A calmodulin-binding protein from *Arabidopsis* has an essential role in pollen germination

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Calmodulin (CaM), a ubiquitous multifunctional calcium sensor in all eukaryotes, mediates calcium action by regulating the activity/function of many unrelated proteins. Although calcium and CaM are known to play a crucial role in pollen germination and pollen tube growth, the proteins that mediate their action have not been identified. We isolated three closely related CaM-binding proteins (*NPG1, NPGR1, and NPGR2*) from *Arabidopsis*. *NPG1* (*No Pollen Germination1*) is expressed only in pollen, whereas the *NPG*-related proteins (*NPGR1* and *NPGR2*) are expressed in pollen and other tissues. The bacterially expressed NPG1 bound three isoforms of *Arabidopsis* CaM in a calcium-dependent manner. To analyze the function of *NPG1*, we performed a reverse genetics screen and isolated a mutant in which *NPG1* is disrupted by a T-DNA insertion. Segregation and molecular analyses of the *NPG1* knockout mutant and a cross with a male sterile mutant indicate that the mutated *NPG1* is not transmitted through the male gametophyte. Expression of *NPG1* in the knockout mutant complemented the mutant phenotype. Analysis of pollen development in the knockout mutant by light microscopy showed normal pollen development. Pollen from *NPG1* mutant in the *quartet* background has confirmed that *NPG1* is dispensable for pollen development. However, germination studies with pollen from the mutant in the *quartet* background indicate that pollen carrying a mutant allele does not germinate. Our genetic, histological, and pollen germination studies with the knockout mutant line indicate that *NPG1* is not necessary for microsporogenesis and gametogenesis but is essential for pollen germination.

Sexual reproduction in flowering plants depends on pollination and fertilization. After landing on the stigma, pollen germinates and delivers sperm cells to the embryo sac by growing through the transmitting tissue in the style (1–4). The mechanisms controlling these processes are poorly understood (5, 6). Lipophilic molecules in the exine wall of pollen are believed to mediate pollen adhesion to stigma (4, 7). Components of the extracellular pollen coat such as tryphine have been shown to be important for pollen germination (8). Pollen lacking tryphine do not absorb water and fail to germinate *in vivo* (8). Flavanols, a specific class of flavanoids, also have a role in pollen germination of certain species (9, 10).

Numerous reports have implied a central role for calcium in pollen germination and pollen tube growth and guidance (2, 3, 11–15). Calcium is an essential constituent of *in vitro* pollen germination media, and a high calcium concentration has been reported near the germination aperture of hydrated pollen (16, 17). The requirement for calcium for pollen germination and tube growth has been shown by modulating its concentration by using chemicals that interfere with calcium transport (15, 17). Calcium measurement studies with fluorescent indicator dyes have demonstrated that cytosolic calcium forms a steep tip-focused gradient (18–21). The concentration of calcium reaches >3 μM at the tip and drops to <0.2 μM within 20 μm of the tip. The growth of the pollen tube is restricted to the tip of the tube, and the growth rate is positively correlated with changes in calcium at the tip (17, 21–23). Disruption of the calcium gradient at the tip results in inhibition of tube growth. Furthermore, the direction of pollen tube growth can be changed by manipulating the calcium gradient (20, 21). These studies indicate that the establishment of a precise tip-focused intracellular Ca^{2+} gradient and its oscillations are important for pollen tube elongation and directional growth. Recent studies have shown that Rho-related GTPases, also called ROPs, control pollen tube polar tip growth by modulating the generation of the tip-localized calcium gradient as well as the assembly of F-actin (23, 24). However, the mechanisms by which ROPs regulate these processes are not known.

Calmodulin (CaM), a universal calcium sensor in eukaryotes, is implicated in regulating diverse cellular process in plants (25–28), including pollen germination and tube growth (29–31), which are enhanced by exogenous application of CaM (32). Furthermore, CaM is localized to the germinal aperture and the cytoplasm near the germination bubble in hydrated pollen and in the cytosol of the pollen tube, where it forms a gradient similar to the tip-focused Ca^{2+} gradient (29). In another report, CaM showed uniform localization at the tip but formed a V-shaped collar behind the apical region, which dissipated on growth arrest (30). Despite numerous reports on the role of calcium and CaM in pollen germination and tube growth, the proteins that mediate calcium/CaM action have not been identified.

In this study, we cloned three closely related CaM-binding proteins (*NPG1*, *NPGR1* and *NPGR2*) from *Arabidopsis*. *NPG1 (NO POLLEN GERMINATION*1) is expressed exclusively in pollen, whereas *NPG*-related genes (*NPGR1* and *-2*) are expressed in pollen and several other tissues. The function of *NPG1* was investigated by using an *Arabidopsis* mutant in which the gene is disrupted. Studies with the mutant show that *NPG1* is not necessary for male meiosis and pollen development. However, *NPG1* is essential for pollen germination, and the mutant allele is not transmitted through the male gametophyte.

