pollen tube was recently compared with axon guidance in vertebrates (Palanivelu and Preuss, 2000) and, by analogy, pollen tube growth likely involves both short-distance signals such as adhesion molecules, and long-distance signals such as attractants and repellents. The pistil may not always promote growth and guidance of the pollen tube. Maternal selection of the male has been reported in plants (Marshall, 1998), suggesting the existence of rapidly evolving recognition mechanisms between the male and the female, in analogy to recog-

nitiation mechanisms between pathogens and their hosts, which, interestingly, also involve LRR-type receptors (reviewed in van der Biezen and Jones 1998). Finally, male competition may also operate in plants (Marshall *et al.*, 1996) suggesting that pollen tubes may signal to other pollen tubes. Our studies indicate there are at least ten similar pollen receptor kinases in the *Arabidopsis* genome (Figure 8). Now that the *Arabidopsis* genome sequence is completed, it will be possible to systematically generate mutants in each member of this sub-family and determine the role each plays during pollen tube growth.

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