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Characterization of a Pollen-Expressed Receptor-like Kinase Gene of *Petunia inflata* and the Activity of Its Encoded Kinase

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From a pollen tube cDNA library of *Petunia inflata*, we isolated clones encoding a protein with structural features and biochemical properties characteristic of receptor-like kinases. It was designated PRK1 for pollen receptor-like kinase 1. The cytoplasmic domain of PRK1 is highly similar to the kinase domains of other plant receptor-like kinases and contains nearly all of the conserved amino acids for serine/threonine kinases. The extracellular domain of PRK1 contains leucine-rich repeats as found in some other plant receptor-like kinases, but overall its sequence in this region does not share significant similarity. Characterization of a gene encoding PRK1 revealed the presence of two introns. During pollen development, *PRK1* mRNA was first detected in anthers containing mostly binucleate microspores; it reached the highest level of mature pollen and remained at a high level in in vitro-germinated pollen tubes. The recombinant cytoplasmic domain of PRK1 autophosphorylated on serine and tyrosine, suggesting that PRK1 may be a dual-specificity kinase. Monospecific immune serum to the recombinant extracellular domain of PRK1 detected a 69-kD protein in microsomal membranes of pollen and pollen tubes. The characteristics of PRK1 suggest that it may play a role in signal transduction events during pollen development and/or pollination.

INTRODUCTION

Cellular signal perception and transduction in response to external stimuli are essential for the growth and development of multicellular organisms. These processes have been extensively characterized in animal systems. In many cases, they involve membrane-associated receptor proteins whose ligand binding domains interact with extracellular signals and whose kinase domains transduce the signals to cytosolic targets. Most receptor kinases from animal systems are tyrosine kinases, with a few serine/threonine kinases (Ullrich and Schlessinger, 1990; Lin et al., 1992).

In higher plants, the first cDNA encoding a putative receptor-like kinase was identified in maize by Walker and Zhang (1990). Subsequently, cDNAs for other putative receptor-like kinases were identified in *Brassica oleracea* (Stein et al., 1991), *B. campestris* (Goring and Rothstein, 1992), and Arabidopsis (Chang et al., 1992; Tobias et al., 1992; Walker, 1993). All of the putative receptor-like kinases identified contain an extracellular domain for potential ligand binding, a membrane-spanning domain, and a cytoplasmic domain with consensus sequences for serine/threonine-type kinases. Three of these putative kinases have been shown to have serine/threonine kinase activity associated with their cytoplasmic domains (Chang et al., 1992; Goring and Rothstein, 1992; Stein and Nasrallah, 1993). Thus, the identification and characterization of plant receptor-like kinases strongly suggest the existence of transmembrane signal reception and transduction events in plants.

Based on the amino acid sequences of their extracellular domains, the plant receptor-like kinases reported so far are of three types. The extracellular domain of one type is similar in sequence to *S* locus glycoproteins (SLGs) of *Brassica* (Nasrallah and Nasrallah, 1993). Among this type of receptor-like kinases are *S* locus receptor kinases (SRKs) of *Brassica*, which are specific to reproductive tissues and have been implicated, along with SLGs, in controlling self-incompatibility interactions between pollen and stigma (Stein et al., 1991). Other receptor-like kinases of this type are found in vegetative tissues, but their functions have not yet been determined (Walker and Zhang, 1990; Tobias et al., 1992; Walker, 1993). Another type of receptor-like kinase is characterized by extracellular domains containing leucine-rich repeats (LRRs), a motif occurring in both receptor and nonreceptor proteins of animals that may be involved in protein–protein interactions. The presence of LRR motifs provides support for the idea that these receptor-like kinases are involved in ligand binding. Two Arabidopsis receptor-like kinases of this type, a transmembrane kinase (TMK1) (Chang et al., 1992) and a receptor-like protein

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kinase (RLK5) (Walker, 1993), were found to be expressed in a variety of tissues, including vegetative and reproductive tissues, suggesting that they may play a general role in cell signaling. The third type of receptor-like kinase contains epidermal growth factor repeats in the extracellular domain. An Arabidopsis receptor-like kinase of this type, PRO25, is specifically expressed in green leaves and may play a role in assembly or regulation of light-harvesting chlorophyll *a/b*-binding protein (Kohorn et al., 1992).

Previously, both calcium-dependent and -independent protein kinase activities have been observed in both soluble and pelletable fractions of germinated pollen of tobacco (Polya et al., 1986). In addition, phosphorylation has been implicated as essential for pollen tube growth in the pistil (Kandasamy et al., 1993). It is likely that membrane-associated kinases are involved in pollen development and pollination. Here, we report the isolation and characterization of cDNA and genomic DNA clones encoding a receptor-like kinase of *Petunia inflata*. This kinase, designated pollen receptor-like kinase 1 (PRK1), although sharing sequence similarity in the cytoplasmic domain with previously reported plant receptor-like kinases, is unlikely to be a functional homolog of any of them. Unlike other receptor-like kinases, expression of the *PRK1* gene is specific to the male reproductive tissue, with the highest level in mature pollen. Furthermore, results from an autophosphorylation assay showed that PRK1 appears to have both serine/threonine and tyrosine kinase activities. These characteristics of PRK1 make it a likely candidate as a transducer of signals during pollen development and/or pollination.

RESULTS

Isolation of cDNA Clones Encoding a Putative Receptor-like Kinase from a Pollen Tube cDNA Library of *P. inflata*

Our goal was to identify *P. inflata* genes that are specifically expressed in pollen tubes in an attempt to identify genes whose products are required for pollen germination and/or tube growth. A cDNA library was constructed using poly(A)$^+$ RNA isolated from in vitro–germinated pollen tubes. This library was separately screened with cDNA probes derived from poly(A)$^+$ RNA of pollen and in vitro–germinated pollen tubes. The screening yielded 10 cDNA clones that consistently hybridized more strongly to the pollen tube cDNA probe than to the pollen cDNA probe. All the positive clones were either partially or completely sequenced, and the GenBank and EMBL data bases were searched to identify any known genes with which these cDNAs shared sequence similarity. One of the clones, 1-Aa, contained a 1.2-kb cDNA fragment that encoded an open reading frame with significant sequence similarity with the cytoplasmic domain of plant receptor-like kinases.

The pollen tube cDNA library was rescreened using the 1.2-kb cDNA as a probe to search for possible cDNA clones containing longer inserts. Eight additional clones were isolated that contained cDNA inserts ranging from 1.3 to 2.4 kb. Partial nucleotide sequences of these cDNAs were determined, and sequence comparison revealed that they, as well as the 1.2-kb cDNA, were all likely derived from the same mRNA. The longest cDNA, designated *PRK1* cDNA, was completely sequenced. The nucleotide and the deduced amino acid sequences are shown in Figure 1. Excluding the poly(A) tract, *PRK1* cDNA is 2355 bp in length. A putative polyadenylation signal AATAAA was found 22 nucleotides upstream from the poly(A) tract. The longest open reading frame contains 720 amino acids.

The first methionine is followed by a sequence of 32 amino acids that contains structural features common to signal peptides. From the putative cleavage site of the signal sequence (between Ser-33 and Ser-34), small and neutral amino acids are located at positions −1 and −3, and proline, the helix breaker, is located at −6 position (von Heijne, 1983). Ten potential N-linked glycosylation sites (Asn-X-Ser/Thr) were found throughout the sequence. Hydropathy analysis of the protein sequence predicted a membrane-spanning region of 22 amino acids (residues 329 to 350) followed by three positively charged amino acids (Arg-Arg-Arg), characteristic of stop transfer sequences (Weinstein et al., 1982). Therefore, we concluded that PRK1 consists of an extracellular domain (328 amino acids), a membrane-spanning region (22 amino acids), and a cytoplasmic domain (370 amino acids).

Comparison of the Amino Acid Sequences of PRK1 and Other Plant Receptor-like Kinases

Sequence similarity between PRK1 and other plant receptor-like kinases is limited to the region of the cytoplasmic domain implicated in kinase activity. An alignment of the amino acid sequences of the kinase domains of six plant receptor-like kinases and the corresponding domain (residues 432 to 699) of PRK1 is shown in Figure 2. The kinase domain is divided into 11 subdomains according to Hanks et al. (1988). Forty-four perfectly conserved residues were found.

