Isolation and Characterization of Kinase Interacting Protein 1, a Pollen Protein That Interacts with the Kinase Domain of PRK1, a Receptor-Like Kinase of Petunia[1]

Andrea L. Skirpan, Andrew G. McCubbin, Takeshi Ishimizu, Xi Wang, Yi Hu, Peter E. Dowd, Hong Ma, and Teh-hui Kao*

Departments of Biochemistry and Molecular Biology (A.L.S., A.G.M., X.W., P.E.D., T.-h.K.) and Biology and the Life Sciences Consortium (Y.H., H.M.) and Intercollege Graduate Degree Program in Plant Physiology (H.M., T.-h.K.), The Pennsylvania State University, University Park, Pennsylvania 16802; and Graduate School of Science, Osaka University, 1–1 Machikaneyama-cho, Toyonaka 560–0043, Japan (T.I.)

Many receptor-like kinases have been identified in plants and have been shown by genetic or transgenic knockouts to play diverse physiological roles; however, to date, the cytosolic interacting proteins of relatively few of these kinases have been identified. We have previously identified a predominantly pollen-expressed receptor-like kinase of petunia (*Petunia inflata*), named PRK1, and we have shown by the antisense RNA approach that it is required for microspores to progress from the unicellular to bicellular stage. To investigate the PRK1-mediated signal transduction pathway, *PRK1-K* cDNA, encoding most of the cytoplasmic domain of PRK1, was used as bait in yeast (*Saccharomyces cerevisiae*) two-hybrid screens of pollen/pollen tube cDNA libraries of petunia. A protein named kinase interacting protein 1 (KIP1) was found to interact very strongly with PRK1-K. This interaction was greatly reduced when lysine-462 of PRK1-K, believed to be essential for kinase activity, was replaced with arginine (the resulting protein is named PRK1-K462R). The amino acid sequence of KIP1 deduced from full-length cDNA contains an EF-hand Ca^{2+}-binding motif and nine predicted coiled-coil regions. The yeast two-hybrid assay and affinity chromatography showed that KIP1 interacts with itself to form a dimer or higher multimer. *KIP1* is present in a single copy in the genome, and is expressed predominantly in pollen with a similar temporal pattern to *PRK1.* In situ hybridization showed that *PRK1* and *KIP1* transcripts were localized in the cytoplasm of pollen. PRK1-K phosphorylated KIP1-NT (amino acids 1–716), whereas PRK1-K462R only weakly phosphorylated KIP1-NT in vitro.

Since the cloning of the first plant receptor-like kinase (RLK) gene (Walker and Zhang, 1990), there have been a large number of such genes identified from a variety of plant species (for review, see Becraft, 1998; Torii and Clark, 2000). These RLKs were initially classified into three classes based on the nature of the proteins related to their extracellular domains. They are the S-domain class, the Leu-rich repeat class, and the epidermal growth factor-like class (Braun and Walker, 1996). Subsequent identification of additional classes of RLKs (e.g. an RLK of tobacco with the extracellular domain similar to chitinases; see Kim et al., 2000) has revealed the presence of a wider variety of RLKs in plants. Functional studies using transgenic approaches or mutant analyses have shown that plant RLKs play diverse physiological roles, including control of shoot and floral meristem size by CLAVATA1 of Arabidopsis (Clark et al., 1997), mediation of a brassinosteroid signal transduction pathway by BRI1 of Arabidopsis (Li and Chory, 1997), mediation of race-specific recognition of bacterial pathogens by Xa21 of rice (Song et al., 1995), and control of the stigma's ability to recognize and reject self pollen in self-incompatibility interactions by SRK of *Brassica rapa* (Takasaki et al., 2000).

To understand the signaling cascade mediated by an RLK, it is imperative to identify its ligand(s) and substrate(s). The yeast (*Saccharomyces cerevisiae*) two-hybrid protein-protein interaction screen and expression cDNA library screening have been successfully used to identify cytosolic proteins that interact with the kinase domain of several RLKs. For example, a protein phosphatase, KAPP, of Arabidopsis interacts with CLAVATA1 (Williams et al., 1997; Stone et al., 1998) and with several other RLKs of Arabidopsis (Stone et al., 1994; Braun et al., 1997); three *Brassica napus* proteins, ARC1 (which contains an Arm repeat) and two thioredoxins, interact with SRK (Bower et al., 1996; Gu et al., 1998; Stone et al., 1999). Identification of the ligand(s) of RLKs has proven more difficult, and to date, putative ligands of only a few plant RLKs have been reported. Among them are CLAVATA3, a putative ligand for CLAVATA1 (Clark et al., 1995; Trotochaud et al., 2000); SCR (or

[1] This work was supported by the U.S. Department of Agriculture (grant nos. 96–35304–3635 and 99–35304–8004 to T.-h.K.), by the National Science Foundation (grant nos. MCB–9728772 and IBN–0077832), by the Biology Department and the Life Sciences Consortium of The Pennsylvania State University (to H.M.), by a pre-doctoral Fellowship for Students with Disabilities from the National Institutes of Health (to P.E.D.), and by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science (to T.I.).

* Corresponding author; e-mail txk3@psu.edu; fax 814–863–9416.

SP11), a putative ligand for SRK (Schopfer et al., 1999; Takayama et al., 2000; Shiba et al., 2001); and brassinolide, a putative ligand for Xa21 (He et al., 2000; Wang et al., 2001).

PRK1 of petunia (*Petunia inflata*) was the first pollen-expressed RLK to be identified (Mu et al., 1994). The deduced amino acid sequence predicts a protein consisting of an extracellular domain including five Leu-rich repeats, a transmembrane domain, and a cytoplasmic domain. A recombinant PRK1-K protein that contained most of the cytoplasmic domain of PRK1 was shown to autophosphorylate on Ser and possibly Tyr residues (Mu et al., 1994). During pollen development, the *PRK1* message is first detected in anthers around the developmental stage when microspores undergo pollen mitosis I to produce bicellular microspores. The message level then increases and reaches its highest point in mature pollen, and it remains high in in vitro-germinated pollen tubes.

The physiological role of PRK1 in pollen development was examined in an antisense RNA experiment (Lee et al., 1996). Petunia plants were transformed with a construct containing the *LAT52* promoter of tomato (Twell et al., 1990) fused to a 0.6-kb cDNA segment encoding approximately 58% of the extracellular domain of PRK1 in antisense orientation. The transgenic plants in which the message level of the endogenous *PRK1* gene was reduced produced approximately equal amounts of normal and aborted pollen, as would be expected when the function of a gametophytic gene is knocked out. Moreover, the aborted pollen grains were arrested at the unicellular stage of microspore development at the time that normal microspores have completed mitosis to become bicellular. Thus, PRK1 appears to be required for the progression of microspores from the unicellular stage to the bicellular stage during pollen development. Since *PRK1* is expressed in mature pollen, it cannot be ruled out that PRK1 is also involved in late stages of pollen development and/or post-pollination events. It is interesting that antisense *PRK1* transgenic plants that showed the pollen abortion phenotype also showed abnormal embryo sac development, with the two polar nuclei failing to migrate and fuse to form the central cell (Lee et al., 1997). However, although the *PRK1* transcript was detected in the ovary, its level was much lower than in pollen, making it difficult to definitively establish the cause-and-effect relationship between down-regulation of *PRK1* and the embryo sac phenotype (Lee et al., 1997). Two other pollen-expressed RLKs, LePRK1 and LePRK2, have recently been identified in tomato, but they are thought to be involved in post-pollination events rather than in pollen development because their transcripts are not detected until pollen is nearly mature and because they are localized to the wall/membrane of pollen tubes (Muschietti et al., 1998).

To gain a better understanding of how PRK1 mediates a signal transduction pathway essential for pollen development, we set out to use the yeast two-hybrid screen to identify pollen proteins that interact with the cytosolic domain of PRK1. Such a screen of a tobacco flower cDNA library previously identified a protein showing sequence similarity with the β-subunit of translation initiation factor 2B (eIF2B-β) of human and yeast (Park et al., 2000). However, since this is a tobacco protein, its physiological relevance to the function of PRK1 is unknown. In this report we describe the identification and characterization of a petunia pollen-specific protein named kinase interacting protein 1 (KIP1), which interacts very strongly with PRK1-K.

