PRK1, a Receptor-like Kinase from *Petunia inflata,* is Essential for Post-meiotic Development of Pollen and Embryo Sac

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We previously identified and characterized a predominantly pollen-expressed gene of *Petunia inflata* that encodes a receptor-like kinase named PRK1. The extracellular domain of PRK1 contains leucine-rich repeats which have been implicated in protein-protein interactions, and the cytoplasmic domain was found to autophosphorylate on serine and tyrosine. To investigate the function of PRK1 in pollen development, we transformed *P. inflata* plants with a construct containing the promoter of a predominantly pollen-expressed gene of tomato, *LAT52*, fused to an antisense PRK1 cDNA corresponding to part of the extracellular domain of PRK1. Three transgenic plants were found to each produce approximately equal amounts of normal and aborted pollen. Analysis of the inheritance of the transgene inserts in two of the transgenic plants, ASRK-13 and ASRK-20, to their progeny revealed that certain transgene inserts cosegregated with the pollen abortion phenotype. Microscopic examination of the aborted pollen grains showed that their outer wall, the exine, was essentially normal, but that their cytoplasm contained only starch-like granules. Staining of the nuclei of the microspores at different stages of anther development revealed that the microspores of the transgenic plants developed normally until the uninucleate stage. However, at subsequent stages half of the microspores completed mitosis and developed into normal binucleate pollen, but the other half initially remained uninucleate, then lost their nuclei. Analysis of the amounts of PRK1 mRNA and the antisense PRK1 transcript suggested that the pollen abortion phenotype most likely resulted from down-regulation of the *PRK1* gene by the antisense *PRK1* transgene. These results suggest that PRK1 plays an essential role in a signal transduction pathway that mediates post-meiotic development of microspores.

In flowering plants, pollen grains develop in the cavity of the anther locule, and this developmental process has been well characterized at anatomical and cytological levels (Esau, 1977; Shivanna and Johri, 1985; McCormick, 1993). Each pollen mother cell undergoes meiosis to produce a tetrad of microspores which are enclosed within a callosic wall. After the microspores are released from the tetrad, they enlarge and undergo an asymmetric division to give rise to a vegetative and a generative cell. For taxa producing trinucleate pollen, the generative cell undergoes a second mitotic division to produce two sperm cells. The tapetum, the innermost wall layer of the anther which surrounds the locule, is thought to play a pivotal role in pollen development, including supplying nutrients for developing pollen and precursors for exine formation, and secreting β-1,3-glucanase for the breakdown of the callose wall around the microspores (Pacini et al., 1985; Shivanna and Johri, 1985; Dickinson, 1992). This important role is evidenced by the findings that a number of sporophytic male-sterile mutants have defects in the structure of the tapetal cell (Chaudhury, 1993), and that ablation of the tapetum in transgenic plants by the toxic action of a bacterial ribonuclease results in the abortion of pollen development (Mariani et al., 1990).

Pollen development thus requires intimate

interactions between the gametophyte and the sporophytic tissue of the tapetum; however, so far the molecules involved and the underpinning molecular mechanisms for these interactions are essentially unknown. In many cases of cell-cell interactions, signals released from one cell are perceived and transduced across the plasma membrane of another cell by receptor kinases, which span the plasma membrane (Ullrich and Schlessinger, 1990). Ligand binding to the extracellular domain of the receptor kinase alters the activity of the intracellular kinase domain. Thus, the extracellular signal is transduced to affect cytosolic targets through a cascade of events including changes in protein phosphorylation patterns. Such receptor kinases present attractive targets for mediating the exchange of the information from tapetal cells that regulates pollen development in the anther.

We recently isolated and characterized a predominantly pollen-expressed gene of *Petunia inflata* that encodes a receptor-like kinase named PRK1 (Mu et al., 1994). The extracellulardomain of PRK1 contains leucine-rich repeats which have been implicated in protein-protein interactions and represent putative ligand binding sites. Similar leucine-rich motifs have also been found in other plant receptor-like kinases (Chang et al., 1992; Walker, 1993). The cytoplasmic domain of PRK1 contains conserved features of protein kinases, and the recombinant protein produced in *E. coli* was found to autophosphorylate serine and, uniquely for any plant receptor-like kinase reported so far, tyrosine (Mu et al., 1994). Since receptor kinases of animals often have tyrosine kinase activity that is integral to their signal transduction activity (Ullrich and Schlessinger, 1990; Fanti et al., 1993; Johnson and Vaillancourt, 1994), the characteristics of PRK1 make it an attractive candidate to mediate the tapetal/pollen signaling that regulates normal pollen development.

To investigate whether PRK1 indeed plays a role in regulating pollen development, we transformed *P. inflata* plants with an antisense PRK1 cDNA encoding part of the extracellular domain driven by a predominantly pollen-expressed *LAT52* promoter of tomato, and examined the effect of inhibition of PRK1 synthesis on pollen development in the transgenic plants.

MATERIALS AND METHODS

Construction of Antisense PRK1 Gene

The 0.6-kb SacI-BamHI fragment encoding approximately 58% of the extracellular domain of PRK1 was released from pPRK1 (Mu et al., 1994) and cloned into pBluescript KS+ vector (Stratagene, La Jolla, CA) to yield pBSB. pLAT52-7 (Twell et al., 1991) was digested with NcoI, made blunt ended by Klenow enzyme, and digested with SalI to release the 0.6-kb SalI-NcoI fragment containing the LAT52 promoter. This fragment was then cloned into the SalI and BamHI (which had been made blunt ended) sites of pBSB. The 1.2-kb SalI-SacI fragment containing the antisense PRK1 cDNA fused to the *LAT52* promoter was released and cloned into the SalI and SacI sites of a Ti-plasmid vector pBI101 (Clontech, Palo Alto, CA). The recombinant Ti plasmid was electroporated into *Agrobacterium tumefaciens* LBA4404. Transformation of leaf strips of *P. inflata* plants by *Agrobacterium* and regeneration of transgenic plants were carried out as previously described (Lee et al., 1994).