Among the 10 invariant residues of the serine/threonine type kinases (Lindberg et al., 1992), all but one are conserved in PRK1. They include Gly-443 located in subdomain I and Lys-462 in subdomain II, which have been implicated in Mg/ATP binding, and Asp-576 in subdomain VII, which has been implicated in phosphotransfer (Taylor et al., 1992). The exception is His-558, which does not conform to the invariant Asp at this position. Among the 12 nearly invariant sites (Lindberg et al., 1992), six in PRK1 are identical to the consensus residues at these positions. Thus, the sequence similarity between PRK1 and other serine/threonine kinases suggests that it may be a serine/threonine kinase.

The extracellular domain of PRK1 does not share any significant sequence similarity with the corresponding domain of other plant receptor-like kinases or with any proteins in the data bases. However, the LRRs, which are present in the

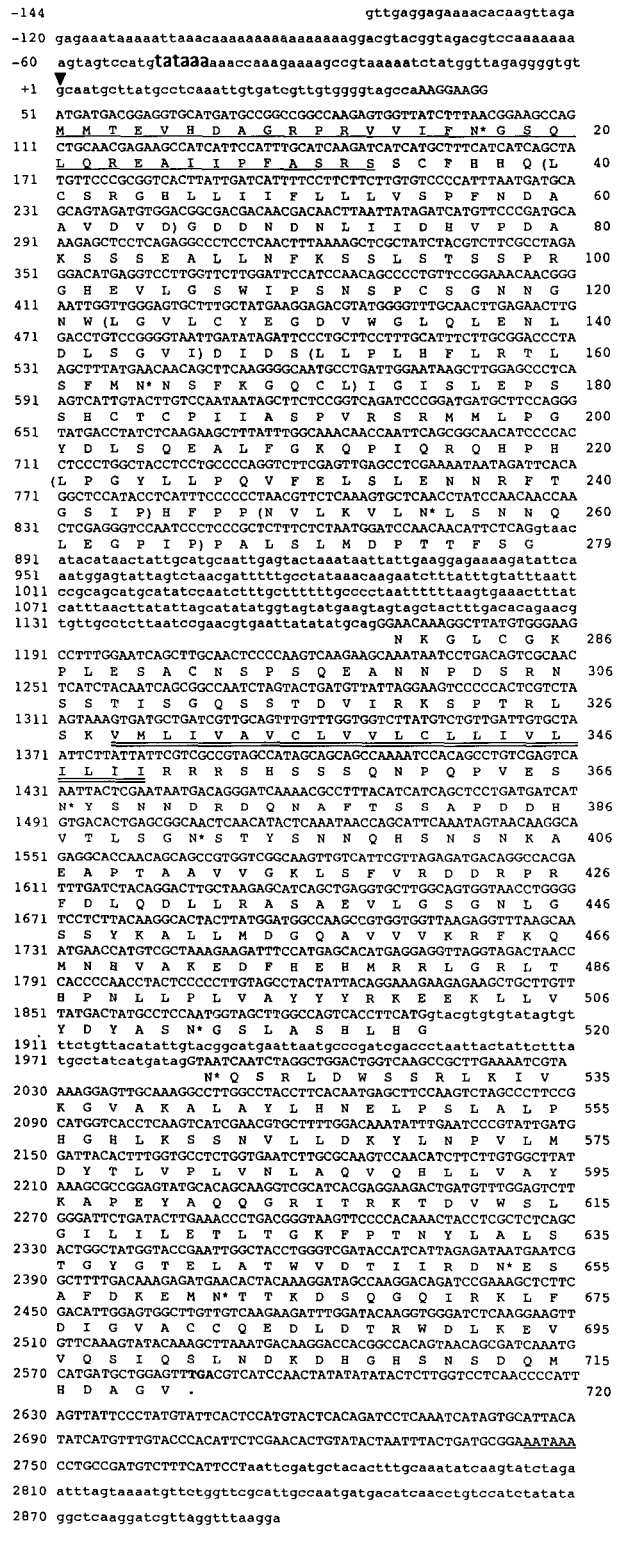

Figure 1. Nucleotide and Deduced Amino Acid Sequences of the *PRK1* Gene of *P. inflata*.

extracellular domain of one type of plant receptor-like kinases, were also found in PRK1; the five LRRs of PRK1 are aligned in Figure 3A. The LRRs have also been found in proteins from a variety of animal species, including vertebrates and invertebrates. These proteins are generally involved in protein–protein interactions and tend to be membrane associated (Rothberg et al., 1990). Figure 3B shows an alignment of the consensus sequence of the LRR motifs of PRK1 with that of six other LRR-containing receptor proteins and two nonreceptor proteins known to be involved in cell adhesion through protein–protein interactions. Three of the receptor proteins shown, Toll, Gp1b, and TRK, a tyrosine protein kinase encoding a putative neurogenic cell surface receptor, are involved in signal perception and transduction (Lopez et al. 1987; Hashimoto et al., 1988; Schneider and Schweiger, 1991).

Isolation and Characterization of Genomic DNA Encoding PRK1

A genomic library of *P. inflata* was screened using *PRK1* cDNA as a probe, and four positive clones were isolated. Restriction mapping indicated that they likely contain an identical genomic DNA fragment. An ~3.0-kb sequence that contains the entire coding region of the *PRK1* gene was determined and is shown in Figure 1. A schematic representation of the structure and restriction sites of the *PRK1* gene is shown in Figure 4A. The gene contains two short introns; intron 1 is 282 bp in length, and intron 2 is 92 bp in length. The coding sequence matches perfectly with the cDNA sequence. The transcription start site, determined by primer extension (results not shown), is 50 bp upstream of the methionine initiation codon. A putative TATA box, TATAAA, was identified 43 to 48 bp upstream from the transcription start site.

To determine the complexity of the *PRK1* gene, HindIII digests of the genomic DNA were hybridized with three different cDNA probes shown in Figure 4B. Four restriction fragments hybridized to the 1.2-kb cDNA (probe 1) encoding the cytoplasmic domain (Figure 4C); however, only two fragments were predicted based on the restriction map (Figure 4A). The 5.0- and

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The cDNA sequence is presented in uppercase letters; the genomic sequence not present in the cDNA is presented in lowercase letters. The putative TATA box is in boldface lettering, and the putative polyadenylation signal is underlined. Numbers to the left refer to nucleotide sequence. The transcriptional initiation site is indicated by a triangle and is assigned +1. The amino acid sequence is presented in the single-letter code below the nucleotide sequence. Numbers to the right refer to the amino acid sequence. The underlined amino acids denote the putative signal peptide; the double-underlined sequences denote the membrane-spanning region. Leucine-rich repeats are enclosed in parentheses. Asterisks indicate potential N-linked glycosylation sites. The translational stop codon is boldface and indicated by a dot. The nucleotide sequence has been submitted to EMBL, GenBank, and DDBJ as accession number L27341.

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                o +    o          o +                +
PRK1     LLRASAEVLGSGNLGSSYKALLMD GQAVVVKRFKQMNH VAKE..DFH.EH  478
ZMPK1    RKF..KVELGRGESGTVYKGVLED DRHVAVKKLENVR. QGKE..VFQAE.  547
TMK1     NNFSSDNILGSGGFGVVYKGELHD GTKIAVKRMENGVI AGKGFAEFKSE.  603
ARK1     NNFSNDNKLGQGGFGIVYKGRLLD GKEIAVKRLSKMSS QGTD..EFMNE.  537
SRK6     ENFSSCNKLGQGGFGIVYKGRLLD GKEIAVKRLSKTSV QGTD..EFMNE.  572
SRK2     EHFSDFNKVGKGGFGVVYKGRLVD GQEIAVKRLSEMSA QGTD..EFMNE.  572
SRK910   ENFSNCNKLGQGGFGIVYKGRLLD GQEIAVKRLSKTSV QGTG..EFMNE.  573
         ------------I---------- -------II------ --------III-

PRK1     MRRLGRL THPNLLPLVAYYYR KEEKLLVYDYASNGSLASHLH.... GNQS  524
ZMPK1    LSVIGRI NHMNLVRIWGFCSE GSHRLLVSEYVENGSLANILF..SE GGNI  595
TMK1     IAVLTKV RHRHLVTLLGYCLD GNEKLLVYEYMPQGTLSRHLFEWSE EGLK  653
ARK1     VRLIAKL QHINLVRLLGCCVD KGEKMLIYEYLENLSLDSHLFDQTR SS..  585
SRK6     VTLIARL QHINLVQVLGCCIE GDEKMLIYEYLENLSLDSYLFGKTR RS..  620
SRK2     VRLMQSF SHNNLVRLLGCCVY EQEKILIYEYLENLSLDSHLFDETR SC..  620
SRK910   VRLIARL QHINLVRILGCCIE ADEKMLVYEYLENLSLDSYLFGNKR SS..  621
         ------- -------IV----- -------------V----------- ----