RESULTS

Identification of KIP1, Which Interacts with the Kinase Domain of PRK1

The yeast two-hybrid protein-protein interaction screen was used to identify pollen proteins that interact with the kinase domain of PRK1 and thus might be involved in the downstream events of the PRK1-mediated signaling pathway. The bait construct, designated pGBT9/PRK1-K, contained the 1.2-kb *PRK1-K* cDNA (Mu et al., 1994) encoding amino acids 408 to 720 of PRK1 (Fig. 1). Because *PRK1* is expressed in mature pollen and in vitro germinated pollen tubes (Mu et al., 1994), both were used as the source of poly(A)$^+$ RNA for the construction of a cDNA library (S_2S_2) in "prey" vector pGAD424. In this system, interaction of bait and prey fusion proteins results in the activation of the GAL4 promoter driving β-galactosidase expression, which

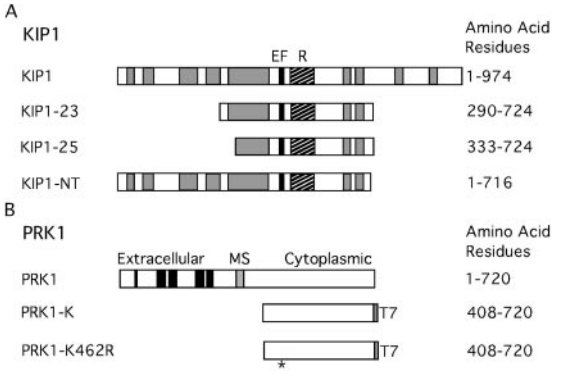

Figure 1. Schematic representation of the structural features of full-length and truncated KIP1 (A) and PRK1 (B) proteins. The drawings are to scale. For KIP1, the nine lightly shaded regions denote coiled-coil regions, R denotes the seven tandem repeats of 11 amino acids, and EF (black region) denotes an EF-hand motif (13 amino acids). For PRK1, MS (lightly shaded region) denotes the membrane-spanning domain, T7 denotes the 11-amino acid T7 tag, the five black regions denote the Leu-rich repeats in the extracellular domain, and an asterisk indicates the Lys-462 residue in the cytoplasmic domain that has been replaced with an Arg in PRK1-K462R. The first and last amino acid residues of each peptide are indicated.

can then be assayed enzymatically. A blue color denotes a positive reaction with the substrate 5-bromo-4-chloro-3 indolyl (3-D-galactopyranoside X-gal). Six positive clones were identified from screening approximately 3×10^6 yeast colonies generated by cotransforming yeast strain HF7c with pGBT9/PRK1-K and the total library cDNA. In the X-gal filter assay, four of these clones turned blue in approximately 1 h and the other two in approximately 5 h. Sequence analysis revealed that the prey cDNAs contained in the former four clones were derived from the same mRNA species, and the prey cDNAs contained in the latter two clones were derived from another mRNA species. The protein encoded by the former class of cDNA, designated KIP1, was chosen for this study for two reasons. First, the X-gal filter assay suggested that KIP1 interacted with PRK1-K more strongly than did the latter class, designated KIP2. Second, clones encoding KIP1 were also isolated from a second yeast two-hybrid screen of a different pollen/pollen tube cDNA library, as described below. Among the four KIP1 clones, those that contained the longest (1.3 kb) and second longest (1.2 kb) cDNA inserts, were designated pGAD424/KIP1-23 and pGAD424/KIP1-25, respectively, and were used in the subsequent experiments described hereafter.

The specificity of the PRK1-K interaction with KIP1-23 and KIP1-25 (spanning amino acids 290–724 and 333–724 of KIP1, respectively; see Fig. 1) was confirmed by cotransforming a different yeast strain, SFY526, with the bait plasmid, pGBT9/PRK1-K, and pGAD424/KIP1-23 or pGAD424/KIP1-25. In SFY526, the *LacZ* reporter gene was under the control of a promoter different from that used to control *LacZ* expression in HF7c. These two promoters share only the GAL4 response elements, thus the observation of two-hybrid interactions in both yeast strains is a strong indication that the activation of *LacZ* expression is due to the specific binding of the DNA-binding domain of GAL4 to its response elements. All the colonies were found to turn blue on X-gal filters in approximately 1 h (as did the HF7c colonies carrying the same constructs). Moreover, transformation of pGBT9/PRK1-K, pGAD424/KIP1-23, or pGAD424/KIP1-25 alone into HF7c or SFY526 did not cause the yeast colonies to turn blue in the X-gal filter assay even after overnight incubation.

A second yeast two-hybrid screen was carried out with the same PRK1-K bait, but with a different pollen/pollen tube cDNA library (S_3S_3) constructed in the vector pGAD424. Screening of approximately 4×10^7 yeast transformants in strain HF7c yielded 144 positive colonies. DNA dot-blot analysis showed that all except one of these prey cDNAs hybridized with *KIP1-23* cDNA. Moreover, PCR of the prey cDNAs of these 143 clones, using a forward primer to *KIP1-23* approximately 700 bp upstream from the 3′ end of the cDNA and a reverse primer to pGAD424 immediately downstream from the cloning site, generated DNA fragments similar in size to the fragment expected from *KIP1-23*. Sequencing of the prey cDNAs isolated from six randomly chosen colonies confirmed that they encoded KIP1 (results not shown). When the prey cDNA (in pGAD424 vector) isolated from the one clone not in the KIP1 class was transformed into SYF526, none of the colonies turned blue. Thus, this clone was not studied further.

Effect of Changing Lys-462 of PRK1-K to Arg on the Interaction of PRK1-K with KIP1

Based on sequence comparison with other kinases, Lys-462 of PRK1 was deemed the most likely candidate for the conserved Lys residue that has been implicated in Mg^{2+}/ATP binding (Hanks et al., 1988; Mu et al., 1994). Changing this Lys to another amino acid has been shown to abolish the kinase activity of many kinases, including several plant RLKs, e.g. SRK of *B. napus* (Goring and Rothstein, 1992). To examine the effect of abolishing (or reducing) the kinase activity of PRK1-K on its interaction with KIP1, the AAG codon for Lys-462 was replaced by AGG for Arg and the resultant construct, designated PRK1-K462R (Fig. 1), was used to assess its interaction with KIP1 in the yeast two-hybrid assay. Yeast colonies carrying pGBT9/PRK1-K462R and pGAD424/KIP1-23 were found to turn very faint blue in the X-gal filter assay only after overnight incubation. A liquid β-galactosidase activity assay was performed using *O*-nitrophenyl β-D-galactopyranoside as substrate to quantify the relative strengths of KIP1 interactions with PRK1-K and with PRK1-K462R. The β-galactosidase activities for yeast cells producing PRK1-K and KIP1-23, and yeast cells producing PRK1-K462R and KIP1-23 were 3.4 ± 0.1 and 0.54 ± 0.1 units, respectively (all negative controls yielded 0.001). Thus, PRK1-K462R interacted much more weakly with KIP1 than did PRK1-K.

Tissue and Temporal Expression Patterns of *KIP1*

RNA gel-blot analysis using *KIP1-23* cDNA as a probe showed that the *KIP1* transcript was approximately 3.4 kb in size and like the *PRK1* transcript, was detectable in mature pollen grains and pollen tubes, but not in style, ovary, petal, leaf, root, or sepal (Fig. 2). The temporal expression pattern of *KIP1* during anther development was also found to be very similar to that of *PRK1*, previously shown by Mu et al. (1994). The transcripts of both genes were first detected in stage 3 anthers (Anther-3) around the time of microspore mitosis and they reached the highest level in stage 5 anthers (Anther-5), which contained nearly mature pollen grains (Fig. 2).

Cloning of Full-Length *KIP1* cDNA and Structural Features of KIP1

As the longest *KIP1* cDNA (*KIP1-23*) obtained from the two yeast two-hybrid screens was shorter than

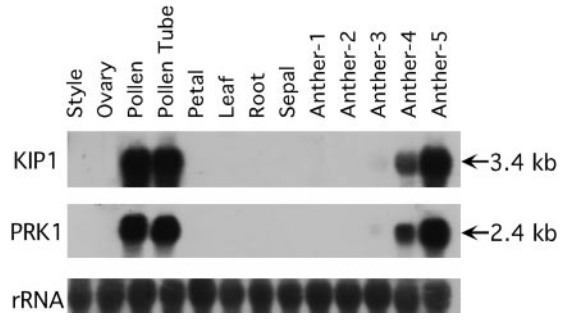

Figure 2. RNA gel-blot analysis of expression of *KIP1* and *PRK1*. Twenty micrograms of total RNA was loaded in each lane. The blot was hybridized, stripped of the probe, and reprobed with each of the following cDNAs in succession: *KIP1-25*, *PRK1*, and *28S rRNA*. Anther-1, Stage 1 anthers from buds that were less than 0.5 cm in length and containing developing microspores in the tetrad configuration; Anther-2, stage 2 anthers from buds between 0.5 and 1.0 cm in length and containing mostly free unicellular microspores; Anther-3, stage 3 anthers from buds between 1.0 and 1.5 cm in length and containing mostly bicellular microspores; Anther-4, stage 4 anthers from buds between 1.5 and 2.0 cm in length and containing essentially all bicellular microspores; Anther-5, stage 5 anthers from purple buds between 2.0 and 2.5 cm in length and containing mature pollen grains. The sizes of *KIP1* and *PRK1* messages are indicated.

the *KIP1* transcript (approximately 3.4 kb) detected by RNA gel-blot analysis, a pollen cDNA library of petunia was constructed and screened to isolate a full-length cDNA clone. The *KIP1* cDNA is 3,374 bp in size (GenBank accession no. AY029758) and consists of an open reading frame (ORF) of 2,922 bp, 120 bp of the 5′non-coding sequence, and 332 bp of the 3′ non-coding sequence, including an 18-bp poly(A) tail. *KIP1* appeared to be present in a single copy in the petunia genome because genomic DNA gel-blot analysis showed that only a single band was detected in *Eco*RI and *Hin*dIII digests when probed with *KIP1-23* cDNA (Fig. 3). Moreover, when *KIP1* cDNA was used as a probe, three *Eco*RI fragments were observed, consistent with the prediction based on the sequence of *KIP1* cDNA (data not shown).