Genomic DNA Gel Blot Analysis

Genomic DNA was prepared from young leaves of *P. inflata* plants as previously described (Lee et al., 1994), except that DNA was further purified by a Cell Culture DNA Kit (QIAGEN, Chatsworth, CA). The genomic DNA (5 μg) was digested with EcoRI, separated on 0.8% agarose gels, and transferred to Biotrans (+) nylon membranes (ICN, Costa Mesa, CA). The 0.6-kb SalI-NcoI DNA fragment containing the LAT52 promoter was

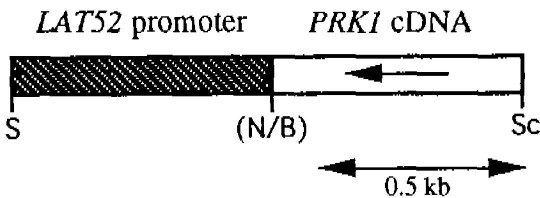

Figure 1. Schematic representation of the antisense PRK1 construct. The *LAT52* promoter of tomato is contained in a 0.6-kb SalI-NcoI fragment (Twell et al., 1991): the PRK1 cDNA is a 0.6-kb SacI- *BamHI* fragment encoding amino acid residues 82 to 274 of the extracellular domain of PRK1 (Mu et al., 1994). The arrow pointing to the left indicates that the PRK1 cDNA was fused with the *LAT52* promoter in antisense orientation. Restriction enzymes sites: S, SalI: N, NcoI: B, BamHI: Sc, SacI. The restriction sites in parentheses are destroyed after ligation.

radiolabeled for use as probe. Prehybridization, hybridization, and washing of the membranes were carried out as previously described (Lee et al., 1994). The membranes were exposed on X-ray films at -70°C for 48 hr with an intensifying screen.

RNA Gel Blot Analysis

Total RNA isolation, electrophoresis of RNA, and transfer of RNA to QIABRANE nylon membrane (QIAGEN) were carried out as previously described (Lee et al., 1994). The membrane was first hybridized at 65°C overnight using the 0.6-kb SacI-BamHI fragment of the PRK1 cDNA as probe (see Figure 1). The membrane was washed twice, 20 min each, at room temperature in 2 × SSC, 0.1% SDS, and then washed twice, 30 min each, at 65°C in 0.1 × SSC, 0.1% SDS. The membrane was exposed on X-ray film with an intensifying screen at -70°C for two different lengths of time, 4 hr and 15 hr. The amount of radioactivity associated with PRK1 mRNA was determined using a Betascope (Betagen, Waltham, MA). The bound radiolabeled probe was then removed from the membrane for hybridization with an rDNA probe that encodes 25S rRNA of *P. inflata* (Mu, J. and Kao, T.-h., unpublished results). The membrane was exposed on X-ray film at -70°C for 1hr with an intensifying screen. The amount of radioactivity associated with 25S rRNA was similarly determined. For each anther developmental stage, the amount of PRK1 mRNA in transgenic plant ASRK-13 relative to that in a wild-type plant was calculated after correction for differences in the total amount of rRNA.

Microscopy

For scanning electron microscopy, freshly collected pollen was placed on stubs coated with double-sided adhesive tape, and coated with 10 nm gold/palladium with BAL-TEC SCD 050 sputter coater. Observations were made with a JEOL 5400 scanning electron microscope. For transmission electron microscopy, anthers were fixed in 1.5% gluteraldehyde, 2.5% formaldehyde, 100 mM phosphate buffer (PB), pH 7.4, for 4 hr, and washed three times for 5 min each time in PB. Subsequently, the tissues were fixed in 1% osmium tetroxide in PB for 6 hr at room temperature, washed in several changes of PB, and dehydrated with a graded ethanol series (50% to 100%). The anthers were then stained in 2% uranyl acetate in 100% ethanol at 4°C overnight, washed in 100% ethanol and then 100% propylene oxide, and infiltrated with Spurrs medium (EM Sciences) and propylene oxide (50%/50%, 75%/25%, 100%, 100% for 8 hr each). The resin was polymerized for 12 hr at 70°C. Thin sections (50 nm) were prepared with LKB III ultramicrotome, and observed on a JEOL 1200 EXII transmission electron microscope. For bright-field micrographs of anther cross sections, thick sections (500 nm) were prepared as above, and observed on a Nikon Diaphot 300 microscope.

DAPI Staining of Microspores

The procedure for DAPI staining was essentially as previously described (Vergne et al., 1987). Freshly collected anthers were placed on microscope slides and dissected to release microspores incitrate

phosphate buffer, pH 4, containing 1% triton X-100 and 1 μg/mL DAPI. Anther debris was removed and a coverslip was applied. After 10 to 15 min incubation in the dark, the DAPI stained microspores were examined under a Dialux 20 EB microscope equipped with epifluorescence.

In Vitro and in Vivo Pollination Assays

For in vitro pollination assays, freshly collected pollen was suspended in pollen germination medium (Brewbaker and Kwack, 1963) at the concentration of 5 mg/mL and incubated for 12 hours at 25°C with gentle shaking. For in vivo pollination assays, the procedure is essentially as described in Muschietti et al. (1994). Stained and squashed pistils were observed under a fluorescent microscope to examine pollen tube growth inside of style.