                           o          oo+ +  +          o
PRK1     RLDWSSRLKIVKGVAKALAYLHNELPSL ALPHGHLKSSNVLLDKYL NPVL  574
ZMPK1    LLDWEGRFNIALGVAKGLAYLHHEC.LE WVIHCDVKPENILLDQAF EPKI  645
TMK1     PLLWKQRLTLALDVARGVEYLHGLA.HQ SFIHRDLKPSNILLGDDM RAKV  703
ARK1     NLNWQKRFDIINGIARGLLYLHQDS.RC RIIHRDLKASNVLLDKNM TPKI  635
SRK6     KLNWNERFDITNGVARGLLYLHQDS.RF RIIHRDLKVSNILLDKNM IPKI  670
SRK2     MLNWQMRFNIINGIARGLLYLHQDS.RF RIIHRDIKASNVLLDKDM TPKI  670
SRK910   TLNWKDRFNITNGVARGLLYLHQDS.RF RIIHRDMKVSNILIDKNM TPKI  671
         -----------VIA--------------- ----------VIB----- ----

          +oo                        o+           + o  o
PRK1     MDYTLVPLVNLA QVQH......LLVAYKAPEYA QQGRITRKTDVWSLGIL  618
ZMPK1    TDFGLVKLLNRG GSTQNVSHVRGTLGYIAPEWV SSLPITAKVDVYSYGVV  695
TMK1     ADFGLVRL.APE GKGSIETRIAGTFGYLAPEYA VTGRVTTKVDVYSFGVI  752
ARK1     SDFGMARIFGRE ETEANTRRVVGTYGYMSPEYA MDGIFSMKSDVFSFGVL  685
SRK6     SDFGMARIFERD ETEANTMKVVGTYGYMSPEYA MYGIFSEKSDVFSFGVI  720
SRK2     SDFGMARIFGRD ETEADTRKVVGTYGYMSPEYA MNGTFSMKSDVFSFGVL  720
SRK910   SDFGMARIFARD ETEANTRKVVGTYGYMSPEYA MDGVFSEKSDVFSFGVI  721
         ----VII----- ---------VIII--------- -------------IX--

PRK1     ILETLTGKFPTNYL ALSTGYGTELATWVDTIIRDNESAFDKEMNTTKDSQ  668
ZMPK1    LLELLTGTRVSELV GGTDEVHSMLRKLVRMLSAKLEGEEQSWIDGYLDSK  745
TMK1     LMELITGRKSLDES QPEESIH..LVSWFK...RMYINK.EASFKKAIDTT  796
ARK1     LLEIISGKRNKGFY NSNRDLN..LLGFVW.RHWKEGNELEIVDPINIDSL  732
SRK6     VLEIVSGKKNRGFY NLDYEND..LLSYVW.SRWKEGRALEIVDPVIVDSL  767
SRK2     LLEIISGKRNKGLC DSDSSLN..LLGCVW.RNWKEGQGLEIVDKVIIDSS  767
SRK910   VLEIVSGKRNRGFY NLNHENN..LLSYVW.SHWTEGRALEIVDPVIVDSL  768
         -------------- ----------------------X-------------

                                    +
PRK1     G......... QIRKLFDIGVACCQEDLDTRWDLKEVVQSI     699
ZMPK1    LNRPVNYV.. QARTLIKLAVSCLEEDRSKRPTMEHAVQTL     784
TMK1     IDLDEETLAS VHTVAELAGHCCARE.PYQRPDMGHAVNIL     835
ARK1     SSKFPTH... EILRCIQIGLLCVQERAEDRPVMSSVMVML     769
SRK6     SSQPSIFQPQ EVLKCIQIGLLCVQELAEHRPAMSSVVWMF     807
SRK2     SPTFRPR... EILRCLQIGLLCVQERVEDRPMMSSVVLML     804
SRK910   SSLPATFQPK EVLKCIQIGLLCVQERAEHRPTMSSVVWML     808
         ---------- --------------XI--------------
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Figure 2. Alignment of the Amino Acid Sequences of the Kinase Domain of Plant Receptor-like Kinases.

The alignment was made to maximize sequence similarity. Conserved residues are in boldface letters. The 11 subdomains, I to XI, described by Hanks and Quinn (1991) and Hanks et al. (1988), are indicated. Crosses denote residues conserved in all serine/threonine–type kinases; open circles denote residues conserved in most serine/threonine–type kinases. The sources of other receptor-like kinase sequences are ZMPK1 of maize (Walker and Zhang, 1990); TMK1 of *Arabidopsis* (Chang et al., 1992); ARK1 of *Arabidopsis* (Tobias et al., 1992); SRK2 and SRK6 of *Brassica* (Stein et al., 1991); and SRK910 of *Brassica* (Goring and Rothstein, 1992).

1.9-kb fragments correspond to the *PRK1* gene, and the 4.2- and 4.0-kb fragments, which hybridized more weakly, probably contain genes encoding proteins with sequence similarity to the putative kinase domain of PRK1. However, only the two HindIII fragments, 1.9 and 1.1 kb, corresponding to the *PRK1* gene hybridized with 0.8-kb cDNA (probe 2), which encodes the extracellular domain. (Two additional small HindIII fragments of the *PRK1* gene were not detected because they ran off the gel.) This suggests that other genes detected with the 1.2-kb cDNA probe may encode kinases whose extracellular domain is absent or different from that of PRK1. When the digests were hybridized with *PRK1* cDNA (probe 3), the sum of the fragments observed with the former two probes was obtained (Figure 4C).

Expression Pattern of the *PRK1* Gene

RNA gel blot analysis using *PRK1* cDNA as a probe revealed a 2.4-kb RNA species, similar in size to *PRK1* cDNA, in both pollen and in vitro–germinated pollen tubes, as shown in Figure 5A. None of the other tissues examined, that is, pistil, petal, leaf, and root, contained this RNA. The finding of *PRK1* mRNA in pollen is unexpected, because the 1-Aa cDNA clone encoding PRK1 was initially isolated by a strategy designed to obtain pollen tube–specific cDNAs. It is possible that labeled cDNAs for PRK1 were underrepresented in the pollen cDNA probe used for differential screening.