Analysis of the deduced amino acid sequence of KIP1 revealed several structural motifs (Figs. 1 and 4). First, the sequence DSSPDQVIALAEI matches the consensus sequence for an EF hand calcium-binding motif. This motif includes the invariant Asp/Glu residue at position 12 that provides the two oxygen atoms required for interaction with calcium ions (Kretsinger, 1976). Second, there are nine alpha-helical coiled-coil regions, a motif involved in protein-protein interactions and responsible for dimerization of some cytoskeletal proteins such as myosins (Titus, 1993; Kinkema et al., 1994). Third, there are seven tandem repeats of an 11-amino acid sequence (A/T)E(G/V)PKNLSTI(K/E) flanked by charged hydrophilic regions. A similar feature exists in the microtubule-associated protein Tau. In Tau, the hydrophilic regions flanking the tandem repeats are responsible for microtubule binding, whereas the repeat region is believed to act as a catalytic domain for microtubule assembly (Trinczek et al., 1995; Preuss et al., 1997).

BLAST searches revealed that the deduced amino acid sequence of KIP1 was most similar to those of two ORFs of Arabidopsis identified from genome-wide sequencing. One of the ORFs, here designated AtORF1 (GenBank accession no. AC006592), encodes 891 amino acids; the other, here designated AtORF2 (GenBank accession no. AC000132), encodes 947 amino acids. Pair-wise comparison shows that AtORF1 is 68.3% similar and 38.6% identical with KIP1, and AtORF2 is 69.4% similar and 36.1% identical with KIP1. These three proteins share blocks of sequence conservation, especially in the regions that were predicted to be coiled-coil in KIP1 (Fig. 4). However, the EF-hand motif and the sequence of seven tandem repeats in KIP1 are not conserved in either Arabidopsis protein. AtORF1 does contain three almost tandem repeats of 15 amino acids, spanning amino acids 494 to 543 (the three boxed regions in Fig. 4), except for amino acids 510 and 527. Although this repeat, (A/E)SD(K/Q)(T/I)DSV(L/P)(D/S)V(L/S)E(N/K)(Q/E), does not share any similarity with the 11-amino acid repeat of KIP1, the position of these repeats is very close to that where the seven tandem repeats of KIP1 are located.

Phosphorylation of KIP1 by PRK1-K in Vitro

To corroborate the interaction between PRK1-K and KIP1 observed in the yeast two-hybrid assay we engineered several expression constructs for producing GST fusion proteins of PRK1-K, PRK1-K462R,

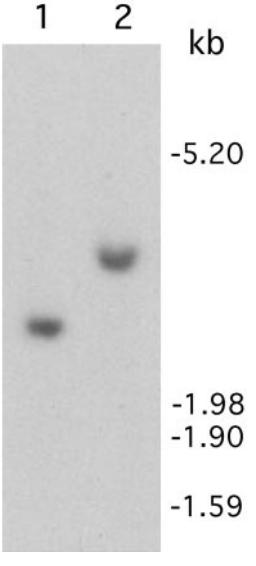

Figure 3. Genomic DNA gel-blot analysis. Each lane contains restriction digests of 12 μg of petunia genomic DNA. Lane 1, *Eco*RI digest; lane 2, *Hin*dIII digest. The blot was probed with *KIP1-23* cDNA. Size markers are shown to the right of the blot.

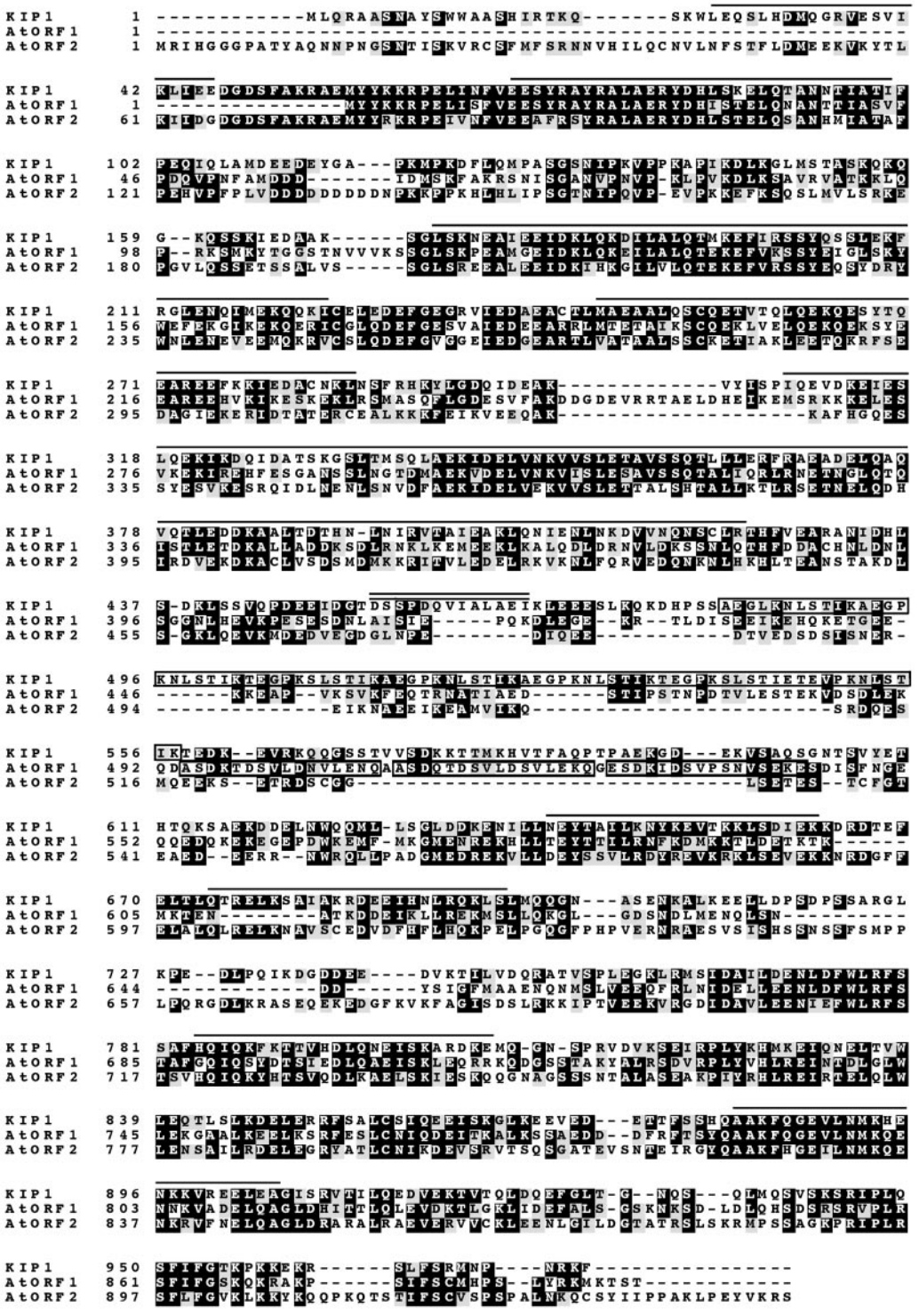

Figure 4. Alignment of the deduced amino acid sequences of KIP1 of petunia and two ORFs of Arabidopsis. AtORF1 corresponds to ORF4 of bacteria artificial chromosome clone F14 M13 (GenBank accession no. AC006592) and encodes 891 amino acids; AtORF2 corresponds to ORF 11 of bacteria artificial chromosome clone F21M12 (GenBank accession no. AC000132) and encodes 947 amino acids. For each aligned position, identical amino acids are shaded in black and conservative changes are shaded in light gray. Hyphens represent gaps that have been introduced to maximize similarity. For KIP1, the nine predicted coiled-coiled regions are overlined; the predicted EF-hand is double-overlined; and the seven 11-amino acid repeats, (A/T)E(G/V)PKNLSTI(K/E), are enclosed in a box. The three boxed regions of AtORF1 correspond to three repeats of 15 amino acids.

and KIP1-NT (encompassing amino acids 1–716 of KIP1; see Fig. 1). Purified recombinant proteins were then used to examine whether PRK1-K could phosphorylate KIP1-NT in vitro. For the PRK1-K and PRK1-K462R fusion proteins, an additional T7 tag was fused to the C terminus (Fig. 1) so that they could be identified by a monoclonal antibody against the tag. GST/PRK1-K/T7tag and GST/PRK1-K462R/T7tag fusion proteins were produced in yeast, as our initial attempts to produce them in several *Escherichia coli* strains resulted in very low yields. On the contrary, production of GST/KIP1-NT fusion protein in *E. coli* resulted in higher yields and fewer contaminating host proteins than in yeast.