RESULTS

Since the PRK1 promoter has not yet been characterized, we used a 0.6-kb *LAT52* promoter of tomato (Twell et al., 1990) to express a 0.6-kb SacI-BamHI cDNA fragment encoding approximately 58% of the extracellular domain of PRK1 (Mu et al., 1994) in antisense orientation (Figure 1). This *LAT52* promoter was chosen because it is active predominantly in the pollen and it confers on the GUS reporter gene an expression pattern similar to that of PRK1 mRNA accumulation during microsporogenesis (Twell et al., 1990, 1991: Mu et al., 1994). The reason that the cDNA encoding the extracellular domain, but not the full-length PRK1 cDNA or the part of PRK1 cDNA encoding the kinase domain, was used in the antisense construct was because we previously showed that the coding sequence for the extracellular domain of PRK1 did not hybridize to any other DNA fragment of the *P. inflata* genome, while the coding sequence for the kinase domain did (Mu et al., 1994). Thus, the use of the cDNA encoding the extracellular domain was unlikely to result in the inhibition of other kinases, which would complicate the interpretation of the results. A recombinant Ti-plasmid containing the antisense PRK1 construct was introduced into *P. inflata* plants via Agrobacterium-mediated transformation, and a total of 61 transgenic plants were obtained.

Phenotypes of Transgenic *P. inflata* Plants

We first examined mature pollen of the transgenic plants for any abnormal phenotype using light microscopy. Three transgenic plants, ASRK-13, ASRK-20, and ASRK-59, were found to each produce approximately equal amounts of normal and aborted pollen. The aborted pollen was smaller in size than the normal pollen, and did not stain with acetocarmine. The rest of the transgenic plants produced mostly normal pollen and a small number (5% or less) of aborted pollen, similar to wild-type plants.

Complexity of Transgene Insertion in Transgenic Plants and Inheritance of Each Insert to the Progeny

We carried out a genomic DNA gel blot analysis using the 0.6-kb SalI-NcoI DNA fragment containing the *LAT52* promoter as probe to determine the presence and number of independent inserts of the transgene (Figure 2a). Two EcoRI fragments each were detected for ASRK-13 and ASRK-20, and five EcoRI fragments for ASRK-59. Since the transgene contained one EcoRI site outside the *LAT52*/antisense PRK1 cDNA region, these results suggest that ASRK-13 and ASRK-20 each contained two independent inserts, and ASRK-59 contained five. ASRK-17, a transgenic plant whose pollen development was not affected, was also examined and found to contain one independent insert of the transgene. A faint hybridizing fragment of 13.7 kb was present in all of the plants, which might represent a *P. inflata* homolog of the *LAT52* gene (shown more clearly in Figure 2b). No

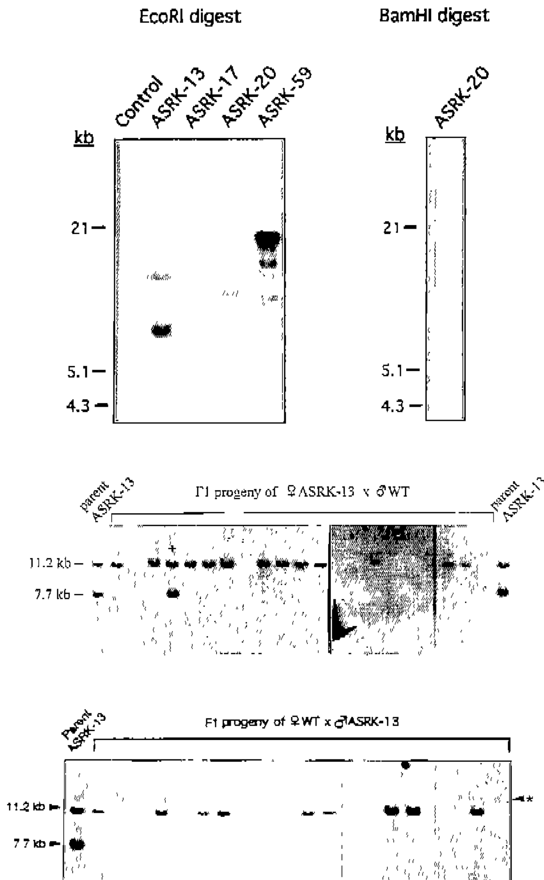

Figure 2. Genomic DNA gel blot analysis of transgenic plants and their progeny. (a) Genomic DNA gel blot showing DNA fragments containing the transgene. The blot containing EcoRI digests of genomic DNA from a wild-type plant (control) and four transgenic plants (as indicated) were hybridized with the 0.6-kb SalI-NcoI fragment containing the *LAT52* promoter. Each hybridized genomic DNA fragment resulted from one cut by EcoRI within the integrated transgene and one cut outside the transgene in the genome. Genomic DNA of ASRK-20 was also digested with BamHI which does not cut within the transgene. (b) Genomic DNA gel blot showing that only one of the two transgene inserts in ASRK-13 was inherited by its progeny when its pollen was used to pollinate a wild-type plant. EcoRI digests of genomic DNA from 20 progeny plants were hybridized with the same DNA probe as in Figure 2(a). The arrow marks a weakly hybridizing 13.7-kb DNA fragment which may be a *P. inflata* homolog of the *LAT52* gene. This hybridizing fragment was present in the original autoradiogram of Figure 2(a) but did not reproduce in the print. (c) Genomic DNA gel blot showing the inheritance of the two transgene inserts of ASRK-13 to the progeny from a cross between it (female) and a wild-type plant (male). The blot was hybridized with the same DNA probe as in Figure 2(a). The single progeny plant that contained the 7.7-kb fragment is indicated with +.

detectable hybridization was observed for a wild-type plant except the weak hybridizing fragment of 13.7 kb.