A

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          L * * L * *L/V* * L * L * N N * * * . G * I P
  L C S R G H L L I I F L L L V S P F N D A A . V D V
    L G V L C Y E G D V W G L Q L . E N L D L S G V I
          L L P L H F L R T L S F M N N S F K . G Q C L
      L P G Y L L P Q V F E L S L E N N R F T . G S I P
                  N V L K V L N L S N N Q L E . G P I P
```

B

Protein	Number of Repeats	LRR Consensus Sequence
Petunia PRK1	5	L**L**L/V**L*L*NN***G*IP
Arabidopsis TMK1	11	L**L** L **L*L**N*a*G*aP
Arabidopsis RLK5	21	L**L** L **L*L**N*LSG*IP
Human Gp1b	7	P**LL**L** L **L*L**N*L**L
Human TRK	3	L **L*a**N*L**a
Drosophila Toll	15	P**LF*H**N L **L*L**N*L**L
Rat LH-CG-R	14	PS*AF*GL** a **L*L**N/T
Human LRG	9	P**LL***** L **L*L**N*L**L
Drosophila Chaoptin	41	P***F**L** L **LDLS*N*L**I

Figure 3. LRRs and Consensus Sequence Alignment.

(A) Alignment of the five LRR motifs of PRK1. Consensus residues are indicated on top of the alignment. Asterisks indicate nonconsensus residues. Dots indicate gaps introduced to maximize similarity.
(B) Comparison of the consensus sequences of LRR motifs of PRK1 and other LRR-containing proteins. The six receptor or receptor-like proteins are grouped with PRK1, and the two nonreceptor proteins are grouped together. The sources of other LRR-containing sequences are TMK1 (Chang et al., 1992); RLK5 (Walker, 1993); Gp1b (Lopez et al., 1987); TRK (Schneider and Schweiger, 1991); Toll (Hashimoto et al., 1988); LH-CG-R (McFarland et al., 1989); LRG (Takahashi et al., 1985); and Chaoptin (Krantz and Zipursky, 1990).

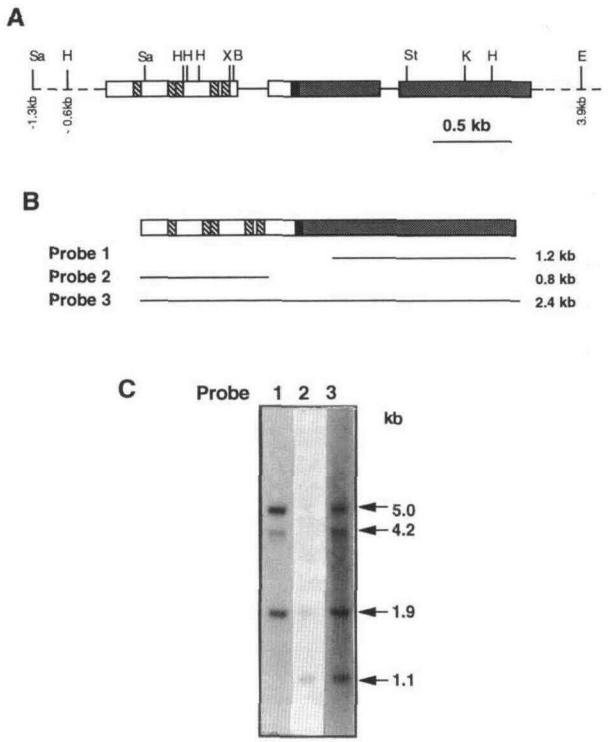

Figure 4. Structure and Genomic Complexity of the *PRK1* Gene.

(A) Schematic representation of the *PRK1* gene. Boxes denote translated regions, straight lines indicate introns, and broken lines denote 5′ and 3′ nontranslated regions and are not drawn to scale. Within the translated regions, open boxes represent the extracellular domain, the blackened box denotes the membrane-spanning region, shaded boxes represent the cytoplasmic domain, and hatched boxes within the open box mark LRRs. B, BamHI; E, EcoRI; H, HindIII; K, KpnI; Sa, SacI, St, StuI; X, XhoI.

(B) Schematic representation of cDNA probes used in DNA gel blot analysis shown in **(C)**. Probe 1 is the 1.2-kb cDNA, which is contained in clone 1-Aa, encoding ~88% of the cytoplasmic domain; probe 2 is the 0.8-kb EcoRI-BamHI cDNA fragment encoding ~84% of the extracellular domain; and probe 3 is the full-length *PRK1* cDNA. Boxes denote the same regions of PRK1 as given in **(A)**.

(C) DNA gel blot analysis. Ten micrograms of HindIII digests of *P. inflata* genomic DNA was loaded in each lane. Of the four fragments indicated by arrows, 5.0, 1.9, and 1.1 kb correspond to the *PRK1* gene; 4.2 kb does not. An additional 4.0-kb fragment, not indicated by an arrow, does not correspond to the *PRK1* gene either.

The developmental expression of the *PRK1* gene was also examined by RNA gel blot analysis (Figure 5B). Anther development was divided into five stages according to flower bud size, and the corresponding development of the microspores was determined microscopically. The earliest stage at which *PRK1* mRNA could be detected was in stage 3 anthers, which contain mostly binucleate microspores. Thereafter, the steady state level increased substantially and reached its highest level in anthers of open flowers, which contain mature pollen. There was an approximately fivefold increase in the amount of *PRK1* mRNA from stage 3 anthers to open flowers. Though slightly decreased in in vitro–germinated pollen tubes, the amount of *PRK1* mRNA remained nearly constant over a 16-hr period after germination commenced (data not shown). Currently, it is not known whether *PRK1* mRNA detected in the anther is exclusively located in the microspore or is also present in the sporophytic tissue of the anther.

Kinase Activity of Recombinant Protein Containing the Cytoplasmic Domain of PRK1

To ascertain whether PRK1 is indeed a serine/threonine kinase as its sequence predicts, the 1.2-kb cDNA (Figure 4B) was expressed in *Escherichia coli* using a pRSET-C expression vector, and the kinase activity of the recombinant protein (PRK1-K) was examined. PRK1-K consists of a short peptide encoded by the vector, followed by the 324–amino acid peptide that contains the entire putative kinase domain of PRK1. A stretch of histidines contained in the vector-encoded peptide allowed rapid purification of PRK1-K on nickel resin. Several proteins from the soluble fraction of *E. coli* extracts were found in the eluates from nickel resin, as shown in Figure 6A (lane 1). Among them was a 40-kD protein with a molecular mass similar to that predicted for the fusion protein. This protein was not detected in the eluates of extracts of *E. coli* cells harboring pPRK1-K(−) with the 1.2-kb cDNA inserted in antisense orientation (Figure 6A, lane 3). In addition, the 40-kD protein cross-reacted with a monoclonal antibody specific for the vector-encoded part of PRK1-K (Figure 6B, lane 1). No cross-reacting proteins were detected in the eluates of extracts of *E. coli* harboring pPRK1-K(−) (lane 3). Thus, the 40-kD protein was identified as the recombinant protein PRK1-K. To further purify PRK1-K, the eluates from the nickel resin were chromatographed on a Mono-Q column, and PRK1-K was eluted at ~120 mM NaCl (Figures 6A and 6B, lane 2).

The Mono-Q purified PRK1-K was subjected to autophosphorylation assay. A single radioactive band was detected by autoradiography, whose position matches that of PRK1-K detected by Coomassie blue staining (Figure 6C). Eluates of extracts from *E. coli* cells containing pPRK1-K(−) did not contain a phosphorylated protein at this position (data not shown). Thus, the radioactive band represented the phosphorylated PRK1-K, which most likely resulted from autophosphorylation, rather than from phosphorylation by a contaminating *E. coli* kinase. This is because in order for a contaminating *E. coli* protein to phosphorylate purified PRK1-K, it would have had to be copurified with PRK1-K, and this would mean that such a protein would have to not only bind nickel resin as PRK1-K does, but also elute at the same salt concentration on the Mono-Q column as PRK1-K does, an unlikely scenario. Further, the high degree of sequence similarity between the "kinase" domain of PRK1 and the corresponding domain of other kinases is strong circumstantial evidence for PRK1 being

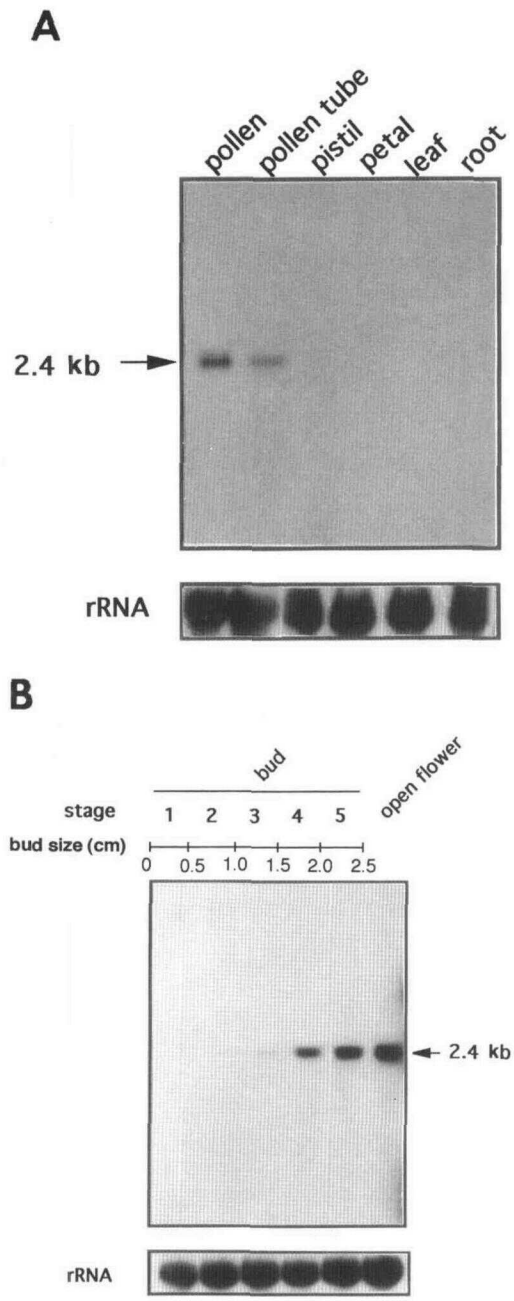

Figure 5. RNA Gel Blot Analysis of the Expression of the *PRK1* Gene.

(A) Tissue-specific expression of the *PRK1* gene. Thirty micrograms of total RNA from each tissue was used. The upper autoradiogram shows the blot hybridized with *PRK1* cDNA. After removing the probe, the blot was hybridized with a 25S rDNA probe from *P. inflata*, and the results are shown in the lower autoradiogram.

(B) Developmental expression of the *PRK1* gene. Thirty micrograms of total RNA from anthers of different sizes of buds, and from open flowers, was used in each lane. The upper autoradiogram shows the blot hybridized with the *PRK1* cDNA probe. After removing the probe, the same blot was hybridized with the 25S rDNA probe, and the results are shown in the lower autoradiogram. Microscopic examination of anther squashes stained with acetocarmine revealed the following correlations between bud size and microspore development: stage 1 buds that were less than 0.5 cm in length showed developing microspores in the tetrad configuration; stage 2 buds between 0.5 and 1.0 cm had free uninucleate microspores; stage 3 buds between 1.0 and 1.5 cm had mostly binucleate microspores; stage 4 buds between 1.5 and 2.0 cm had completed mitotic division; and both stage 5 purple buds between 2.0 and 2.5 cm and open flowers contained mature pollen grains.