Materials and Methods

Cloning of cDNAs. DNAse-treated RNA from pollen was used to synthesize first-strand cDNA with an oligo(dT) primer (33). Primers corresponding to 5′ and 3′ ends of predicted cDNAs were designed on the basis of genomic sequence in the TAIR database. The primers sets are 5′-ATGCTCGGGAATCAATCCGCGG-3′ (forward) and 5′-TTAAAGAATGGTTGAGAAGCTTTC-3′ (reverse) for *NPG1* (At2g43040); 5′-GCAGAATTCATGTTGTGTGCTTGTTCAGGCG-3 (forward) and 5′-GCAGGATTCAAATGAAACTCTGTACCGGAG-3′ (reverse) for *NPGR1* (At1g27460); and 5′-ATGAGGCATAGAGAAGAAGAAGACAAG-3′ (forward) and 5′-TCATCTGAATGGCTCCACTGG-3′ (reverse) for *NPGR2* (gene At4g28600).

PCR was performed in a final volume of 50 μl by using the TaKaRa Ex *Taq*PCR (Takara Shuzo, Kyoto) system according to

Abbreviations: CaM, calmodulin; CBD, CaM-binding domain; NPG, no pollen germination; TPR, tetratricopeptide repeat; DAPI, 4′,6-diamidino-2 phenylindole.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF474176).

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the manufacturer's instructions. The amplified products were resolved by electrophoresis, gel purified, cloned into pGEM-T Easy vector, and sequenced.

Bacterial Expression and Detection of NPG1. A 2.0-kb *Eco*RI fragment of *NPG1* cDNA containing the entire coding region was subcloned into pET28a, and the orientation of the cDNA was confirmed by restriction analysis and sequencing. Induction of fusion protein, its purification on CaM Sepharose column, and detection with horseradish peroxidase CaM isoforms were performed essentially as described (34).

Expression Analysis of *NPG1*. *Arabidopsis* pollen was collected according to Huang *et al.* (35). RNA from pollen and tissues was isolated by using the TRIzol reagent according to the protocol provided by GIBCO/BRL with the following modifications for pollen RNA extraction. Five hundred microliters of TRIzol reagent plus 300 μl of glass beads (710–1,180 μM) were added to 200 μl of pollen and vortexed continuously at a high speed for 15 min, then another 500 μl of TRIzol reagent was added. RNA pellets were dissolved in either deionized formamide (for RNA gel blots) or RNAse-free water (for RT-PCR). Fifty micrograms of total RNA from each tissue was electrophoresed, blotted, hybridized with ^{32}P-labeled cDNA, and washed under high-stringency conditions. Expression analysis of *NPG1*, *NPGR1*, and *NPGR2* was performed by RT-PCR by using the first-strand cDNA prepared from DNAse-treated RNA as described.

Screening of T-DNA Insertion Lines for *NPG1* Mutant. We screened 60,400 T-DNA-tagged *Arabidopsis* mutants generated at the University of Wisconsin *Arabidopsis* knockout facility (36) by using an *NPG1*-specific forward (5′-ACCAAAGAGGGAATTTAGAAGGCGCACTT-3′) or reverse (5′-TACGTAACTCCTAGCTAGGTTGTTTGGCT-3′) primer together with T-DNA left or right border primer. Screening of superpools and pools was performed at the *Arabidopsis* knockout facility. Southern analysis of PCR-amplified products identified a T-DNA insertion in *NPG1* with one primer combination (T-DNA left border and *NPG1* reverse primers). We then reamplified the hybridizing band and sequenced the product to confirm that the insertion was in the coding region of *NPG1*. Final screening and identification of the *NPG1* knockout line were performed at Colorado State University. We refer to *NPG1/npg1* heterozygous plant as a "mutant" because of its pollen phenotype (no pollen germination).

Genetic Crosses. To determine whether the mutated gene is transmitted through pollen, we pollinated a male sterile (*cer6-2*) mutant with pollen from the *NPG1/npg1* plant. Seeds from this cross were germinated on plates with or without kanamycin.

Tetrad analysis was performed by generating a double mutant. The pollen from *qrt/qrt* mutant (*quartet1*) (37) was used to pollinate emasculated flowers of the *NPG1/npg1* plant. We selected the progeny on kanamycin plates for *NPG1/npg1*, *QRT/qrt*, and selfed. Seeds from selfing were grown on kanamycin plates, and plants that showed *qrt* phenotype (*NPG1/npg1*, *qrt/qrt*, one of every four kanamycin-resistant plants) were selected.