We separately pollinated a wild-type plant with pollen from three of the above-mentioned transgenic plants, ASRK-13, ASRK-17, and ASRK-20, and examined the presence or absence of each transgene insert in the genomic DNA of the progeny. For ASRK-13, of the two EcoRI fragments that contained the transgene, the 11.2-kb fragment was present in 14 of the 30 progeny plants analyzed, but the 7.7-kb fragment was not present in any of them (Figure 2b: results from only 20 plants were shown). A faint hybridizing fragment of 13.7 kb was present in all the plants as described in Figure 2a. All the 30 progeny plants showed normal pollen development. Thus, the 7.7-kb EcoRI fragment but not the 11.2-kb EcoRI fragment correlated with pollen abortion.

For ASRK-20, neither of the two EcoRI fragments, 9.9 kb and 21 kb, containing the transgene (Figure 2a) was present in the eight progeny plants analyzed (results not shown), and all the progeny plants showed normal pollen development. The result suggests that the two EcoRI fragments might be linked and that at least one transgene insert is functional to generate the pollen abortion phenotype. For other transgenic plants which do not carry the pollen abortion phenotype, three plants including ASRK-17 showed normal segregation of the transgene in the progeny (results not shown), and all the progeny plants showed normal pollen development as did their parent plants.

We also crossed ASRK-13 and ASRK-20 as female to a wild-type plant, and examined mature pollen of 132 plants in the ASRK-13 and 95 plants in ASRK-20 progeny. One plant from the ASRK-13 progeny and seven plants from the ASRK-20 progeny exhibited the same pollen abortion phenotype (1:1 ratio of normal vs abnormal pollen) as did ASRK-13, ASRK-20, and ASRK-59, while the rest of the ASRK-13 and ASRK-20 progeny

produced normal pollen as wild-type plants.

Genomic DNA gel blot analysis was carried out on 20 of the ASRK-13 progeny plants that showed normal pollen development and the one plant that showed pollen abortion phenotype (Figure 2c). The 11.2-kb EcoRI fragment was present in 12 of the 21 plants analyzed: however, the 7.7-kb fragment was present only in the plant that showed pollen abortion phenotype. For the progeny of ASRK-20, genomic DNA gel blot analysis was carried out on 15 of the plants that showed normal pollen development and the seven plants that showed pollen abortion phenotype. The 15 plants that showed normal pollen development contained neither of the two EcoRI fragments. However, the seven plants that showed pollen abortion phenotype all contained the transgene inserts: six of them contained both of the two EcoRI fragments and one of them contained only the 9.9-kb fragment (results not shown). Thus, these results demonstrated that the transgene insert in the 7.7-kb EcoRI fragment of ASRK-13, and at least the one in the 9.9-kb EcoRI fragment of ASRK-20 cosegregated with the pollen abortion phenotype. In addition, the unexpected finding of the absence of these EcoRI fragments in the majority of the progeny suggested that these EcoRI fragments might also correlate with failure in any of the following processes: female gametophyte development, fertilization, and/or postzygotic development (see Discussion below). Further, the 11.2-kb fragment of ASRK-13 that was not linked to the pollen abortion phenotype did not correlate with the "female" phenotype either. The remainder of this report focuses on the study of the pollen abortion phenotype.

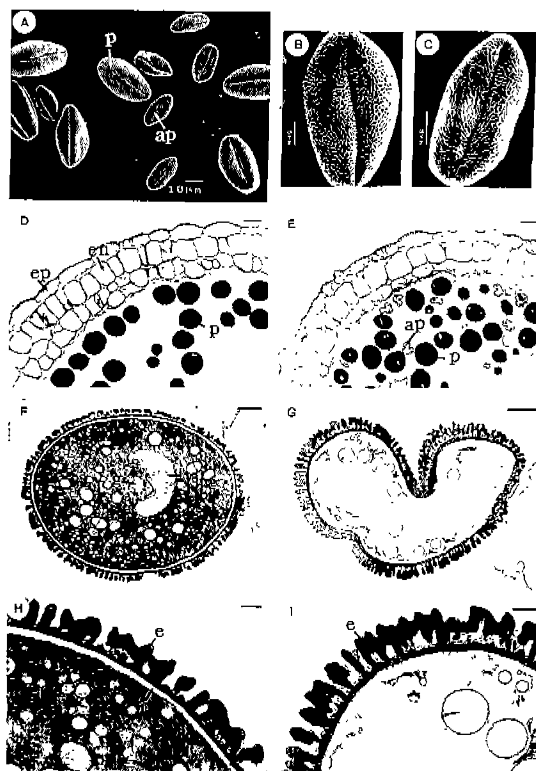

Figure 3. Microscopic examination of normal and aborted pollen. (a) Scanning electron micrograph of pollen grains from ASRK-13. The normal (p) and aborted (ap) pollen are shown at different magnifications to more clearly show the nearly normal exine patterning in the aborted pollen.
(b) and (c) Scanning electron micrographs of a normal pollen grain from a wild-type plant (b) and of an aborted pollen grain from ASRK-13 (c).
(d) and (e) Bright-field micrographs of the cross sections of stage 5 anthers of ASRK-17 (d) and of ASRK-13 (e). Normal microspores are essentially all binucleate at this stage of development (see Figure 4 and Table 1). Abbreviations: ep, epidermis: en, endothecium. Scale bars are 10 mm.
(f) and (g) Transmission electron micrographs of a normal microspore from a stage 5 anther of ASRK-17 (f) and an abnormal microspore from a stage 5 anther of ASRK-13 (g). Abbreviations: g, generative nucleus: v, vegetative nucleus. Scale bars are 2 mm.
(h) and (i) Transmission electron micrographs of a normal microspore from a stage 5 anther of ASRK-17 (h) and an abnormal microspore from a stage 5 anther of ASRK-13 (i). Abbreviations: e, exine: i, intine. Scale bars are 0.5 mm.