a functional kinase. Thus, we concluded that the cytoplasmic domain of PRK1 has kinase activity.

To study the specificity of the kinase activity, the phosphorylated PRK1-K was partially hydrolyzed with acid, and the resulting products were analyzed by one-dimensional thin-layer electrophoresis. γ-^{32}P-labeled spots corresponding to the positions of phosphoserine and phosphotyrosine were detected (Figure 6D). It is unlikely that the radiolabeled spot corresponding to the position of phosphotyrosine was due to partially hydrolyzed peptides of phosphoserine for the following reason. In a parallel experiment, we tested the ability of a serine/threonine kinase, the catalytic subunit of the cAMP-dependent kinase, to phosphorylate casein, and subjected the phosphorylated casein to phosphoamino acid analysis under the same conditions. We detected only the expected radiolabeled spot corresponding to phosphoserine, but did not detect any radiolabeled spot at or near the position to which phosphotyrosine would migrate (results not shown). These results suggest that our hydrolysis procedure did not produce false phosphotyrosine comigrating signals and that PRK1 may have both serine/threonine and tyrosine kinase activities.

Immunological Detection of Native PRK1 in Anthers, Mature Pollen, and in Vitro-Germinated Pollen Tubes

To identify native PRK1 by protein gel blot analysis, we first raised polyclonal antibodies against the extracellular domain of PRK1. The 0.8-kb cDNA fragment (Figure 4B) was expressed in *E. coli* using the pRSET-A expression vector. A 35-kD protein was found at a high level in extracts of *E. coli* cells harboring the recombinant expression vector, but absent from extracts of *E. coli* cells harboring the recombinant expression vector with the 0.8-kb cDNA fragment inserted in antisense orientation (results not shown). The mobility of this protein closely matches that predicted from the open reading frame contained in the construct. The 35-kD protein was thus identified as the recombinant protein and designated PRK1-EC. Most of the PRK1-EC protein was found in inclusion bodies; they were solubilized and subsequently purified by chromatography on nickel resin and preparative SDS-PAGE.

The immune serum raised against PRK1-EC was affinity purified using PRK1-EC to obtain monospecific immune serum.

As shown in Figure 7A, PRK1-EC was found to cross-react with the monospecific immune serum, but not with preimmune serum or with immune serum that had been preincubated with PRK1-EC (blocked immune). As shown in Figure 7B, the monospecific immune serum detected a major protein band of 69 kD from total protein extracts of mature pollen grains. This protein was not detected with preimmune serum, and the degree of its cross-reactivity was much reduced with the monospecific immune serum that had been preincubated with

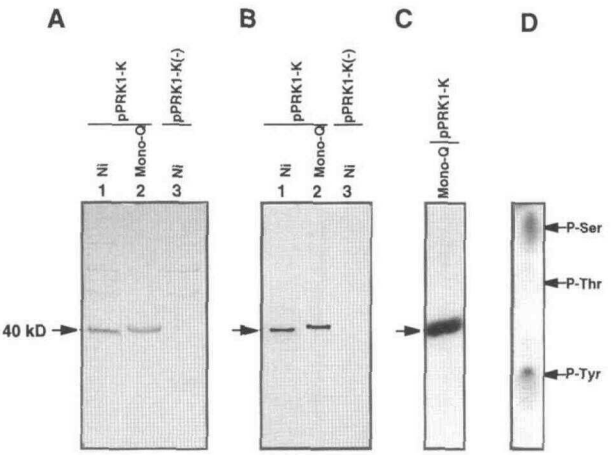

Figure 6. Analysis of Kinase Activity of Recombinant Protein PRK1-K.

(A) Purification of recombinant protein PRK1-K. Extracts of *E. coli* cells harboring pPRK1-K, which contains the 1.2-kb cDNA (Figure 4B) inserted in sense orientation to pSET-C, or pPRK1-K(−), which contains the 1.2-kb cDNA inserted in antisense orientation to pSET-C, were purified by nickel resin, and the bound proteins were eluted and further purified on a Mono-Q column. For pPRK1-K, 1 μg of the proteins eluted from the nickel resins and 1 μg of the protein purified by Mono-Q were used for analysis on a 12% SDS–polyacrylamide gel. For pPRK1-K(−), 0.2 μg of the proteins eluted from the nickel resins was used. Proteins were visualized by Coomassie blue staining. The arrow indicates the 40-kD protein.

(B) Protein gel blot analysis of recombinant protein PRK1-K. Each lane of the 12% SDS–polyacrylamide gel contains 1/20 the amount of the protein used in the corresponding lane in **(A)**. The primary antibody is a T7-tag monoclonal antibody specific to the portion of PRK1-K encoded by pRSET-C. The arrow indicates the position corresponding to the 40-kD protein.

(C) Autoradiogram of autophosphorylation assay of recombinant protein PRK1-K. Ten micrograms of Mono-Q purified PRK1-K was subjected to kinase reactions and electrophoresed on a 12% SDS–polyacrylamide gel. The arrow indicates the position corresponding to the 40-kD protein.

(D) Phosphoamino acid analysis of autophosphorylated PRK1-K. γ-^{32}P-labeled PRK1-K was hydrolyzed with HCl and subjected to one-dimensional thin-layer electrophoresis. Arrows indicate the positions of phosphoserine, phosphothreonine, and phosphotyrosine. Only the portion of the autoradiogram where these three phosphoamino acids are located is shown.

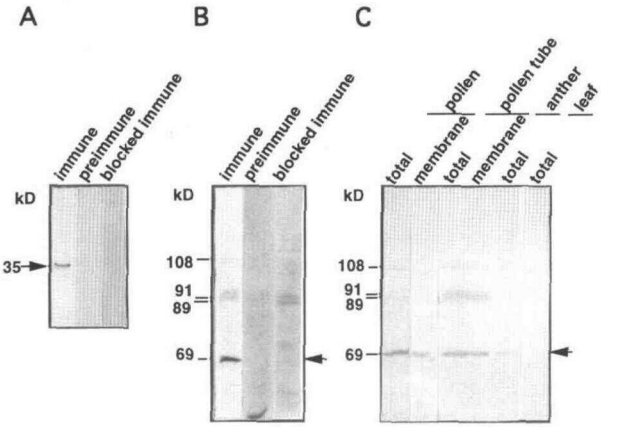

Figure 7. Protein Gel Blot Analysis of Native PRK1.

(A) Specific cross-reactivity of immune serum to recombinant PRK1-EC protein. Each lane of the 8% SDS–polyacrylamide gel contains 12 ng of gel-purified PRK1-EC. The primary antibodies used were monospecific immune serum (immune) that had been affinity purified using PRK1-EC, preimmune serum, and monospecific immune serum that had been preincubated with gel-purified PRK1-EC (blocked immune). The arrow indicates the recombinant PRK1-EC protein.

(B) Identification of the 69-kD protein as the native PRK1. Each lane of the 8% SDS–polyacrylamide gel contains 100 μg of total protein extracted from mature pollen. The primary antibodies used were the same as those described in **(A)**. The arrow indicates the 69-kD protein.

(C) Detection of the 69-kD protein in anther, pollen, in vitro–germinated pollen tubes, and leaf. Each lane contains 100 μg of either total protein of pollen, pollen tubes, stage 5 anthers, and leaf, or proteins in the microsomal membrane fractions of pollen and pollen tubes. The monospecific immune serum was used as the primary antibody. The arrow indicates the 69-kD protein.

PRK1-EC. Three minor bands of 108, 91, and 89 kD were also detected by the monospecific immune serum. However, they cross-reacted with preimmune serum and blocked immune serum (Figure 7B). Thus, none of them is likely to be a candidate for PRK1.

The 69-kD protein was detected in total protein extracts of stage 5 anthers and pollen tubes, consistent with the presence of *PRK1* mRNA in these tissues (Figure 7C). Further, the 69-kD protein was not detected in leaf (Figure 7C) where *PRK1* mRNA was not detected. Also consistent with the membrane location of PRK1 predicted by its primary structural features, the 69-kD protein was found in microsomal membranes of mature pollen and pollen tubes (Figure 7C). Based on these results, the 69-kD protein is considered a possible candidate for native PRK1. The apparent molecular mass of this PRK1 candidate is smaller than that predicted from the sequence of the mature protein (~76 kD); however, this size difference may result from abnormal mobility of the protein on the SDS–polyacrylamide gel, post-translational processing, or proteolytic cleavage during preparation of the extracts.

Detection of Homologs of *PRK1* Gene in Other Species

To determine whether homologs of the *PRK1* gene are present in other plant species, genomic DNA gel blot analysis was carried out on three other solanaceous species, namely *P. hybrida*, *Lycopersicon peruvianum*, and *Solanum chacoense*; one compositaeous species, *Lactura sativa*; and one cruciferous species, *B. oleracea* var *etalica*. Under stringent hybridization conditions, the 1.2-kb cDNA probe (Figure 4B) detected strong hybridizing DNA fragments in all the species examined, except in *Brassica*, as shown in Figure 8. The finding of similar hybridizing patterns suggests that functional homologs of the *PRK1* gene may exist in other species as well. Indeed, a pollen mRNA similar in size to *PRK1* mRNA was detected in *Nicotiana tabacum*, another solanaceous species, by the 1.2-kb cDNA probe (results not shown).