Microprojectile Bombardment of Pollen. The helium-driven PDS-1,000/He particle delivery system (Bio-Rad) was used for the biolistic transformation of midbicellular grains of tobacco pollen (*Nicotiana tabaccum*). Tobacco pollen was resuspended to 1×10^6 cells in 50–100 μl of germination medium (0.1 mg/ml H_3BO_3/0.71 mg/ml $Ca(NO_3)_2$ $4H_2O$/0.2 mg/ml $MgSO_4$ $7H_2O$/0.1 mg/ml KNO_3/10% sucrose/0.5 mg/ml Mes/1.0 mg/ml casein hydrolysate, pH 5.9) and placed in the middle of a 5-cm Petri dish, previously rinsed with the same buffer, to create an even monolayer of cells. Each pollen monolayer was bombarded at least three times, as described (38), and 5 ml of germination medium was added. The plates were then left in the dark without shaking for 12–24 h in a humid chamber at 21 ± 3°C. GFP-expressing pollen were initially scored on the plates and later transferred onto a glass slide for confocal microscopy.

Pollen Germination. *In vitro* germination of *Arabidopsis* pollen was performed in acid-washed depression slides in liquid medium as described (39). About 60–70% of pollen germinated in this medium. Pollen from *qrt/qrt* or *NPG1/npg1*, *qrt/qrt* flowers [stage 12 or 13 (40)] was carefully tapped onto the slides, and the medium was added. Slides were then incubated in a humid chamber at 21 ± 3°C without light for 10–24 h.

Generation and Analysis of Transgenic Plants. The following constructs were used for transient and/or stable expression. A 2.4-kb fragment (NP, *NPG1* Promoter) upstream of the translation initiation codon of the *NPG1* gene was amplified with forward (5′-CATGCCATGGTATGAGTCGAGTGTCTGACTT-3′) and reverse 5′-TCGCCATGGTTCTTCACCTTTTAGACTA-3′) primers carrying an *Nco*I site (underlined). The PCR fragment was cloned into pGEM-TE vector, sequenced, and used to replace the 35S promoter region (*Nco*I-*Nco*I) in a pBA002-GFP binary vector kindly provided by Nam-Hai Chua (The Rockefeller University, New York) to generate NP<GFP>Nos. For complementation of the *NPG1* mutant, another construct was made by replacing the GFP fragment (*Nco*I-*Sma*I) with a full-length blunted *Spe*I-*Nco*I fragment of *NPG1* cDNA in a pBA002-based construct NP<GFP>Nos to create NP<*NPG1*>Nos. The constructs were verified by sequencing, introduced into *Agrobacterium* GV3101 strain, and used to transform *Arabidopsis* plants by using the floral-dip method. Wild-type plants were transformed with NP<GFP>Nos, and *NPG1/npg1* plants were transformed with NP<*NPG1*>Nos. Seeds from infiltrated plants were collected, surface-sterilized, stratified, and selected on appropriate selection plates.

Histology, Light Microscopy, and Confocal Laser Scanning Microscopy. Inflorescences were harvested, dehydrated in a standard ethanol series, fixed (formamide and glutaraldehyde solutions), and embedded in Paraplast tissue medium (41). Samples were sectioned and mounted on polyL-lysine-coated glass slides and then stained. Tissue sections were observed with a Leitz Laborlux S microscope. Images were captured by using Kodak Digital Science DC-120 zoom camera. For nucleus staining, 1 μl of 4′, 6-diamidino-2 phenylindole (DAPI) solution (1 mg/ml) was added to 1–2 ml of mounting buffer (0.1 M Tris·HCl, pH 9.0/50% glycerol), and the pollen was examined in UV light after 10–30 min. GFP images were captured by using a fluorescence microscope with an integrated confocal imaging (FVX-IHRT Fluoview Confocal LSM) system from Olympus (Melville, NY). Images were acquired by using FLUOVIEW software provided by the manufacturer. Scanning electron microscopy was performed essentially as described (8). All images were processed with PHOTOSHOP 5.0 software (Adobe Systems, Mountain View, CA).