Microscopic Examination of Normal and Aborted Pollen Produced by Transgenic Plants

We next examined both the normal and aborted pollen produced in mature flowers of ASRK-13 by scanning electron microscopy. The size of the aborted pollen was estimated to be approximately one-third that of the normal pollen (Figure 3a): however, the pattern of the exine of the aborted pollen (Figure

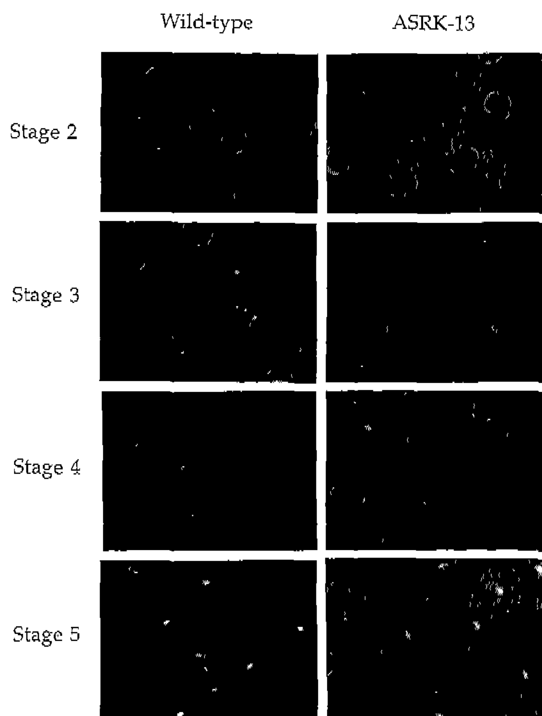

Figure 4. DAPI staining of microspores of a wild-type plant and ASRK-13 at different developmental stages. Stage 2, 3, 4, and 5 anthers are from buds 0.5 to 1.0 cm, 1.0 to 1.5 cm, 1.5 to 2.0 cm, 2.0 to 2.5 cm in length, respectively. Stage 5 anthers are from buds approximately 2 days before flower opening.

3c) did not appear to be significantly different from that of the normal pollen (Figure 3b).

The transverse sections of stage 5 anthers (see the definition of anther developmental stages in the legend to Figure 4) of ASRK-17, ASRK-13, ASRK-20, and ASRK-59 were examined by light microscopy. The results for the latter three transgenic plants were similar and only those for ASRK-13 are shown. The anther of ASRK-17 contained mostly normal pollen which stained with toluidine blue (Figure 3d), while the anther of ASRK-13 contained normal pollen and aborted pollen, with the latter failing to stain with toluidine blue (Figure 3e). The interior of the aborted pollen appeared to be totally degenerated, and this might have caused the cell wall to collapse, because the folding of the wall was highly irregular. Although the tapetal tissue

Table 1. Comparison of microspore development between wild-type and ASRK-13 plants.

	Stage 2*		Stage 3		Stage 4		Stage 5	
Microspore Development	WT (%)	ASRK-13 (%)	WT (%)	ASRK-13 (%)	WT (%)	ASRK-13 (%)	WT (%)	ASRK-13 (%)
Uninucleate	95	99	6	42	0	4	0	0
Binucleate	4	0	89	53	94	48	95	50.5
Aborted	1	0.7	5	15	6	48	5	49.5

*bud developmental stage

surrounding the pollen sac had degenerated at this stage of pollen development, examination of earlier stages of anther development in ASRK-13 did not reveal any morphological abnormality in this tissue (results not shown). The fact that half of the pollen produced by the three transgenic plants developed normally would also suggest that the tapetal functions were not affected by the transgene. This is also consistent with the finding of a nearly normal exine pattern in aborted pollen, because the tapetum is thought to supply the major part of the sporopollenin of the exine (Heslop-Harrison, 1971).

The transverse sections of stage 5 anthers of ASRK-13 and ASRK-17 were further examined by transmission electron microscopy to compare the ultrastructure of the normal pollen (Figure 3f) and the aborted pollen (Figure 3g). The aborted pollen did not contain nuclei or any other organelles except for starch granule-like structures. Again, the exine and the tryphine layers of the aborted pollen appeared to be normal (Figures 3h and 3i).

Examination of the Development of Normal and Abnormal Pollen by DAPI Staining

We next examined nuclear development of the microspores of ASRK-13, ASRK-20, ASRK-59, and a wild-type plant at different stages of anther development using the fluorescent DNA stain 4-6-diamidino-2-phenylindole-2HCl (DAPI). To minimize variations in the development of individual microspores, we examined at least 300 microspores collected from 20 buds of each plant for each developmental stage. Since the DAPI results for the

three transgenic plants were virtually identical, only the results for representative microspores of ASRK-13 and the wild-type plant are shown in Figure 4. In addition, the percentages of uninucleate, binucleate, and aborted microspores at each developmental stage of these two plants are shown in Table 1.