When the same DNA gel blot was hybridized with the 0.8-kb cDNA probe (Figure 4B), the DNA digests of *P. hybrida*, a species closely related to *P. inflata*, were found to hybridize with similar intensity as those of *P. inflata* (data not shown). However, the DNA digests of the other species that hybridized strongly to the 1.2-kb cDNA probe hybridized only weakly to the 0.8-kb cDNA probe (data not shown). These results suggest that PRK1 and its functional homologs may be less conserved in their extracellular domains than in their cytoplasmic (kinase) domains.

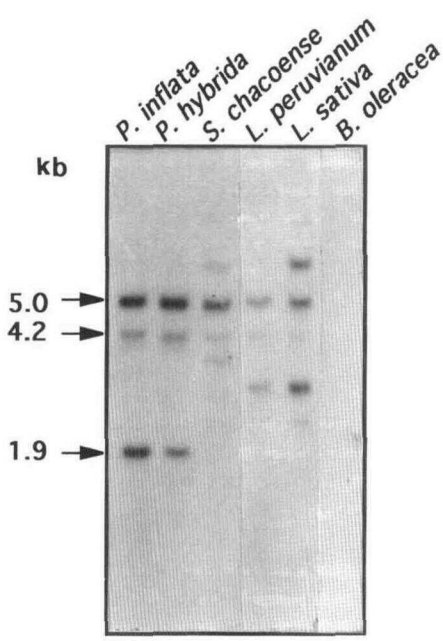

Figure 8. DNA Gel Blot Analysis of Homologs of the *PRK1* Gene in Other Species.

Each lane contains HindIII digests of 10 μg of genomic DNA isolated from leaves. The blot was hybridized with the 1.2-kb cDNA probe (Figure 4B). Of the three fragments indicated by arrows, 5.0 kb and 1.9 kb correspond to the *PRK1* gene, and 4.2 kb corresponds to a related gene with sequence similarity only to the region of the *PRK1* gene encoding the kinase domain. The species examined were *P. inflata*, a wild species of petunia; *P. hybrida*, a cultivated species of petunia; *Solanum chacoense*, a wild species of potato; *Lycopersicon peruvianum*, a wild species of tomato; *Lactura sativa*, lettuce; and *Brassica oleracea* var *etalica*, cauliflower.

DISCUSSION

PRK1, a receptor-like kinase from *P. inflata* that we have characterized here, shares sequence similarity with other plant receptor-like kinases in the region of the cytoplasmic domain that has been implicated in kinase activity. Nine of the 10 invariant residues characteristic of serine/threonine kinases (Lindberg et al., 1992) are among the 44 residues conserved among PRK1 and other plant receptor-like kinases. Not surprisingly, recombinant protein containing the kinase domain of PRK1 was found to autophosphorylate on serine, as has been demonstrated for three other plant receptor-like kinases (Chang et al., 1992; Goring and Rothstein, 1992; Stein and Nasrallah, 1993). However, unlike these plant receptor-like kinases, PRK1 was also found to autophosphorylate on tyrosine.

The results of the autophosphorylation assay thus raise the intriguing possibility that PRK1 may be a dual-specificity kinase that has both serine/threonine and tyrosine kinase activities. Kinases of this type have recently been discovered in mammals, yeast, *Dictyostellium* (for a review, see Lindberg et al., 1992), and plants (Hirayama and Oka, 1992). As is true with PRK1, all the other dual-specificity kinases are more similar to serine/threonine kinases in sequence than to tyrosine kinases. Comparison of the sequences of several such kinases led Seger et al. (1991) to suggest that the subdomain XI may be indicative of dual-specificity kinases. Figure 9 shows a sequence alignment of this domain of PRK1 and several dual-specificity kinases. In some of the sites where consensus amino acids have been implicated, PRK1 contains amino acids that are either identical or similar to the consensus amino acid. In addition, in some sites that are not part of the consensus sequence, PRK1 also contains amino acids similar to these dual-specificity kinases. However, because not all the consensus amino acids are found in PRK1, this comparison by itself does not conclusively show that PRK1 is a dual-specificity kinase. In addition, the validity of using subdomain XI as a criterion for dual-specificity kinases has recently been questioned, because this domain is not located in close proximity to the active site and therefore may not be directly involved in determining substrate specificity (Lindberg et al., 1992). At present, no other consensus sequence motifs for dual-specificity kinases have been identified.

However, it is not clear whether results from the autophosphorylation assay, using only the kinase domain of PRK1, truly reflect the physiological activity of PRK1. It should also be cautioned that phosphorylation patterns exhibited by kinases

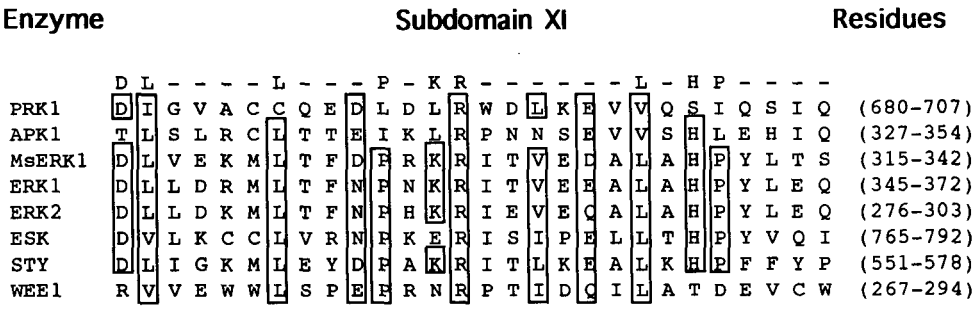

Figure 9. Alignment of the Amino Acid Sequences of Subdomain XI of PRK1 and Other Dual-Specificity Kinases.

The conserved residues identified by Seger et al. (1991) are shown atop the alignment. Identical or similar amino acids are boxed. The sources of other dual-specificity kinase sequences are APK1 of *Arabidopsis* (Hirayama and Oka, 1992); MsERK1 of alfalfa (Duerr et al., 1993); ERK1 and ERK2 of rat (Seger et al., 1991); ESK of mouse (Douville et al., 1992); STY of mouse (Howell et al., 1991); and WEE1 of yeast (Featherstone and Russell, 1991).

expressed in *E. coli* may not truly reflect their native phosphorylation patterns. For example, when expressed in *E. coli*, two serine/threonine–type kinases have been shown to have tyrosine kinase activity, albeit at much lower levels than their serine/threonine kinase activity (Stern et al., 1991). Although the level of tyrosine phosphorylation exhibited by the *E. coli*–expressed PRK1-K is comparable to the level of serine phosphorylation, we cannot completely rule out the possibility that the observed tyrosine kinase activity was due to aberrant phosphorylation with the *E. coli*–expressed PRK1-K.

Regardless, the physiological relevance of the dual specificity exhibited by PRK1-K will have to be demonstrated with the identification of its phosphorylated substrates. For one dual-specific kinase, WEE1 of yeast, which autophosphorylates on serine and tyrosine, several lines of evidence suggest that its physiological function does indeed involve tyrosine phosphorylation of its substrate (Lindberg et al., 1992). If PRK1 indeed behaves as a dual-specificity kinase in vivo, it would be a unique receptor-like kinase because all the previously reported receptor-like kinases from both animal and plant systems are either tyrosine-type kinases or serine/threonine-type kinases.

The previously reported plant receptor-like kinases appear to participate in diverse physiological events based on their different patterns of tissue distribution. Among them, only SRKs of *Brassica* are exclusively found in reproductive tissues (Stein et al., 1991), as is PRK1. Whereas SRKs have been implicated in self-incompatibility interactions, the function of PRK1 is most likely different from that of SRKs for the following reasons. First, *SRK* mRNA is present much more abundantly in the pistil than in the anther, whereas *PRK1* mRNA is exclusively present in the anther and male gametophyte. Second, the *SRK* gene is highly polymorphic to and located at the *S* locus, which controls sporophytic self-incompatibility, whereas the *PRK1* gene does not display any *S* allele–associated polymorphism (results not shown). Third, SRK and PRK1 do not share any sequence similarity in their extracellular domains, indicating that they most likely interact with different ligands.

Based on the timing of gene expression during microsporogenesis, the *PRK1* gene belongs to the class of "late" pollen genes whose transcripts are first detected around the time of microspore mitosis and continue to accumulate as pollen matures (Mascarenhas, 1990). It is generally thought that the late pollen genes may be required for pollen maturation and/or pollen tube growth. These processes involve intimate chemical and physical interactions between microspores and the nurse tissue of the anther and between pollen tubes and the transmitting tissue of the style. It is highly likely that they would require a complex network of signal transduction pathways. And indeed phosphorylation events have been shown to occur during pollen development and pollen tube growth (Polya et al., 1986; Kyo and Harada, 1990). Although the precise function of PRK1 remains to be determined, its characteristics suggest that it may serve as a transducer of signals during pollen development and/or pollination.