GST/PRK1-K/T7tag was found to autophosphorylate (Fig. 5, A and B, lane 1) as had been previously shown using PRK1-K fused to a 6×-His tag (Mu et al., 1994). The ability of GST/PRK1-K462R/T7tag to autophosphorylate was greatly reduced (Fig. 5, A and B, lane 2) when compared with that of GST/PRK1-K/T7tag, but not completely abolished. Incubation of GST/KIP1-NT alone in the kinase reaction did not result in any detectable phosphorylation of this protein (Fig. 5, A and B. lane 3). A strongly phosphorylated GST/KIP1-NT band was detected when GST/PRK1-K/T7tag was co-incubated with GST/KIP1-NT (Fig. 5, A and B, lane 4), whereas GST/KIP1-NT was very weakly phosphorylated by GST/PRK1-K462R/T7tag (Fig. 5, A and B, lane 5).

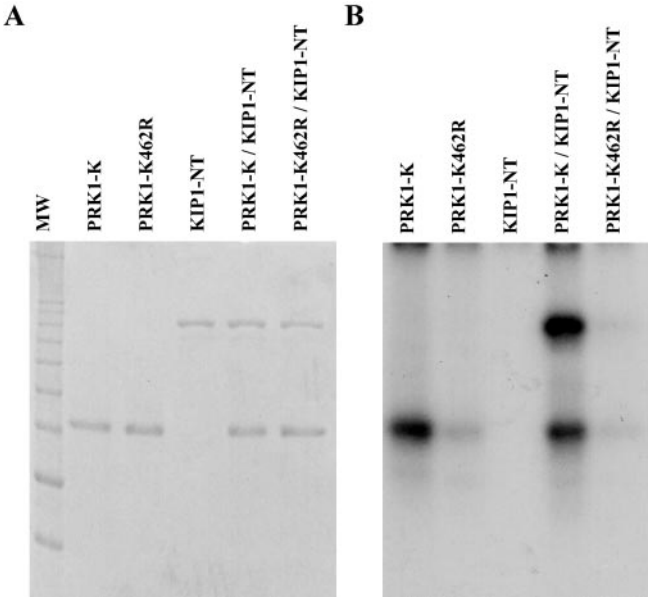

Figure 5. Phosphorylation of KIP1 by PRK1-K. GST fusion proteins of PRK1-K, PRK1-K462R, and KIP1-NT were purified by glutathione Sepharose 4B columns and were used for the phosphorylation assay in the presence of [^{32}P]ATP. Aliquots of five different reaction mixtures, as indicated, were electrophoresed on a 10% (w/v) SDS-polyacrylamide gel that was stained with Coomassie Blue (A) and dried for autoradiography (B). MW, Molecular mass markers.

In Situ RNA Hybridization of *KIP1* and *PRK1*

To determine whether *PRK1* and *KIP1* are specifically expressed in pollen and not in the sporophytic tissue of the anther, in situ hybridization was carried out on anthers collected from stage 4 buds. To prevent hybridization to RNA transcripts of other kinases that share sequence similarity with the kinase domain of PRK1, antisense and sense RNA probes for *PRK1* were obtained by in vitro transcription of a 537-bp DNA fragment of *PRK1* cDNA encoding amino acids 82 to 261 in the extracellular domain of PRK1. We had previously shown that this part of *PRK1* hybridized to a single genomic fragment (Mu et al., 1994). For *KIP1* (a single-copy gene), antisense and sense RNA probes were obtained by in vitro transcription of a 507-bp DNA fragment encoding amino acids 184 to 353 of KIP1. The in situ hybridization results (Fig. 6) clearly showed that *PRK1* and *KIP1* transcripts were present in the pollen grain cytoplasm. In contrast, the signals detected in the anther wall with the antisense *PRK1* and *KIP1* probes were similar to those detected with the corresponding sense probes and were considered non-specific. The distribution of both transcripts appeared to be rather uniform throughout the pollen cytoplasm.

Interaction between KIP1 and Itself

To gain insight into the cellular function of KIP1 and its role in the PRK1-mediated signaling pathway, the yeast two-hybrid screen was used to identity protein(s) with which KIP1 interacts. *KIP1-25* cDNA was released from the prey vector pGAD424 and was ligated into the pGBT9 vector to make a bait construct. The bait DNA and prey S_2S_2 pollen/pollen tube cDNA library were transformed into yeast strain HF7c. Approximately 1×10^5 yeast transformants were screened and four colonies were found to turn blue in approximately 4 h. Sequencing of the cDNAs contained in these four colonies revealed that they were identical to *KIP1-23* or *KIP1-25* cDNA. This two-hybrid interaction was found to also occur in yeast strain SYF526.

The KIP1-KIP1 interaction was further confirmed by affinity chromatography. *KIP1-23* cDNA was cloned into two expression vectors, pGEX-5X-1 and pRSET-C, for the production of GST/KIP1-23 fusion protein and 6×-His-tag/KIP1-23 fusion protein, respectively, in *E. coli. KIP1-23* cDNA was also cloned in antisense orientation into pGEX-5X-1; the resulting protein consists of GST fused to a 22-amino acid peptide encoded by the short ORF of the antisense *KIP1-23* cDNA. Total protein extracts from *E. coli* cells harboring the GST/*KIP1-23* cDNA construct and GST/antisense *KIP1-23* cDNA construct were passed through two separate glutathione Sepharose 4B columns to generate a GST/KIP1-23 affinity column and a GST (+ 22 amino acids unrelated to KIP1) affinity column (as a negative control).

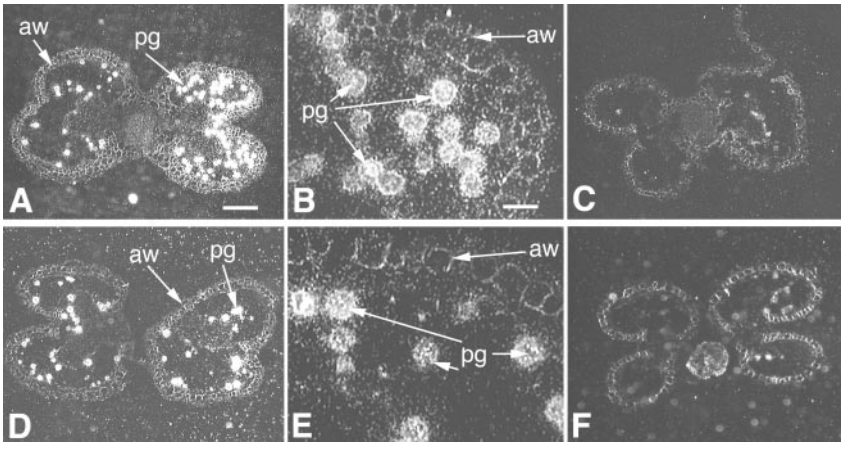

Figure 6. RNA in situ hybridization of anther sections of petunia. Sections of stage 4 anthers were hybridized with [^{35}S]-labeled *KIP1* antisense (A and B), *KIP1* sense (C), *PRK1* antisense (D and E), and *PRK1* sense (F) probes. A, C, D, and F are of the same magnification; bar = 200 μm. B and E are of the same magnification; bar = 50 μm. pg, Pollen grain; aw, anther wall.