Stage 2 anthers (from buds 0.5 to 1.0 cm in length) of the wild-type plant and ASRK-13 contained almost entirely free uninucleate microspores, and there was no visible difference in the size and staining pattern between the microspores of these two plants. Thus, the microspores of the transgenic plants developed normally up to the free uninucleate stage. Stage 3 anthers (from buds 1.0 to 1.5 cm in length) of the wild-type plant contained mostly binucleate microspores, each with a larger and more weakly stained vegetative nucleus and a more compact and brighter generative nucleus. However, in stage 3 anthers of ASRK-13, only approximately half of the total microspores were binucleate: the rest either remained uninucleate (32% of the total) or were aborted with no nuclear staining detected (15% of the total). In stage 4 anthers (from buds 1.5 to 2.0 cm in length) of the wild-type plant, all except for a small number of presumably naturally aborted microspores were binucleate, indicating completion of microspore mitosis. In stage 4 anthers of ASRK-13, the number of binucleate microspores remained approximately the same as in stage 3: however, nearly all of the uninucleate microspores observed in stage 3 appeared to have lost their nuclei, resulting in approximately half of the total microspores lacking a nucleus. Stage 5 anthers (from buds 2.0 to 2.5 cm in length: 2 days before flower opening) of the wild-type plant contained mature binucleate pollen, and those of ASRK-13 contained approximately equal amounts of normal and aborted pollen.

Steady-State Levels of PRK1 mRNA at Different Stages of Anther Development in Transgenic and Wild-type Plants

To confirm that the pollen abortion phenotype indeed resulted from down-regulation of the endogenous PRK1 gene by the antisense PRK1 transgene, we used the 0.6-kb SacI-BamHI fragment of the PRK1 cDNA contained in the transgene construct (Figure 1) as probe to examine the steady-state levels of PRK1 mRNA and the antisense PRK1 transcript at different stages of anther development in ASRK-13 and a wild-type

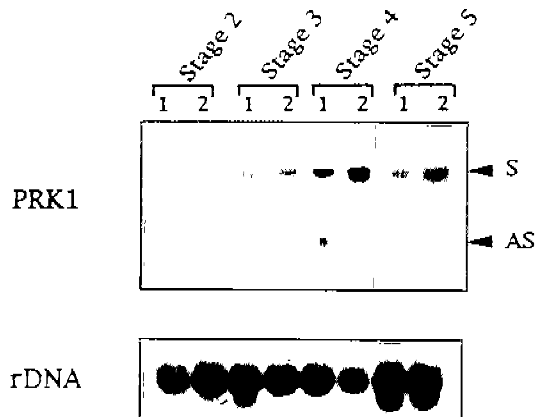

Figure 5. RNA gel blot analysis of the amounts of PRK1 mRNA and antisense PRK1 transcript in transgenic and wild-type plants.
Total RNA was extracted from anthers of ASRK-13 and a wild-type plant at 4 different developmental stages as indicated. Thirty micrograms of total RNA were used for each lane. For each stage, lane 1 contains total RNA from ASRK-13 and lane 2 contains total RNA from the wild-type plant.
(Top) The autoradiograms show the blot that was hybridized with the 0.6-kb SacI-BamHI fragment of the PRK1 cDNA (see Figure 1). S denotes PRK1 mRNA: AS denotes antisense PRK1 transcript. The autoradiogram containing RNA samples from stage 2 through stage 4 was obtained from exposure of the blot on X-ray film for 15 hr at -70°C. The autoradiogram containing RNA samples from stage 5 was obtained from exposure of the blot on X-ray film for 4 hr at -70°C.
(Bottom) The autoradiogram shows rehybridization of the same blot with an rDNA probe encoding the 25S rRNA of *P. inflata*, after the bound probe was removed.

plant (Figure 5). Separatcly, the same experiment was also carried out with ASRK-20 plant. PRK1 mRNA (marked "S" in Figure 5) could be distinguished from the antisense PRK1 transcript (marked "AS" in Figure 5) because its size, 2.4 kb, was much larger than that of the antisense PRK1 transcript, 0.7 kb. The level of PRK1 mRNA at each lane was determined using a Betascope (Betagen, Waltham, MA).

At stage 2 of anther development, PRK1 mRNA was not detected in either ASRK-13 (Figure 5: lane 1 of Stage 2) or the wild-type plant (lane 2 of Stage 2), nor was the antisense PRK1 transcript detected in ASRK-13. These results are consistent with the finding described above that the microspores of ASRK-13 developed normally up to this stage (Figure 4). The antisense PRK1 transcript was first detected in stage 3 anthers of ASRK-13 (lane 1 of Stage 3), a developmental stage which coincided with the first detection of abnormality in microspore development in ASRK-13. PRK1 mRNA was also first detected in stage 3 anthers of ASRK-13 (lane 1 of Stage 3) and the wild-type plant (lane 2 of Stage 3), with the amount of PRK1 mRNA in ASRK-13 being approximately 70% that of the wild-type plant. The expression of the antisense PRK1 transgene in stage 3 anthers of ASRK-13 thus coincided with the reduction of the amount of PRK1 mRNA relative to the wild-type level. As shown in Table 1 discussed above, most of the microspores contained in stage 3 anthers of ASRK-13 were either uninucleate or binucleate, and only 15% of the microspores had lost their nuclei. Thus, the 30% reduction in the amount of the PRK1 mRNA would suggest that those microspores carrying the active antisense PRK1 transgene but remaining uninucleate contained reduced levels of PRK1 mRNA. Since these microspores lost their nuclei and most of their cytoplasmic contents in subsequent stages of anther development (Figure 4), we concluded that the reduction in the amount of PRK1 mRNA in the affected microspores preceded the disintegration of their cellular contents. Therefore, the pollen abortion phenotype most likely resulted from the down-regulation of the PRK1 gene by the antisense PRK1 transgene.