Results and Discussion

Isolation of *NPG1* and *NPG*-Related cDNAs from *Arabidopsis* Pollen. To investigate the role of CaM-binding proteins in pollen germination and tube growth, we identified three genes (At2g43040, At1g27460, and At4g28600) in the recently completed *Arabidopsis* genome sequence that are closely related to a gene encoding pollen-specific maize CaM-binding protein (34). We cloned the corresponding cDNAs from *Arabidopsis* pollen RNA by RT-PCR. An expected size fragment (≈2.3 kb) for each gene was obtained and verified by sequencing. Amplification of RNA

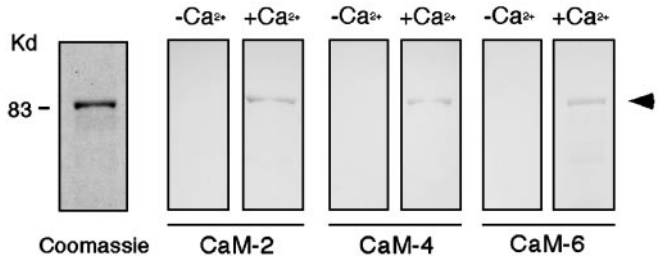

Fig. 1. NPG1 is a CaM-binding protein. Binding of bacterially expressed and purified NPG1 (Coomassie) to horseradish peroxidase-labeled *Arabidopsis* CaM isoform-2 (CaM-2), -4 (CaM-4), and -6 (CaM-6) in the absence ($-Ca^{2+}$) or presence of calcium ($+Ca^{2+}$) is shown.

itself did not yield any products, and amplification from genomic DNA with the corresponding primers yielded a larger-size product due to introns. Comparison of the At2g43040 (named *NPG1* for no pollen germination; see below) cDNA sequence with the genomic sequence has revealed the presence of four introns in the coding region of the *NPG1* gene (Fig. 6, which is published as supporting information on the PNAS web site, www.pnas.org). *NPG1* encodes a protein of 704 aa (Fig. 6). The deduced amino acid sequence of NPG1 (GenBank accession no. AF474176) differs from the predicted protein in the *Arabidopsis* genome database at the C terminus due to inaccurate prediction of the last two introns. The predicted amino acid sequences of the two *NPG*-related proteins (*NPGR1* coded by At1g27460 and *NPGR2* coded by At4g28600) are the same as their predicted sequences in the database. NPG1, NPGR1, and NPGR2 share significant sequence similarity among themselves and with maize pollen-specific CaM-binding protein (39–56% identity and 56–70% similarity) (Fig. 7, which is published as supporting information on the PNAS web site). The NPG1 amino acid sequence showed 56% identity and 70% similarity to the maize CaM-binding protein. Analysis of the predicted amino acid sequence of *Arabidopsis* NPG1 and the related proteins using SMART (http://smart.embl-heidelberg.de) revealed the presence of six tetratricopeptide repeats (TPRs) (Figs. 6 and 7). TPR domains consist of a degenerate 34-aa region and are implicated in protein–protein interaction (42, 43). The second TPR overlaps with the CaM-binding domain (CBD) (Fig. 6; see below). A homolog of *NPG1* or *NPGR*s has not been found in any nonplant system, including the yeast, fly (*Drosophila melanogaster*), worm (*Caenorhabditis elegans*), and human (*Homo sapiens*) genomes, whose sequences have been completed, suggesting these represent a small family of plant-specific CaM-binding proteins.

NPG1 and NPGRs Are CaM-Binding Proteins. To demonstrate that NPG1 is a CaM-binding protein, we expressed the coding region of *NPG1* as a T7-tag fusion by using an *E. coli* expression system and used the bacterially expressed protein in CaM-binding studies. The bacterially expressed NPG1 bound CaM-Sepharose beads in a calcium-dependent manner and eluted with EGTA-containing buffer (data not shown). The purified NPG1 bound three *Arabidopsis* CaM isoforms in a calcium-dependent manner in blot overlay assays (Fig. 1). These results confirm that NPG1 is a CaM-binding protein. The interaction of NPG1 with CaM only in the presence of calcium suggests that the function of NPG1 is modulated *in vivo* by calcium and CaM. To map the CBD, we expressed a truncated version (amino acids 448–496) of NPG1, which contains a putative CBD, in *E. coli* and tested for its ability to bind CaM. The truncated protein bound CaM in a manner similar to the full-length protein, indicating that the CBD is located between amino acids 448 and 496 (M.G., S.-B. Shin, and A.S.N.R., unpublished work). This region is highly conserved among these three proteins (see Fig. 7*A*). The proteins coded by the full-length cDNAs of *NPGR1* and *NPGR2* also bind CaM in a calcium-dependent manner (M.G., S.-B. Shin, and A.S.N.R., data not shown).