Total protein extract from *E. coli* cells harboring the 6×-His-tag/*KIP1-23* cDNA construct was then passed through both columns. GST/KIP1-23 and GST (+ 22 amino acids) were eluted from their respective columns by 20 mM glutathione and were analyzed by SDS-PAGE. An anti-GST antibody (Fig. 7A) and a T7-tag monoclonal antibody (Fig. 7B) were used to detect the fusion proteins, GST/KIP1-23 and 6×-His-tag/KIP1-23 (which contained the T7-tag sequence), respectively. For the GST/KIP1-23 affinity column, GST/KIP1-23 and 6×-His-tag/KIP1-23 fusion proteins were detected (Fig. 7, A and B, lane 1), whereas for the GST (+ 22 amino acids) affinity column, GST (+ 22 amino acids), but not 6×-His-tag/KIP1-23, was detected (Fig. 7, A and B, lane 2). These results suggest that the retention of 6×-His-tag/KIP1-23 on the GST/KIP1-23 affinity column was due to the interaction between the KIP1-23 part of the GST/KIP1-23 fusion protein and the KIP1-23 part of the 6×-His-tag/KIP1-23 fusion protein.

DISCUSSION

Results from our previous antisense RNA experiment have suggested that PRK1, a predominantly pollen-expressed RLK of petunia, regulates a signal transduction pathway necessary for unicellular microspores to progress through pollen mitosis I to generate bicellular microspores. In this report we describe the use of the yeast two-hybrid protein-protein interaction screen to identify KIP1, a pollen-specific protein of petunia, which interacts with the cytosolic kinase domain of PRK1. Since the yeast two-hybrid screen is prone to yielding false positives we have used several criteria to validate the interaction between PRK1 and KIP1.

First, multiple independent clones encoding KIP1 were isolated from each of the two two-hybrid screens using different pollen/pollen tube cDNA libraries of petunia. In contrast, for KIP2, two independent positive clones were isolated from the first two-hybrid screen, but none was isolated from the second screen. Second, for each two-hybrid screen, interactions of a similar strength (as judged by the time it took for the colony to turn blue in X-gal filter assay) were observed in two different yeast strains containing different GAL4-responsive promoters driving the expression of the reporter protein, thus making the positive results unlikely to be due to promoter-specific artifacts. Third, pGBT9/PRK1-K, pGAD424/KIP1-23, or pGAD424/KIP1-25 alone did not yield positive results in either yeast strain. Fourth, most importantly, the recombinant PRK1-K, the kinase domain of PRK1, was found to phosphorylate KIP1 in vitro.

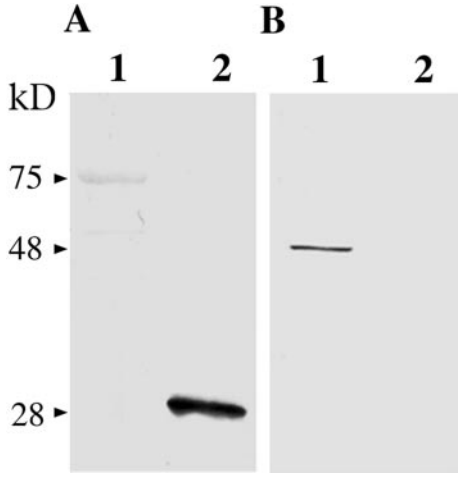

Figure 7. Protein gel-blot analysis of the interaction between 6×-His-tag/KIP1-23 and GST/KIP1-23. The blot shown in A was incubated with an anti-GST antibody and the blot shown in B was incubated with a T7-tag monoclonal antibody that reacted with an 11-amino acid sequence present at the N-terminal end of the fusion protein 6×-His-tag/KIP1–23. For both blots, lane 1 represents the fraction eluted from the GST/KIP1-23 affinity column over which total protein extract from *E. coli* cells harboring the 6×-His-tag/*KIP1-23* cDNA construct had been passed, and lane 2 represents the fraction eluted from the GST (+ 22 amino acids from antisense *KIP1-23* cDNA) affinity column over which total protein extract from *E. coli* cells harboring the 6×-His-tag/*KIP1–23* cDNA construct had been passed. The protein bands indicated with arrows are GST/KIP1-23 (75 kD) and GST (+ 22 amino acids; 28 kD), and 6×-His-tag/KIP1-23 (48 kD).

Although PRK1 and KIP1 have not been shown to interact in planta, we believe, based on the following observations, that the interaction can occur in vivo and is most likely physiologically relevant. First, RNA gel-blot analysis shows that *PRK1* and *KIP1* transcripts are detected only in pollen, and that the temporal expression patterns of these two genes during anther development are very similar, with the first detection around the stage of pollen mitosis I. (It should be noted that very low levels of the *PRK1* transcript were previously detected in ovaries when poly(A)$^+$ RNA, instead of total RNA, was used for RNA gel-blot analysis; see Lee et al., 1997.) Second, the in situ hybridization results show that the transcripts of *PRK1* and *KIP1* are present in the cytoplasm of the pollen grain and not in the sporophytic tissue of the anther. Third, the yeast-two hybrid and in vitro phosphorylation assays show that reducing the kinase activity of PRK1-K leads to reduction in its interaction with KIP1 and its ability to phosphorylate KIP1, respectively. These results taken together suggest that KIP1 and PRK1 are present in the developing pollen at the stage when PRK1 is required, and that optimal interaction between KIP1 and PRK1 requires the native enzymatic activity of PRK1. Thus, the PRK1 and KIP1 interaction is most likely physiologically relevant. Nonetheless, the involvement of KIP1 in the PRK1-mediated signaling pathway will ultimately have to be confirmed by transgenic experiments. If *KIP1* is represented as a single copy in the genome, antisense and dominant negative approaches can be used to reveal whether suppression of the production, or the normal function, of KIP1 will result in the same developmental abnormality of microspores as that exhibited by antisense *PRK1* transgenic plants.

BLAST searches have revealed that KIP1 is most similar to two predicted proteins of Arabidopsis. Since these two proteins have unknown functions, they cannot be used to deduce the biochemical nature and physiological function of KIP1. However, KIP1 contains several structural motifs that may shed light on these attributes. First, the presence of nine coiled-coil regions, a motif responsible for dimerization of some cytoskeletal proteins, coupled with the finding from the yeast two-hybrid assay and affinity chromatography that KIP1 interacts with itself, suggests the possibility that KIP1 is a dimeric protein in vivo. Second, the presence of an EF-hand motif, found in many Ca^{2+}-sensor proteins such as calmodulin, and Ca^{2+}-buffer proteins such as parvalbumin (Ikura, 1996), suggests that KIP1 may be a Ca^{2+}-binding protein. It will be of interest to investigate whether KIP1 binds Ca^{2+}, and if so, whether Ca^{2+} binding modulates its interaction with itself and/or with PRK1. Third, the presence of seven tandem repeats of an 11-amino acid segment, flanked by charged hydrophilic regions, is reminiscent of a similar feature possessed by the microtubule associated protein Tau. In Tau, the hydrophilic regions are responsible for microtubule binding and the repeat region is believed to act as a catalytic domain for microtubule assembly (Trinczek et al., 1995; Preuss et al., 1997).

Since the two Arabidopsis ORFs do not contain all these structural features described above (Fig. 4), despite the overall sequence similarity, they may not be functional homologs of KIP1. BLAST searches have also yielded three expressed sequence tags, one from tomato (GenBank accession no. BE354499) and two from *Lycopersicon pennellii* (GenBank accession nos. BG137170 and BG138234), that share a high degree of sequence similarity with different short regions of KIP1. BE354499 shares 92.1% identity with amino acids 1 to 135, BG137170 shares 77.6% identity with amino acids 159 to 301, and BG138234 shares 78.3% identity with amino acids 317 to 467. Since these expressed sequence tags are very short and do not include the EF-hand or the repeat regions of KIP1, it is also not known whether any of them represent a functional homolog of KIP1.

It remains to be determined whether KIP1 is associated with microtubules, which play important roles in karyokinesis and cytokinesis during microspore mitosis. If KIP1 is found to be associated with microtubules, it will be of interest to examine whether the PRK1-mediated signal transduction pathway regulates microtubule dynamics via phosphorylation of KIP1.

MATERIALS AND METHODS

Plant Material

Petunia (*Petunia inflata*) plants of S_2S_2 and S_3S_3 self-incompatibility genotypes (Ai et al., 1990) were used in this study.