The amounts of PRK1 mRNA in both ASRK-13 and the wild-type plant increased as anther development progressed through stage 4 and stage 5. The amount of PRK1 mRNA in ASRK-13 relative to that in the wild-type plant decreased to approximately 50% in both stage 4 and 5 anthers (compare lanes 1 and 2 of Stage 4, and lanes 1 and 2 of Stage 5). The observation of half the wild-type level of PRK1 mRNA at these two late stages of anther development in ASRK-13 is consistent with the finding that only half of the microspores produced by this transgenic plant were normal and the other half had lost their nuclei and most of their cytoplasmic contents.

DISCUSSION

Among a number of plant receptor-like kinases that have been reported so far (Walker and Zhang, 1990: Goring and Rothstein, 1992: Stein et al., 1991: Chang et al., 1992: Tobias et al., 1992: Walker, 1993: Dwyer et al., 1994: Mu et al., 1994), the *PRK1* gene of *Petunia inflata* we previously identified is the only one that is predominantly expressed in pollen (Mu et al., 1994). PRK1 mRNA is first detected in stage 3 anthers and remains present throughout subsequent stages of pollen development. It is also present in in vitro-germinated pollen tubes. We therefore speculated that PRK1 might serve as a signal transducer during late stages of pollen development and/or pollination (Mu et al., 1994). In this report, we have investigated the function of PRK1 during pollen development. We have used an antisense RNA approach to examine the effect of abolishing the production of PRK1 on pollen development in transgenic plants.

Since the promoter of the *PRK1* gene has not yet been characterized, we used a heterologous promoter, the promoter of the tomato *LAT52* gene to express

an antisense PRK1 cDNA (Figure 1) in transgenic *P. inflata* plants. Both the *LAT52* gene and the *PRK1* gene are predominantly expressed in pollen, and they exhibit a similar developmental expression pattern during microsporogenesis. The results obtained would be expected to reflect most, if not all, of the physiological functions of PRK1 (see discussion below). Among the 61 transgenic plants analyzed, three, ASRK-13, ASRK-20, and ASRK-59, were found to produce approximately equal amounts of normal and aborted pollen.

Progeny analysis using ASRK-13 and ASRK-20 as male and a wild-type plant as female revealed that a particular transgene (s) were not transmitted to the progeny (Figures 2a, 2b, and Results), thus suggesting that the aborted pollen grains were the ones carrying these transgene inserts. Analysis of the progeny from reciprocal crosses showed that these transgene inserts indeed cosegregated with the pollen abortion phenotype: the plants that inherited either the 7.7-kb EcoRI fragment from ASRK-13, or the 9.9-kb or both of the EcoRI fragments from ASRK-20 exhibited the pollen abortion phenotype, while the ones that did not inherit any of these transgene inserts exhibited normal pollen phenotype.

Though it is possible that the pollen abortion phenotype exhibited by the three transgenic plants may be due to insertion of the transgene into genes of the transgenic plants that are essential for pollen development, that is highly unlikely. The probability of having three independent transgenic plants exhibiting the same phenotype caused by random insertions of the transgene into their respective genomes would be extremely low.

Furthermore, the following observations suggest that down-regulation of the endogenous *PRK1* gene by the antisense *PRK1* transgene is most likely the cause of the pollen abortion phenotype. First, the 1:1 ratio of normal vs aborted pollen is what would be expected for plants that contain an active antisense transgene that targets a gametophytically-expressed gene, such as the *PRK1* gene. (If insertion mutagenesis were the cause of the phenotype, the transgenes in all three transgenic plants would have to be inserted into genes that not only are required for pollen development but also act gametophytically, a very unlikley scenario.) Second, the timing of the first detection of abnormality in microspore development (see Stage 3 in Figure 4) coincides with the first detection of PRK1 mRNA and the antisense PRK1 transcript (see lanes 1 and 2 of Stage 3 in Figure 5). That is, microspores develop normally up until stage 2 of anther development when neither PRK1 mRNA nor the antisense PRK1 transcript is detected. Third, the reduction of PRK1 mRNA in stage 3 anthers of ASRK-13 coincides with the appearance of the antisense PRK1 transcript. Fourth, the finding that in stage 3 anthers of ASRK-13, the amount of PRK1 mRNA is reduced by 30% from that of the wild-type level (lanes 1 and 2 of Stage 3 in Figure 5), but most of the microspores (85% of the total) retain their nuclei (e.g., only 15% of the microspores have lost their nuclei), suggests that those uninucleate microspores carrying the active transgene (contained in the 7.7-kb EcoRI fragment) have reduced levels of PRK1 mRNA. Fifth, the further reduction of the amount of PRK1 mRNA in stage 4 and stage 5 anthers of ASRK-13 to approximately 50% the wild-type level is consistent with the increase in the percentage of the microspores that have lost their nuclei from 15% in stage 3 anthers to 50% in both stage 4 and stage 5 anthers. Since the additional microspores that lose their nuclei and eventually become aborted when anthers develop from stage 3 through stage 4 to stage 5 are those uninucleate microspores that contain the active transgene, the reduction of PRK1 mRNA in the affected microspores precedes, and is not the result of, the disintegration of their nuclei and most of their cytoplasmic contents.