One approach to addressing the function of PRK1 is to study the effect of inhibition of the synthesis of PRK1 on pollen development or pollination. Experiments are underway to use antisense RNA methodology to inhibit the synthesis of PRK1 in transgenic plants. If PRK1 is shown to be involved in these processes, identification of the ligands and substrates of PRK1 will further delineate the role of PRK1 in mediating the signal transduction events.

METHODS

Construction and Screening of a Pollen Tube cDNA Library

Five hundred milligrams of pollen collected from open flowers of *Petunia inflata* was equally distributed into 75 culture tubes (17 × 100 mm). Two milliliters of germination medium (Harris et al., 1989) was added to each tube, and germination was carried out at 30°C for 16 hr with gentle rotating. Pollen tubes were sedimented by centrifugation at

10,000*g* for 10 min at 4°C, and the residual supernatant was removed by passing through a 0.2-μM filter (Millipore, Bedford, MA). Total RNA and poly(A)$^+$ RNA were isolated using the methods described by Kheyr-Pour et al. (1990). Approximately 4.4 mg of total RNA and 10 μg of poly(A)$^+$ RNA were obtained. One microgram of poly(A)$^+$ RNA was used for the construction of a cDNA library using the procedures described by Kheyr-Pour et al. (1990), except that a λ ZAPII vector was used (Stratagene, La Jolla, CA). Infection of *Escherichia coli* host strain XL1-blue yielded a primary library with a titer of ~2 × 10^5 plaque-forming units. The primary library was amplified to obtain a total of 2.3 × 10^{10} plaque-forming units. Two sets of duplicated nitrocellulose filters, each containing ~1 × 10^5 plaques from the amplified library, were prehybridized at 42°C for 5 hr in 50% formamide, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, and 200 μg/mL salmon sperm DNA. Each set of filters was hybridized with ^{32}P-labeled cDNA probes derived from either pollen poly(A)$^+$ RNA or pollen tube poly(A)$^+$ RNA at 42°C for 20 hr. The filters were washed four times with 2 × SSC, 0.1% SDS at room temperature for 20 min, and then with 0.2 × SSC, 0.1% SDS at 65°C for 1 hr.

Screening of a *P. inflata* Genomic Library

A λ phage library of *P. inflata* genomic DNA previously constructed (Coleman and Kao, 1992) was used in screening for genomic clones encoding pollen receptor-like kinase 1 (PRK1). Nitrocellulose filters containing a total of ~1 × 10^5 plaques were prehybridized, and hybridized with *PRK1* cDNA as described above.

DNA Sequencing

cDNA inserts were excised from λ ZAPII vector and recirculated to form recombinant pBluescript SK− phagemid DNA according to the procedure recommended by the manufacturer (Stratagene). Genomic DNA was released from λ GEM-11 vector by digestion with SacI and subcloned into the SacI site of pBluescript SK− vector. Plasmid DNA was prepared using Qiagen Tip-20 columns (QIAGEN Inc., Chatsworth, CA) and sequenced directly using Taq DNA polymerase (Promega, Madison, WI). Both universal sequencing primers and synthetic primers were used for sequencing.

Primer Extension

An oligonucleotide 5′-CGTTGCAGCTGGCTTCCG-3′, complementary to the sequence between nucleotides 101 and 118 of the *PRK1* gene (see Figure 1), was used in primer extension experiments. The oligonucleotide was end labeled with polynucleotide kinase (New England Biolabs, Beverly, MA). Forty micrograms of total pollen RNA was coprecipitated with 0.16 pmol of the labeled oligonucleotide (10^5 cpm), and the pellet was redissolved in 20 μL of water. Primary extension was carried out at 37°C for 1 hr in a 50-μL solution containing 10 mM Tris-HCl, pH 8.3, 15 mM KCl, 0.6 mM $MgCl_2$, 1 mM each of deoxyribonucleotide triphosphates, 20 units of moloney murine leukemia virus reverse transcriptase (Stratagene), and 20 units of RNase inhibitor. After treatment with ribonucleases, the reaction mixture was extracted with phenol, and the nucleic acids were precipitated with ethanol. The pellet was redissolved in 3 μL of water and 4 μL of gel loading dye (Promega) and electrophoresed on an 8% polyacrylamide/6 M urea sequencing gel. A sequencing reaction using the same primer and *PRK1* cDNA as template was run alongside.

RNA and DNA Gel Blot Analyses

Total RNA was isolated from various tissues of *P. inflata* as previously described (Ai et al., 1990). Approximately 30 μg of total RNA from each tissue was electrophoresed on 1.2% formaldehyde/agarose gels (Sambrook et al., 1989) and transferred to Biotrans nylon membranes (ICN, Costa Mesa, CA). Membranes were prehybridized for 2 hr at 65°C in a solution containing 5 × Denhardt's, 5 × SSC, 0.1% SDS, and 200 μg/mL salmon sperm DNA and then hybridized with *PRK1* cDNA at 65°C overnight. Washing was carried out in 0.2 × SSC, 0.1% SDS at 65°C for 1 hr with two changes of solution. After autoradiography, the amounts of radioactive probes hybridized were quantified using a Betascope (Betagen, Waltham, MA). The membranes were boiled in 0.1 × SDS solution for 5 min to remove the original probes and then hybridized with a DNA probe encoding 25S rRNA of *P. inflata*. The amounts of radiolabeled 25S rRNA were quantified by the Betascope, and the relative amounts of RNA hybridized to the *PRK1* cDNA probe were determined after correction for differences in the amounts of 25S RNA. Genomic DNA was isolated from leaves using a method previously described (Coleman and Kao, 1992). Ten micrograms of genomic DNA was digested with restriction enzymes for 4 hr and electrophoresed on a 0.7% agarose gel. The DNA was transferred to Biotrans nylon membranes, and the membranes were prehybridized and hybridized with radiolabeled cDNA probes as described above.

SDS-Polyacrylamide Gel Electrophoresis

Proteins dissolved in 1 × Laemmli sample buffer (Laemmli, 1970) were loaded onto either 12 or 8% SDS–polyacrylamide gels. The protein bands were visualized by staining with Coomassie Brilliant Blue R 250.

Synthesis and Purification of a Recombinant Protein Corresponding to the Extracellular Domain of PRK1 in *E. coli*

The 0.8-kb EcoRI-BamHI cDNA fragment (Figure 4B) was blunt ended with the Klenow fragment of DNA polymerase I and then ligated to an XbaI linker with the sequence 5′-CTAGTCTAGACTAG-3′. Addition of the XbaI linker to the 3′ end introduced a translation termination codon (the underlined TAG sequence) in phase with the reading frame encoded by the 0.8-kb cDNA fragment. After ligation to an EcoRI linker with the sequence 5′-CGGAATTCCG-3′ and digestion with EcoRI, the fragment was inserted into the EcoRI site of pRSET-A vector (Invitrogen, San Diego, CA). The recombinant vector, pPRK1-EC, with the cDNA inserted in sense orientation, was sequenced across the site of insertion to confirm that the expected reading frame had been generated during the construction. The reading frame begins with a vector-encoded short sequence, which contains a stretch of six histidines, and is followed by a 272–amino acid sequence of the extracellular domain of PRK1. Both pPRK1-EC and pPRK1-EC(−), which contains the cDNA inserted in antisense orientation, were used to separately transform *E. coli* BL21 (DE3)pLyS (Novagen, Madison, WI; this strain will be abbreviated as BL21).

BL21 cells harboring pPRK1-EC or pPRK1-EC(−) were grown in a 4-L culture with vigorous shaking at 37°C until A_{600} reached 0.3. Isopropyl-β-D-thiogalactopyranoside was added to the culture to a final