Construction of Bait Plasmids for Yeast (*Saccharomyces cerevisiae*) Two-Hybrid Screens

pGBT9, a GAL4-binding domain vector, and pGAD424, a GAL4-activation domain vector, were purchased from CLONTECH (Palo Alto, CA). To construct bait plasmid pGBT9/PRK1-K, the 1.2-kb cDNA encoding approximately 88% of the cytoplasmic kinase domain of PRK1 (Mu et al., 1994) was ligated to the *Sal*I site of pGBT9 to generate an in-frame fusion between the sequence of the DNA-binding domain of GAL4 and that of *PRK1-K*. To construct pGBT9/PRK1-K462R, the full-length *PRK1* cDNA (2,355 bp; Mu et al., 1994) was used as a template for the amplification of the entire cytosolic region. The four primers used were: PKTS-1 (5′-TCGTCGCCGTAGCCATAGC-3′), a kinase domain forward primer; PKTAS-2 (5′-GCGCCATATGTCATCCACCCATTTGCTGTCCACCAGTCATGCTAGCCATAACTCCAGCATCATGCATTTG-3′), a kinase domain reverse primer including a T7 tag sequence at the end; PK2S-3 (5′-GTGGTGGTTAGGAGGTTTAAG-3′), a forward primer used to incorporate point mutation; and PK2AS-2 (5′-CTTAAACCTCCTAACCACCAC-3′), a reverse primer used to incorporate point mutation. PKTS-1 and PK2AS-2 were

used to generate a 346-bp fragment corresponding to the 5′ portion of the cytosolic region, and PK2S-3 and PKTAS-2 were used to generate an 845-bp fragment corresponding to the 3′ portion. Overlap extension with PKTS-1 and PKTAS-2 as primers and the two PCR fragments mentioned above as the templates was used to amplify the entire 1,160-bp fragment encoding the cytosolic region of PRK1. This fragment was cloned into pGEM-T Easy (Promega, Madison, WI) to generate pPRK1-K462R.

pGBT9/PRK1-K was digested with *Bst*EII to release a 350-bp fragment that contained the codon for Lys-462. This fragment was replaced with the corresponding *Bst*EII fragment released from pPRK1-K462R, and the resulting pGBT9/PRK1-K462R construct was sequenced. A third bait plasmid, pGBT9/KIP1-25, was constructed by digesting pGAD424/KIP1-25, isolated from the yeast two-hybrid screen, with *Eco*RI and *Pst*I, and ligating the resulting 1.2-kb fragment into the *Eco*RI and *Pst*I sites of pGBT9 to create in-frame fusion with the sequence for the DNA-binding domain of GAL4. All the amplification reactions were carried out as follows. A 50-μL reaction mixture (20 mM Tris-HCl, pH 8.4, 2 mM $MgCl_2$, and 50 mM KCl) containing 10 ng of DNA template, 2.5 μM each of the upstream and downstream primers, 0.1 mM each of dATP, dGTP, dCTP, and dTTP, and 2.5 units of Display *Taq* DNA polymerase (PGC Scientific, Gaithersburg, MD) was denatured at 93°C for 2 min, and was subjected to 30 cycles of denaturation at 93°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 30 s. In the final cycle, the extension was for 10 min.

Construction of Yeast Two-Hybrid Libraries

Freshly collected pollen of petunia was incubated in an in vitro pollen germination medium, containing 20 mM MES [2-(*N*-morpholino)-ethanesulfonic acid], pH 6.0, 0.07% (w/v) $Ca(NO_3)_2 \times 4$ water, 0.02% (w/v) $MgSO_4 \times 7$ water, 0.01% (w/v) KNO_3, 0.01% (w/v) H_3BO_3, and 10% (w/v) Suc, at 30°C for 3 h with shaking at 250 rpm. Total RNA was isolated from the pollen/pollen tubes using TRIzol reagent (Life Technologies, Rockville, MD), and poly(A)$^+$ RNA was isolated from the total RNA by the PolyATract mRNA Isolation System IV (Promega). Double-stranded cDNAs were synthesized from 1 μg of poly(A)$^+$ RNA using the SUPERSCRIPT Choice System (Life Technologies) except that a primer, named RT1 (5′-CGGATATCGAATTCTCGATTTTTTTTTTTTTTTTT-3′), was used for first-strand cDNA synthesis. After ligation to an *Eco*RI/*Not*I linker (5′-GAATTCGCGGCCGCGTCGAC-3′), the double-stranded cDNAs were digested with *Eco*RI and *Xho*I, ligated into the *Eco*RI and *Sal*I sites of pGAD424, and used to transform *Escherichia coli* DH5α. Two separate prey libraries were constructed, one using plants of S_2S_2 genotype and the other plants of S_3S_3 genotype; the titers of the libraries were 1.3×10^6 and 5×10^5, respectively.

Yeast Two-Hybrid Library Screening

Yeast strains HF7C and SFY526 were purchased from CLONTECH. A modified lithium acetate method (Gietz et al., 1992) was used in all the yeast transformation experiments. HF7c cells were transformed with 0.1 μg of bait plasmid DNA, and the transformants were subsequently transformed with 250 and 500 μg of total pGAD424 library cDNA for S_2S_2 and S_3S_3 libraries, respectively, and 20 mg of denatured herring testes carrier DNA (CLONTECH). Yeast transformants that produced interacting proteins were selected by plating on synthetic dropout (SD) without Leu, Trp, and His. Colonies were assayed using an X-gal filter lift method (Breeden and Nasmyth, 1985).

Construction and Screening of a Pollen cDNA Library

A pollen cDNA library of petunia was constructed in λZAPII (Stratagene, La Jolla, CA) essentially as described by Mu et al. (1994). Prior to packaging, a small aliquot of the ligation mixture (containing total cDNA ligated to λZAPII) was removed and used as a template for 5′-RACE. Primer 1A (5′-ACCAAGATACTTATGCCTGAA-3′), designed based on a sequence near the 5′ end of *KIP1–25C* cDNA (a 23-kb cDNA isolated from a pollen cDNA library previously described by Mu et al. [1994]), was used as the reverse primer, and T3 primer (Stratagene) was used as the forward primer. PCR conditions were the same as described under "Construction of Bait Plasmids for Yeast Two-Hybrid Screens." An approximate 1.3-kb PCR fragment was cloned into pGEM-T Easy and was radiolabeled with ^{32}P using the RTS RadPrime DNA Labeling kit (Life Technologies). The procedures for library screening and filter washing were the same as those described by Mu et al. (1994).

DNA- and RNA-Blot Analyses

Genomic DNA was isolated from young leaves of petunia plants by the Plant DNAzol reagent (Life Technologies), following the procedure recommended by the manufacturer. Two samples of genomic DNA (12 μg each) were digested overnight; one with *Eco*RI and the other with *Hin*dIII. The digests were separated by electrophoresis on a 0.7% (w/v) agarose gel and transferred to a charged nylon membrane, Biodyne B (Life Technologies). Total RNA was isolated by the TRIzol reagent (Life Technologies) and electrophoresed as previously described (Mu et al., 1994). The membranes were prehybridized in 10% (w/v) Dextran sulfate, 1 M NaCl, and 1% (w/v) SDS, hybridized in the same solution plus a ^{32}P-labeled probe, and washed in 2× SSC.

Quantitative Assay of β-Galactosidase Activity

The assay was performed on yeast colonies grown to mid-log phase in the Z buffer according to Miller (1972). β-galactosidase activity was calculated using the following equation: β-galactosidase units = $1{,}000 \times [OD_{420}/t \times V \times OD_{600}]$, where t = time (min) of incubation and V = volume (milliliters) of culture added to the Z buffer.

DNA Sequence Analysis

Cycle sequencing reactions were performed at the Nucleic Acid Facility of The Pennsylvania State University

using 3′-BigDye-labeled dideoxynucleoside triphosphates (dye terminators) and were run on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Data were analyzed using the ABI PRISM XL Sequencing Analysis 3.3 Program. Nucleotide sequences were assembled and analyzed using DNA Strider 1.2.1. Database searches were conducted with the BLAST program at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). Alignments of amino acid sequences were made by the CLUSTAL W method (www.clustalw.genome.ad.jp) using a K-tuple value of 1, a gap penalty value of 3, and a window size of 5. Alignments were shaded using Boxshade, version 3.21 (www.ch.embnet.org/software/BOX_form.html). Coiled-coils regions were predicted using COILS, version 2.1 (www.ch.embnet.org/software/COILS_form.html). Boundaries of coiled-coils regions were defined using an MTDIK matrix, with no weights and a 21-residue window. Using these criteria, it was assumed that residues with probabilities >50% are part of a coiled-coil segment.