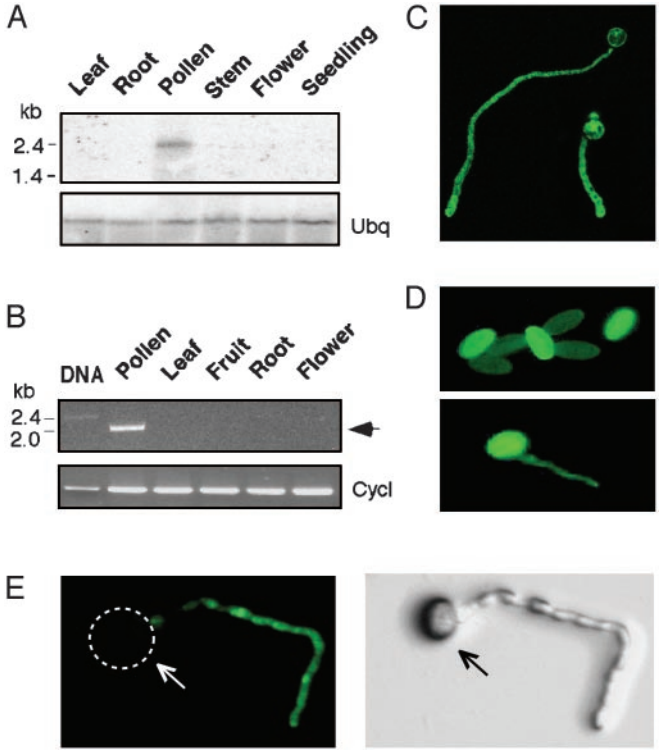

Fig. 2. *NPG1* is expressed only in pollen. (*A*) Northern blot analysis. The blot was first hybridized with *NPG1* cDNA (*Upper*) and then with *Arabidopsis* ubiquitin (Ubq) (*Lower*). (*B*) Amplification of *NPG1* transcript in pollen and tissues by RT-PCR (*Upper*). Amplified product using the same primers with genomic DNA (DNA) is also shown. The presence of cDNA template in all reactions was confirmed by amplification of the cyclophilin gene (*Lower*). (*C*) Expression of GFP fused to the *NPG1* promoter. Shown is GFP expression in transiently transformed germinating tobacco pollen. (*D*) Expression of GFP fused to the *NPG1* promoter in mature (*Upper*) or germinated pollen (*Lower*) from a heterozygous transgenic *Arabidopsis* plant. (*E*) (*Left*) Expression of GFP in stably transformed pollen at 6 h of germination. (*Right*) Bright-field picture.

***NPG1* Is Expressed Specifically in Pollen.** To determine the expression of *NPG1*, RNA from different tissues/organs was analyzed by Northern analysis. As shown in Fig. 2*A*, *NPG1* transcripts were detected only in pollen. The transcript size (≈2.5 kb) is consistent with the expected size. Equal amounts of RNA on the blot were verified by hybridizing with ubiquitin (Fig. 2*A Lower*). To eliminate the possibility that *NPG1* may be expressed at very low levels, we performed RT-PCR analysis. The results from the RNA blot analysis were confirmed by RT-PCR, where the *NPG1* transcript was detected only in pollen (Fig. 2*B*). The amplified transcript was verified by hybridization with *NPG1* (data not shown). We have further confirmed pollen-specific expression of *NPG1* by fusing its promoter to GFP and analyzing the promoter activity. In transient expression assays with tobacco pollen, a very strong expression of GFP was found in pollen tubes (Fig. 2*C*). In transgenic *Arabidopsis* plants, GFP was seen in mature and germinating pollen (Fig. 2*D*). The earliest expression of GFP was detectable in pollen at stages 12–13 of flower development (sepals open, petals can be seen between the sepals; data not shown) (40). During the later stages of pollen germination, the expression of GFP is mostly confined to the tube (Fig. 2*E*), GFP was not detected in any other tissues except pollen. To determine the expression of *NPGRs,* we performed RT-PCR analysis by

using the first-strand cDNA from different tissues and gene-specific 5′ and 3′ primers for each gene. The amplified products were hybridized with corresponding cDNA to verify that the amplified products are real. Unlike *NPG1*, *NPGR1* and *NPGR2* are expressed in suspension culture cells, pollen, flowers, and fruits (Fig. 8, which is published as supporting information on the PNAS web site). In addition, *NPGR2* is expressed in leaves also (Fig. 8). In the case of *NPGRs*, additional smaller bands were detected, which may have been the products of alternative splicing (data not shown). Together, these results clearly show that *NPG1* is expressed only in pollen and pollen tubes.