It was unexpected that the antisense PRK1 transcripts was detected in stage 4 and particularly in stage 5 anthers of ASRK-13, since by stage 5, most, if not all, of the mutant microspores of ASRK-13 have lost their nuclei and cytoplasmic

contents (Figure 5 and Figure 3e). However, we found that the transgene contained in the 11.2-kb EcoRI fragment of ASRK-13, which is expected to be carried by half of the normal microspores present in these two stages of anther, could express the antisense transcripts. RNA gel blot analyses with the plants carrying only the 11.2-kb fragment (progeny of ASRK-13: Fig. 2b) showed that the antisense PRK1 transcripts was present at 25 to 30% level of ASRK-13 plants in stage 3 anthers, and at similar level in stage 5 anthers (results not shown). These results suggest that the antisense mRNA detected in stage 4 and particularly stage 5 of ASRK-13 plants was mostly originated from the transgene contained in the 11.2 kb fragment. The likely explanation why the transgene in the 11.2-kb fragment could not generate the pollen abortion phenotype is that the level of antisense PRK1 transcripts might not be high enough to exert the effect. It has been suggested that the degree of inhibition of gene expression correlates with the level of antisense mRNA produced. If a cell does not produce enough antisense transcripts, the remaining sense mRNA might make enough protein to show a normal phenotype. It may also explain why we obtained only three transgenic plants carrying the phenotype, because in stage 3 of anther development the *LAT52* promoter does not show a very strong activity (Twell et al., 1990).

The observation that the affected microspores developed normally up to the uninucleate stage but became arrested at the stage and subsequently lost their nuclei (Figure 4) suggests that PRK1 is required for post-meiotic development of microspores. Though any specific function(s) of PRK1 is not defined yet, it appears to play such an essential role for normal pollen development that when the synthesis of PRK1 protein was inhibited in stage 3, it resulted in the immediate arrest of pollen development at the stage. The continuing presence of PRK1 mRNA in the later stage of microspore development (Figure 5: Mu et al., 1994) suggests that PRK1 might be required constantly in those developmental stages for one type or a variety of cell signaling. It is not known whether PRK1 is involved in a signal transduction pathway for microspore mitosis. If that is true, then the passage of microspores through normal post-meiotic development to form binucleate microspores may require the perception and transduction of a signal, maybe from tapetum, by PRK1. Since PRK1 has both serine/threonine and tyrosine kinase activities (Mu et al., 1994), it is conceivable that this putative signal transduction pathway may involve phosphorylation/dephosphorylation of microspore proteins. Whether the disintegration of the nucleus and the cytoplasm in the microspore that fail to progress down the developmental path is caused by a programmed cell death mechanism remains to be studied.

Progeny analysis revealed unexpectedly that the transgene inserts carried by ASRK-13 and ASRK-20 which did not transmit to the progeny through male also in the majority of cases failed to transmit to progeny through female (Figure 2c). The reduced transmission of the transgene through female is not likely due to its instability resulting from a complex rearrangement during integration, because both of ASRK-13 and -20 of which transgene inserts integrated into independent genomic loci showed the same phenomenon. Especially, in ASRK-13, only the 7.7-kb fragment that was linked to the pollen abortion phenotype was poorly inherited through female. In contrast, ASRK-17, a transgenic plant which did not carry the pollen abortion phenotype, showed normal transmission of the transgene through female as well as male (results not shown). The *LAT52* promoter has been shown to drive the expression of the *LAT52*-GUS fusion gene in immature and mature seeds of transgenic tomato plants (Twell et al., 1991), and more importantly, transgenic tobacco plants expressing the *LAT52* promoter-Diphtheria toxin fusion gene have been found to show reduced transmission of the transgene through female (D. Twell, 1995). Additionally, the RNA gel blot analysis using poly A+ RNA revealed

that the PRK1 mRNA is present in mature ovules of Petunia inflata (unpublished results, Lee and Kao). This "female" phenotype observed here is currently being investigated and is beyond the scope of this report. If true, PRK1 may also be involved in female gametophyte development, fertilization, and/or postzygotic development (embryogenesis and/or endosperm development).

Despite the fact that the aborted pollen grains have lost their nuclei and nearly all the cytoplasmic contents (Figures 3f, 3g), the morphology of their outer wall and the exine patterning appear similar to those of normal pollen (Figures 3b, 3c, 3h, 3i). It has been shown that the sporopollenin of the exine is tapetal in origin, but the 'blueprint' of the exine pattern is encoded within the gametophyte and is related to specific features in the pollen cytoplasm (Heslop-Harrison, 1968: Dickinson and Potter, 1976). Our observation of almost normal exine patterning in the aborted pollen grain suggests that the events associated with laying down the template of exine patterning occur prior to microspore mitosis.

Although a number of pollen development mutants, including pre-meiotic, meiotic, and post-meiotic mutants of microspores, have been identified, virtually all are sporophytic in nature because of the relative ease of screening for male sterile phenotype (Albertsen & Phillips, 1981: Chaudhury, 1993: Chaudhury et al., 1994). Mutations in genes acting in gametophytes, such as the PRK1 gene, result in reduction of the amount, but not complete absence, of normal pollen and thus require more elaborate strategies to be identified. Identification of the molecule(s) that interact(s) with the extracellular domains of PRK1, the molecule(s) that interact with the cytoplasmic kinase domain of PRK1, and other components of a PRK1-regulated signal transduction cascade will provide an insight into the precise role of this gametophytic-acting gene, PRK1, in the post-meiotic developmental process of microspores, and will allow us to examine the hypothesis of the "signaling" role of the tapetum.

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