Construction of Expression Plasmids for Recombinant Protein Production in *E. coli* and Yeast

To construct pGEX/KIP1-25 for producing GST/KIP1-25 fusion protein in *E. coli*, *KIP1-25* cDNA was released from pGAD424/KIP1-25 and was ligated to pBluescript SK$^-$ (Stratagene) to generate pKIP1-25. pKIP1-25 was digested with *Eco*RI and *Sma*I to release the *KIP1-25* cDNA fragment, which was ligated to the *Eco*RI and *Sma*I sites of pGEX-5x-1 (Amersham Pharmacia Biotech, Piscataway, NJ) to create in-frame fusion between *KIP1-25* cDNA and the coding sequence for GST. The pGEX/antisenseKIP1-25 construct was similarly generated except that *KIP1-25* cDNA was released from pKIP1-25 by digesting with *Bam*HI and *Xho*I, and the resulting *KIP1-25* cDNA fragment was ligated to the *Bam*HI and *Xho*I sites of pGEX-5x-1 in antisense orientation. To produce GST/KIP1-NT in *E. coli*, *KIP1* cDNA was amplified by PCR using SALI-ATG forward primer (5′-AACGCGTCGACCTATGTTGCAGAGAGCTGCCAGC-3′) and reverse primer 2A (5′-GATCA CCAAGATACTTATGCCTGAA-3′). The resulting PCR product was digested with *Sal*I and *Hin*dIII to generate an approximate 550-bp fragment (encoding amino acids 1–184 of KIP1). This fragment was ligated to the *Sal*I and *Hin*dIII sites of pBluescript SK$^-$ and sequenced. *KIP1* cDNA was digested with *Hin*dIII and *Bam*HI to release the approximate 1,600-bp fragment that encodes amino acids 185 to 716 of KIP1. This fragment was ligated with the 550-bp *Sal*I/*Hin*dIII fragment in pBluescript SK$^-$ to generate *KIP1-NT* cDNA, which encompasses amino acids 1 to 716. *KIP1-NT* cDNA was then cloned into pGEX-5x-1 at the *Sal*I-*Not*I sites, creating an in-frame fusion with the sequence for GST.

To make the pYEX/PRK1-K/T7tag construct, pGBT9/PRK1-K was digested with *Eco*RI and *Kpn*I to generate a fragment encoding amino acids 408 through 639 of PRK1. PCR was performed on the full-length *PRK1* cDNA using the primers PKTS-1 and PKTAS-2 (see "Construction of Bait Plasmids for Yeast Two-Hybrid Screens") to amplify the 1,160-bp fragment encoding amino acids 351 to 720 of PRK1 plus the T7 tag. The PCR product was cloned into pGEM-T Easy, and the recombinant plasmid DNA was digested with *Kpn*I and *Sal*I to generate a fragment encoding amino acids 640 to 720 of PRK1 plus the T7 tag. This fragment, along with the *Eco*RI/*Kpn*I fragment mentioned above, was purified and ligated into the *Eco*RI/*Sal*I-digested pYEX-4T-3 vector to generate pYEX/PRK1-K/T7tag. The pYEX/PRK1-K462R/T7tag construct was similarly made, except that pGBT9/PRK1-K462R was used in place of pGBT9/PRK1-K.

Purification of Recombinant Proteins

For pGEX constructs, *E. coli* cells (BL21-Codon Plus [DE3]-RIL strain obtained from Stratagene) were transformed with each construct and a single transformant was inoculated into a 10-mL medium for overnight growth. One millimeter of the overnight culture was used to inoculate a 100-mL medium and the cells were grown at 37°C to an OD_{600} of 0.5. Isopropylthio-β-galactoside was then added to a final concentration of 0.5 mM, and the cells were grown for 3 h at 30°C. *E. coli* cells were harvested by centrifugation and the pellet was resuspended in 5 mL of GST-binding buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol (DTT), and 0.4% (w/v) Triton X-100 (Harper et al., 1994), plus lysozyme (0.75 mg mL^{-1}) and 1 mM phenylmethylsulfonyl fluoride. The suspension was incubated on ice for 15 min and was sonicated to lyse the cells. The supernatant was cleared by centrifugation and was incubated with approximately 250 μL of Glutathione Sepharose 4 Fast Flow resin (Amersham Pharmacia Biotech) for 2 h at 4°C. The resin was washed extensively in GST wash buffer containing 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.4% (w/v) Triton X-100, and the bound protein was eluted with 50 mM Tris-HCl, pH 8.0 and 20 mM glutathione.

For pYEX constructs, yeast strain Y57 (provided by Joseph Reese, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park) was transformed with each construct and plated on synthetic media lacking uracil. Transformed yeast colonies were then used to inoculate 50 mL of SD-Leu, and the culture was grown at 30°C overnight with shaking at 250 rpm. The overnight culture was used to inoculate 2 L of SD-Leu medium, and then grown at 30°C overnight with shaking at 200 rpm. Cells were harvested and transferred to 2 L of fresh SD-Leu media and grown for 2 h. The culture was induced with 0.5 mM copper sulfate for 1 h. Yeast pellets were then frozen into liquid nitrogen and stored at −80°C. Yeast cells were lysed in liquid nitrogen using a blender (Waring, East Windsor, NJ) according to Ausubel et al. (1993). Lysed yeast powder was resuspended in ice-cold phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) with 1 mM phenylmethylsulfonyl fluoride at twice the cell paste volume. The lysate was centrifuged at 5,000*g* for 15 min at 4°C and 1 mL of Glutathione Sepharose 4 Fast Flow resin was

added. Protein was bound to resin for 4 h at 4°C and washed once in phosphate-buffered saline and three times with GST wash buffer. Protein was eluted with 50 mM Tris-HCl, pH 8.0, and 20 mM glutathione.

Protein Gel-Blot Analysis

Protein gel electrophoresis and blotting was carried out essentially as described by McCubbin et al. (1997). Anti-T7 tag monoclonal antibody (1:10,000; Novagen, Madison, WI) or anti-GST antibody (1:1,000; Amersham Pharmacia Biotech) was used as a primary antibody. Immunoreactive proteins were visualized with Bio-Rad (Hercules, CA) AP-Conjugate Substrate Kit after the blots had been incubated with alkaline phosphatase conjugated goat anti-mouse IgG (Life Technologies) and alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma, St. Louis), respectively.

Phosphorylation Assay

Purified recombinant proteins were incubated in 99 μL of kinase buffer {Horn and Walker, 1994; 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.6, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT} and 1 μL of [^{32}P] ATP (>3,000 Ci mmol^{-1}, 10 mCi mL^{-1}; ICN, Costa Mesa, CA) at room temperature for 1 h. To the reaction mixture was added 180 μL of water and 20 μL of Strataclean resin (Stratagene), and the mixture was gently mixed for 20 min. The supernatant was removed by aspiration and 70 μL of sample buffer containing 50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS), 1% (v/v) β-mercaptoethanol, and 0.1% (w/v) bromphenol blue was added to the resin. The sample was heated to 95°C for 3 min and was loaded on 10% (w/v) SDS-polyacrylamide gels. Proteins were resolved by electrophoresis at 200 V for 3 h. Gels were stained with Coomassie Blue R-250, dried, and exposed to x-ray film (Eastman-Kodak, Rochester, NY) at −80°C overnight.

In Situ RNA Hybridization

Tissue preparation and hybridization were performed essentially as previously described (Drews et al., 1991; Flanagan and Ma, 1994). Anthers were collected from stage 4 buds and were fixed in formaldehyde-acetic acid (3.7% [v/v] formaldehyde, 5% [v/v] acetic acid, and 50% [v/v] ethanol). Fixed samples were dehydrated with a graded series of ethanol/aqueous solutions, cleared with a series of graded ethanol/xylene solutions, and embedded in paraffin (Paraplast Plus; Oxford Labware, St. Louis). Embedded anthers were sliced into 8-μm sections and were placed onto slides coated with poly-L-Lys. The sections were then dewaxed in xylene and rehydrated by passing them through graded ethanol/aqueous solutions and rinsing them in water. The sections were then treated with proteinase K and acetylation reaction, and were dehydrated again.

To generate *KIP1* probes for hybridization, a 507-bp *Hin*dIII-*Xba*I fragment (encoding amino acids 184–353) was released from pKIP1-NT by *Xba*I and *Hin*dIII digestion, and the fragment was cloned into pBluescript SK$^-$ at *Xba*I and *Hin*dIII sites. The *KIP1* fragment was released from the recombinant pBluescript SK$^-$ using *Sal*I and *Not*I, and was cloned into pGEM-T Easy. Radiolabeled (^{35}S) single-stranded RNAs were synthesized using an in vitro transcription kit from Promega. The antisense probe was synthesized by T7 RNA polymerase from the plasmid DNA linearized at the *Hin*dIII site, and the sense probe was synthesized by SP6 RNA polymerase from the plasmid DNA linearized at the *Xba*I site. To generate *PRK1* probes, full-length *PRK1* cDNA was digested with *Sac*I and *Xho*I, and the 537-bp fragment (encoding amino acids 82–261 of PRK1) was ligated into pGEM-T Easy at *Sac*I and *Sal*I sites. The antisense probe was synthesized by T7 RNA polymerase from the plasmid DNA linearized at the *Sac*I site, and the sense probe was synthesized by SP6 RNA polymerase from the plasmid DNA linearized at the *Not*I site in the multiple cloning site of pGEM-T Easy. Dehydrated sections were hybridized with each probe in the hybridization solution overnight, washed, and exposed with emulsion as previously described (Drews et al., 1991; Flanagan and Ma, 1994). Slides with emulsion were developed after 2 weeks of exposure; bright and dark field images were recorded using a compound microscope (Nikon, Tokyo) and a digital camera (Optronics, Goleta, CA), and were processed using Photoshop (Adobe Systems, Mountain View, CA).