Isolation of an *NPG1* Mutant. Because *NPG1* is expressed exclusively in pollen, it is likely to function in one or more of the following processes: pollen development, germination, and/or pollen tube growth. To analyze the function of *NPG1*, we isolated a knockout mutant by screening a mutant library (60,400 independent kanamycin-resistant lines), which was generated by T-DNA insertion, by using a reverse genetics screen (36, 44). A plant with a T-DNA insertion in *NPG1* was identified by PCR amplification of a 0.8-kb product by a reverse primer of *NPG1* and T-DNA left border primer (Fig. 3 *A* and *B*). Sequence analysis of the PCR product amplified from the insertion allele revealed that the T-DNA interrupted the coding region (1,566 bp) after 461 aa in exon 3 (Fig. 3*A*). Any protein product from this allele would lack the CBD and five putative TPRs (Fig. 3*A*) known to be involved in protein–protein interaction. Amplification of both the wild-type and mutated genes from the plant suggested that it was a heterozygous plant (Fig. 3*B*). To confirm that the mutant plant is heterozygous and to determine the number of T-DNA insertions, we isolated DNA from wild type and mutant and digested with a restriction enzyme (*Bam*HI), which does not cut *NPG1* but has a single site in T-DNA. Therefore, it is expected that a Southern analysis probed with *NPG*1 should produce a single hybridizing band in wild type and three bands in the heterozygote. Furthermore, if there is a single insertion, then the Southern analysis with DNA from the mutant probed with the T-DNA should produce two hybridizing bands. As shown in Fig. 3*C*, Southern analysis with *NPG1* and T-DNA probes confirmed that the plant was heterozygous with a single T-DNA insertion in the mutant allele (Fig. 3*C*).

The Mutant Allele Is Not Transmitted Through Male Gametophyte. Heterozygous mutant plants were selfed to obtain homozygous plants. Analysis of the progeny from this cross revealed two significant findings. First, the ratio of kanamycin-resistant to kanamycin-sensitive plants was 1:1 (476:444) instead of the expected 3:1. Second, Southern and PCR analyses of the kanamycin-resistant progeny with primers that amplify the wild type and mutant allele showed that a subset of randomly selected kanamycin-resistant plants (50 plants) were heterozygous (data not shown). A distorted segregation ratio and an inability to produce homozygous plants, together with pollen-specific expression of *NPG1*, suggested that the mutated gene is not transmitted through the male gametophyte.

To further confirm that the *npg1* allele is not transmitted through pollen, we used a convenient male sterile mutant (*cer6-2*) (8) to cross with an *NPG1/npg1* plant (Fig. 3*D*). As expected, we did not obtain any kanamycin-resistant progeny from this cross (Fig. 3*D*). However, pollination of emasculated *NPG1/npg1* flowers with pollen from wild-type plants produced the expected 1:1 kanamycin-resistant/sensitive progeny (data not shown) demonstrating that transmission of the *npg1* allele through female gametes is not affected. These results confirm that the mutant allele is not transmitted through pollen.

Complementation of Mutant with *NPG1* cDNA. To demonstrate that the mutant phenotype is caused by a disruption of the *NPG1* gene, *NPG1/npg1* plants were transformed with an *NPG1* cDNA driven by the *NPG1* promoter in a vector that confers resistance to BASTA. Plants resistant to both kanamycin and BASTA were selected. Pollen from 48 kanamycin- and BASTA-resistant plants was used to pollinate a male sterile (*cer6-2*) mutant to test transmission of the *npg1* allele. From this cross, we obtained progeny resistant to both selective agents from 37 of 48 total crosses (an example is shown in Fig. 3*E*). The reason for not seeing complementation in some lines is most likely the variations in the expression of introduced *NPG1* gene. The presence of introduced cDNA in the kanamycin- and BASTA-resistant progeny was verified by amplifying the cDNA from genomic DNA (data not shown). These results provide evidence that expression of the introduced *NPG1* cDNA complements the mutant *NPG1* allele and that the mutant phenotype is indeed due to the T-DNA insertion in the *NPG1* gene.