concentration of 0.5 mM to induce the production of recombinant protein. Three hours postinduction, BL21 cells were harvested, and the pellet was resuspended in 40 mL of 20 mM Tris-HCl, pH 8.0, 200 mM KCl, 20% glycerol. After sonication, the insoluble proteins were collected by centrifugation at 39,000*g* for 20 min, and the pellet was solubilized by a solution containing 6 M guanidine, 100 mM NaH_2PO_4, 20 mM Tris-HCl, pH 8.0. The solubilized proteins were mixed with nickel resin according to the procedure recommended by the manufacturer (Novagen). After extensive washing to remove unbound proteins, the recombinant protein was eluted from the resin using a solution containing 8 M urea, 20 mM Tris-HCl, 100 mM NaH_2PO_4, pH 4.5. The eluates were concentrated with a stirred cell (Amicon, Beverly, MA) using filters with a molecular weight cutoff of 10,000 and then subjected to SDS-PAGE. The gel was stained with 0.3 M $CuCl_2$ (Harlow and Lane, 1988), and the 35-kD PRK1-EC recombinant protein was electroeluted from gel slices using an Elutrap electroseparation system (Schleicher & Schuell, Keene, NH).

Synthesis and Purification of a Recombinant Protein Corresponding to the Cytoplasmic Domain of PRK1 in *E. coli*

The 1.2-kb cDNA (Figure 4B) was inserted into the EcoRI site of pRSET-C vector in sense and antisense orientations to yield recombinant plasmids pPRK1-K and pPRK1-K(−), respectively. Both plasmids were used separately to transform BL21 cells. The conditions for the growth of *E. coli* cultures were the same as those described above, except that the cultures were grown at 30°C to increase the proportion of soluble recombinant protein and that isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. Four hours after induction, cells were harvested. The soluble proteins were separated from the insoluble proteins by centrifugation at 39,000*g* for 20 min. The soluble proteins were bound to nickel resin, and the resin was washed with an imidazole gradient according to the procedure recommended by the manufacturer (Novagen). The recombinant protein PRK1-K was eluted with a solution containing 100 mM imidazole, 20 mM Tris-HCl, pH 8.0, 200 mM KCl, 20 mM glycerol. For purification of the recombinant protein by a Mono-Q column, the proteins purified by the nickel resin were first diluted twofold with the addition of 20 mM Tris-HCl, pH 8.0, and then diluted another twofold with the addition of 20 mM Tris-HCl, pH 8.0, 10% glycerol. The proteins were chromatographed on a Mono-Q column (HR5/5; Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 10% glycerol. The bound proteins were eluted with a linear gradient of 0 to 500 mM KCl in the same buffer at a flow rate of 1 mL/min. Protein-containing fractions were analyzed by SDS-PAGE to identify the fractions containing the recombinant protein PRK1-K.

Production of Polyclonal Antibodies against the Extracellular Domain of PRK1

Preimmune serum was collected from three New Zealand White rabbits prior to immunization. Approximately 750 μg of the gel-purified recombinant protein PRK1-EC was mixed with an adjuvant consisting of monophosphoryl lipid A + trehalose dimycolate + cell wall skeleton (RIBI Immunochem Research, Inc., Hamilton, MT), and the emulsion was injected subcutaneously at multiple sites on each of the three rabbits. The rabbits were boosted twice with half the amount of the antigen used for initial immunization. The serum was collected and euthanasia of the animals was performed according to the guidelines of the Institutional Animal Care and Use Committee at The Pennsylvania State University.

Preparation of Monospecific Immune Serum

Approximately 1 mg of eluates from nickel resin, which contained the recombinant PRK1-EC protein, was electrophoresed on a preparative 12% SDS–polyacrylamide gel and electroblotted to a nitrocellulose membrane (Millipore). The membrane was stained with Ponceau S reagent according to the manufacturer's procedure (Sigma). The membrane piece containing PRK1-EC was excised and rinsed with PBS buffer to remove the dye. The membrane slice was blotted with 3% BSA in PBS buffer for 2 hr and subsequently blotted with 1 mL of immune serum that had been diluted with 30 mL of 3% BSA in PBS buffer for 2 hr. After removal of immune serum, the membrane slice was washed with 0.1% Tween 20 in PBS buffer for 20 min with four changes of solution. The monospecific immune serum was eluted by treating the membrane with 500 mL of 0.2 M glycine, pH 2.8, containing 1 mg/mL BSA for 5 min with gentle shaking. The supernatant was removed and immediately neutralized using 1 M Tris, pH 8.0.

Protein Gel Blot Analysis

Microsomal membranes were prepared from mature pollen and in vitro–germinated pollen tubes, and total protein or proteins in the microsomal fractions were extracted from mature pollen, in vitro–germinated pollen tubes, and stage 5 anthers using the methods described by Chang et al. (1992). Protein concentration was determined by the Bradford method (Bradford, 1976) using reagents from BioRad (Hercules, CA). Approximately 100 μg of the protein for each sample was electrophoresed on SDS–polyacrylamide gels and electroblotted to nitrocellulose membranes in an electroblotting buffer (Harlow and Lane, 1988). The membranes were blotted with 3% BSA in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, and subsequent steps were performed as described by Harlow and Lane (1988). Either a T7-tag monoclonal antibody specific to the portion of the recombinant protein encoded by pRSET-C vector (Novagen) or the monospecific immune serum to PRK1-EC was used as primary antibody. Either goat anti–mouse polyclonal antibody or goat anti–rabbit polyclonal antibody conjugated with alkaline phosphatase (Life Technologies, Inc., Gaithersburg, MD) was used as a secondary antibody. Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine (Life Technologies, Inc.) were used as substrates for alkaline phosphatase for color development.

Kinase Assay and Phosphoamino Acid Analysis

Ten micrograms of the purified recombinant protein PRK1-K was used for kinase assay. The reaction was performed in a 100-μL solution (30 mM Tris-HCl, pH 7.4, 20 mM Hepes, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP) with 50 μCi of γ-^{32}P-ATP (3000 Ci/mmol). The reaction was performed at 30°C for 30 min and terminated by the addition of one tenth the volume of 10% Nonidet P-40 and the addition of trichloroacetic acid to a final concentration of 12.5%. The reaction mixtures were placed on ice for at least 1 hr and centrifuged in a microcentrifuge at 4°C for 15 min. The pellet was washed with cold 100% ethanol

twice and air dried. It was dissolved in 1 × Laemmli sample buffer and electrophoresed on a 12% SDS–polyacrylamide gel. As a positive control, the catalytic domain of cAMP-dependent kinase was used to phosphorylate casein using a procedure recommended by the manufacturer (Sigma) and similarly processed.

After blotting, the Immobilon-P membranes (Millipore) were autoradiographed, and radiolabeled protein bands corresponding to PRK1-K and casein were excised. The proteins were partially hydrolyzed in 6 N HCl at 110°C for 1 hr (Kamps and Sefton, 1989). The supernatant containing protein hydrolysates was lyophilized and redissolved in water; the process was repeated four times. The samples were dissolved in 4 μL of distilled water and 2 μL of cold phosphoamino acid standards (2 mg/mL each of phosphoserine, phosphothreonine, and phosphotyrosine). The samples were spotted onto 100-mm thin-layer chromatography plates (EM Science, Gibbstown, NJ). One-dimensional thin-layer electrophoresis was performed using a HTLE 7000 unit (CBS Scientific, Del Mar, CA) at 1300 V for 50 min in the pH 3.5 electrophoresis buffer with 0.5 mM EDTA (Boyle et al., 1991). The positions of the three phosphoamino acids used as markers were visualized by spraying the thin-layer chromatography plates with 0.2% ninhydrin (Sigma) in absolute ethanol followed by heat treatment. The thin-layer chromatography plates were autoradiographed for 8 days to visualize labeled phosphoamino acid(s).

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