ACKNOWLEDGMENTS

We thank Balasulojini Karunanandaa, Yong-Yoon Chung, and Cheryl Granger for contributions to the initial phase of this work, and Carmen Zuniga for help with yeast two-hybrid screens. The clones described and the DNA sequence for *KIP1* are available from the corresponding author upon request.

Received February 6, 2001; returned for revision March 27, 2001; accepted April 23, 2001.

LITERATURE CITED

Ai Y, Singh A, Coleman CE, Ioerger TR, Kheyr-Pour A, Kao T-h (1990) Self-incompatibility in *Petunia inflata*: isolation and characterization of cDNAs encoding three *S* allele-associated proteins. Sex Plant Reprod **3:** 130–138

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1993) Current Protocols in Molecular Biology, Vol 2. Wiley Interscience, New York, pp 13.13.5–13.13.9

Becraft PW (1998) Receptor kinases in plant development. Trends Plant Sci **3:** 384–388

Bower MS, Matias DD, Fernandes-Carvalho E, Mazzurco M, Gu T, Rothstein SJ, Goring DR (1996) Two members of the thioredoxin-h family interact with the kinase do-

main of a *Brassica S* locus receptor kinase. Plant Cell **8:** 1641–1650

Braun DM, Stone JM, Walker JC (1997) Interaction of the maize and *Arabidopsis* kinase interaction domain with a subset of receptor-like protein kinases: implication for transmembrane signaling in plants. Plant J **12:** 83–95

Braun DM, Walker JC (1996) Plant transmembrane receptors: new pieces in the signaling puzzle. Trends Biol Sci **21:** 70–73

Breeden L, Nasmyth K (1985) Regulation of the yeast HO gene. *In* Cold Spring Harbor Symposia on Quantitative Biology, Vol 50. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 643–650

Clark SE, Running MP, Meyerowitz EM (1995) *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same process as *CLAVATA1*. Development **121:** 2057–2067

Clark SE, Williams RW, Meyerowitz EM (1997) The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell **89:** 575–585

Drews GN, Bowman JL, Meyerowitz EM (1991) Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETALA2* product. Cell **65:** 995–1002

Flanagan CA, Ma H (1994) Spatially and temporally regulated expression of the *Arabidopsis* MADS-box gene *AGL2* in wild-type and mutant *Arabidopsis* flowers. Plant Mol Biol **26:** 581–595

Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res **20:** 1425–1425

Goring DR, Rothstein SJ (1992) The *S*-locus receptor kinase gene in a self-incompatible *Brassica napus* line encodes a functional serine/threonine kinase. Plant Cell **4:** 1273–1281

Gu T, Mazzurco M, Sulaman W, Matias D, Goring DR (1998) Binding of an arm repeat protein to the kinase domain of the *S*-locus receptor kinase. Proc Natl Acad Sci USA **95:** 382–387

Hanks S, Quinn AM, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:** 42–52

Harper JF, Huang J-F, Lloyd SJ (1994) Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain. Biochemistry **33:** 7267–7277

He Z, Wang Z-Y, Li J, Zhu Q, Lamb C, Ronald P, Chory J (2000) Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. Science **288:** 2360–2363

Horn MA, Walker JC (1994) Biochemical properties of the autophosphorylation of RLK5, a receptor-like kinase from *Arabidopsis thaliana*. Biochim Biophys Acta **1208:** 65–74

Ikura M (1996) Calcium binding and conformational response in EF-hand proteins. Trends Biol Sci **21:** 14–17

Kim YS, Lee JH, Yoon GM, Cho HS, Park S-W, Suh MC, Choi D, Ha HJ, Liu JR, Pai H-S (2000) CHRK1, a chitinase-related receptor-like kinase in tobacco. Plant Physiol **123:** 905–915

Kinkema M, Wang H, Schiefelbein J (1994) Molecular analysis of the myosin gene family in *Arabidopsis thaliana*. Plant Mol Biol **26:** 1139–1153

Kretsinger RH (1976) Calcium-binding proteins. Annu Rev Biochem **45:** 239–266

Lee H-S, Chung Y-Y, Das C, Karunanandaa B, van Went JL, Mariani C, Kao T-h (1997) Embryo sac development is affected in *Petunia inflata* plants transformed with an antisense gene encoding the extracellular domain of receptor kinase PRK1. Sex Plant Reprod **10:** 341–350

Lee H-S, Karunanandaa B, McCubbin A, Gilroy S, Kao T-h (1996) PRK1, a receptor-like kinase of *Petunia inflata* is essential for post-meiotic development of pollen. Plant J **9:** 613–624

Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell **90:** 929–938

McCubbin AG, Chung Y-Y, Kao T-h (1997) A mutant S3 RNase of *Petunia inflata* lacking RNase activity has an allele-specific dominant negative effect on self-incompatibility interactions. Plant Cell **9:** 85–95

Miller JH (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Mu JH, Lee HS, Kao T-h (1994) Characterization of a pollen expressed receptor like kinase gene of *Petunia inflata* and the activity of its encoded kinase. Plant Cell **6:** 709–721

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

Park S-W, Yu SH, Kim MI, Oh SC, Kao T-h, Pai H-s (2000) Interaction of PRK1 receptor-like kinase with a putative eIF2B β-subunit in tobacco. Mol Cell **10:** 626–632

Preuss U, Biernat J, Mandelkow E (1997) The "jaws" model of microtubule interaction examined in CHO cells. J Cell Sci **110:** 789–800

Schopfer CR, Nasrallah ME, Nasrallah JB (1999) The male determinant of self-incompatibility in *Brassica*. Science **266:** 1697–1700

Shiba H, Takayama S, Iwano M, Shimosato H, Funato M, Nakagawa T, Che F-K, Suzuki G, Watanabe M, Hinata K et al. (2001) A pollen coat protein, SP11/SCR, determines the pollen *S*-specificity in the self-incompatibility of *Brassica*. Plant Physiol **125:** 2095–2103

Song W-Y, Wang G-L, Chen L-L, Kim H-S, Pi L-Y, Holsten T, Gardner J, Wang B, Zhai W-X, Zhu L-H et al. (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science **270:** 1804–1806

Stone JM, Collinge MA, Smith RD, Horn MA, Walker JC (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine/threonine receptor kinase. Science **266:** 793–795

Stone JM, Trotochaud AE, Walker JC, Clark SE (1998) Control of meristem development by CLAVATA1 receptor kinase and KAPP protein phosphatase interactions. Plant Physiol **117:** 1217–1225

Stone SL, Arnoldo M, Goring DR (1999) A breakdown of *Brassica* self-incompatibility in ARC1 antisense transgenic plants. Science **286:** 1729–1731

Takasaki T, Hatakeyama K, Suzuki G, Watanabe M, Isogai A, Hinata K (2000) The *S* receptor kinase determines self-incompatibility in *Brassica* stigma. Nature **403:** 913–916

Takayama S, Shiba H, Iwano M, Shimosato H, Che F-S, Kai N, Watanabe M, Suzuki G, Hinata K, Isogai A (2000) The pollen determinant of self-incompatibility in *Brassica campestris*. Proc Natl Acad Sci USA **97:** 1920–1925

Titus MA (1993) Myosins. Curr Opin Cell Biol **5:** 77–81

Torii KU, Clark SE (2000) Receptor-like kinases in plant development. Adv Bot Res **32:** 225–267

Trinczek B, Biernat J. Mandelkow EM, Mandelkow E (1995) Domains of tau protein, phosphorylation, and dynamic instability of microtubules. Mol Biol Cell **6:** 1887–1902

Trotochaud AE, Jeong S, Clark SE (2000) CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase. Science **289:** 613–617

Twell D, Yamaguchi J, McCormick S (1990) Pollen specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. Development **109:** 705–713

Walker JC, Zhang R (1990) Relationship of a putative receptor kinase from maize to the *S*-locus glycoproteins of *Brassica*. Nature **345:** 743–746

Wang ZY, Seto H, Fujioka S, Yoshida S, Chory J (2001) BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature **410:** 380–383

Williams RW, Wilson JM, Meyerowitz EM (1997) A possible role for kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. Proc Natl Acad Sci USA **94:** 10467–10472