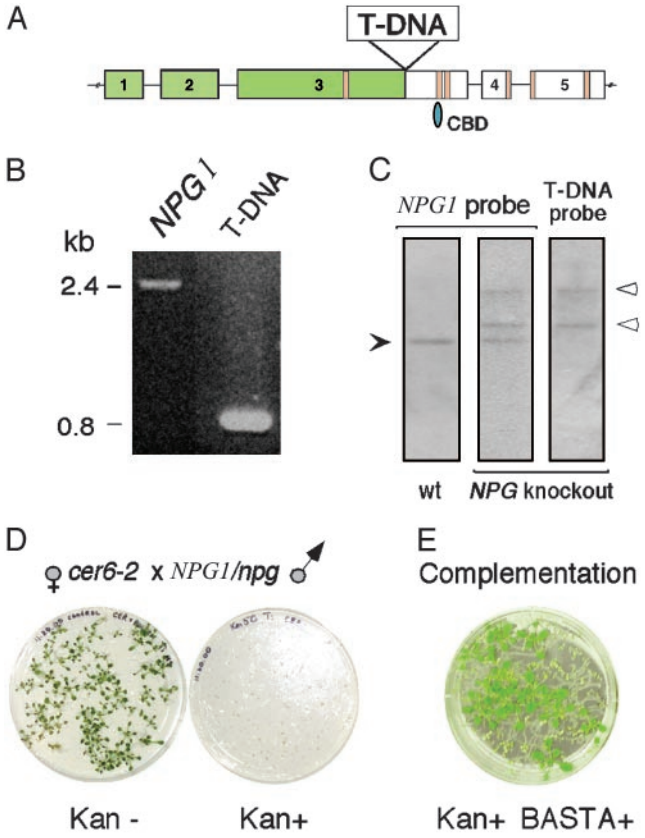

Fig. 3. *NPG1* mutant characterization and complementation. (*A*) Schematic diagram of *NPG1* gene showing the site of T-DNA insertion positioned at 1,566 bp. Numbered boxes represent exons. Coding region upstream of T-DNA is shown in green. The position of the CBD, as defined by deletion analysis, is indicated as a blue oval underneath exon 3. TPR motifs are shown as pink boxes. (*B*) PCR with the DNA from a *NPG1/npg1* plant using gene-specific forward and reverse primers (*NPG1*) and with *NPG1* reverse primer and T-DNA left border (T-DNA). (*C*) Southern blot analysis with DNA from wild type and *NPG1* mutant. Genomic DNA of wild type (wt) and *NPG1* mutant digested with *Bam*HI (single site in T-DNA and no site in *NPG1*) was hybridized first with the full-length *NPG1* cDNA (*NPG* probe) and then with T-DNA that includes the *Bam*HI site (T-DNA probe). (*D*) Analysis of progeny from a cross between male sterile mutant (*cer6-2*) and *NPG1* mutant for kanamycin-resistant seedlings. Kan-, plates without kanamycin; Kan+, plates with 50 μg/ml kanamycin. (*E*) Complementation of *NPG1* mutation. Pollen from *NPG1/npg1* plants additionally containing a *NPG1* cDNA driven by the *NPG1* promoter was used to pollinate flowers of a male sterile mutant *cer6-2*. Seeds from this cross were grown on a medium containing kanamycin and BASTA (Kan$^+$ BASTA$^+$).

Pollen Development Is Normal in the Mutant. Because the *npg1* allele is not transmitted through pollen, we envisioned two possible

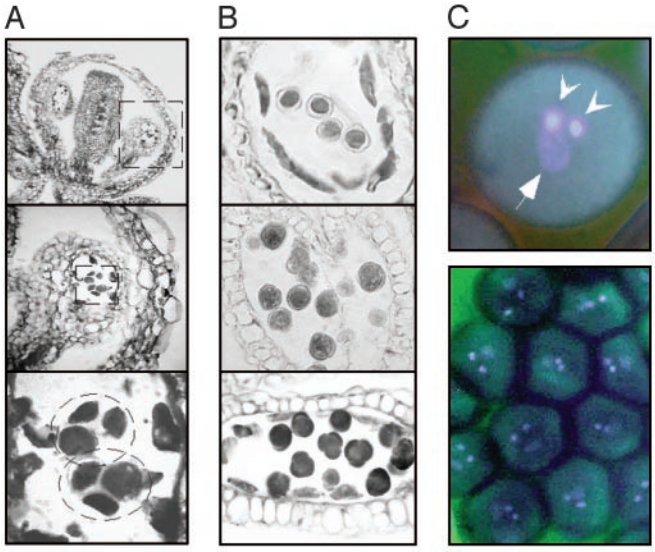

Fig. 4. Pollen development in *NPG1* mutant. (*A*) Sections of *Arabidopsis* flower [stages 7–8 according to Sanders *et al.* (45)] from *NPG1* mutant (*Top*), magnified view of the anther (*Middle*), and tetrad (*Bottom*). (*B*) Pollen development through stages 9 (*Top*), 10 (*Middle*), and 11 (*Bottom*) (45). (*C*) DAPI staining of pollen from *NPG1* mutant. White arrow indicates the diffusely staining vegetative nucleus, and arrowheads indicate two densely staining sperm cell nuclei (*Upper*). All DAPI-stained pollen grains from the mutant (*NPG1/npg1*) have one vegetative nucleus and two sperm nuclei (*Lower*).

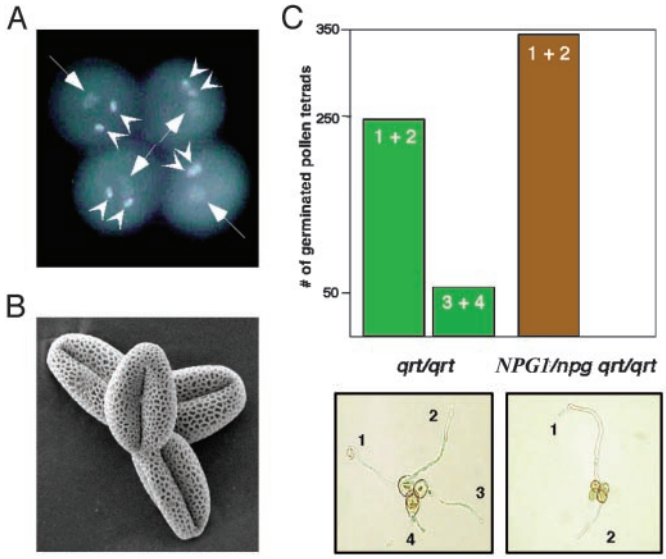

Fig. 5. Pollen development and germination in *NPG/npg, qrt/qrt* plant. (*A*) DAPI staining of a tetrad from an *NPG1* mutant (*NPG1/npg1*) in the *qrt* background. White arrows point to the vegetative nucleus and two sperm nuclei in each pollen grain of a tetrad. (*B*) SEM image of a tetrad from the *NPG1/npg1, qrt/qrt* plants. (*C*) Germination of tetrads from *qrt/qrt* and *NPG1/npg1, qrt/qrt* plants. The number of germinated pollen from each tetrad was counted. Tetrads with 1 or 2 germinating pollen (1 + 2) and 3 or 4 germinating pollen (3 + 4) from the *quartet* mutant (*qrt/qrt*) and the *npg1* mutant in *quartet* background (*NPG1/npg1, qrt/qrt*) are shown (*Upper*). The maximum number of germinating pollen in a tetrad from *qrt/qrt* is four (*Lower Left*), whereas the maximum number for an *NPG1/npg1, qrt/qrt* mutant is two (*Lower Right*).

scenarios by which the mutated allele (*npg1*) is not transmitted through pollen. One is that haploid cells that inherit the mutant allele after meiosis (half of the haploid cells) do not develop into pollen. Alternatively, the haploid cells carrying *npg1* could develop normally but not be functional due to a defect in later events of pollen germination and/or tube growth.

To address the first possibility, we analyzed the pollen development in wild-type and *NPG1/npg1* plants by light microscopy. We found that in the mutant normal tetrads are formed after meiosis, and the microspores developed into morphologically normal pollen (Fig. 4 *A* and *B*) (45). Pollen from *NPG1/npg1* plants was indistinguishable from wild type in shape, size, and DAPI staining, which showed all three nuclei (a single diffusely staining vegetative nucleus and two condensed sperm nuclei per pollen) (Fig. 4*C*). These results indicate that pollen development is normal in *NPG1/npg1* plants. To further confirm these results, we used the *quartet1 (qrt/qrt)* mutant of *Arabidopsis*. In the *qrt*1 mutant, the four microspores/pollen grains produced after male meiosis remain attached as tetrads due to fusion of the outer walls of the four meiotic products of the pollen mother cell (37). Therefore, any abnormalities in pollen development can be determined by analyzing the tetrads (46). We generated *NPG1/npg1, qrt/qrt* as described in *Materials and Methods*. On the basis of our earlier microscopic observations, we expect to see normal development of all four pollen grains, including those that carry the *npg1* allele in the double mutant. The tetrads in the double mutant are normal in appearance and indistinguishable from tetrads of the *qrt* mutant (Fig. 5 *A* and *B*). Furthermore, DAPI staining revealed the presence of the vegetative and two sperm nuclei in each pollen grain in the tetrad (Fig. 5*A*). Scanning electron micrographs of tetrads from the double mutant show that all four pollen grains in a tetrad are indistinguishable in their morphology (Fig. 5*B*). These studies clearly indicate that *NPG1* is not essential for male meiosis and pollen development.

Pollen Carrying the Mutant Allele Does Not Germinate. To address the role of *NPG1* in pollen germination and/or tube growth, we used the tetrads from *NPG1/npg1, qrt/qrt*, and *qrt/qrt* flowers (40) for *in vitro* germination (39). If pollen germination is affected in the double mutant, we expect to see germination of no more than two of the four pollen grains in a tetrad, whereas up to four pollen grains can germinate from *qrt/qrt* plants. The germination of pollen in tetrads from the *qrt/qrt* mutant ranged from one to four, whereas only one or two pollen grains germinated from tetrads from the double mutant (Fig. 5*C*). One of five germinating tetrads from *qrt/qrt* plants showed germination of three or four pollen grains. In contrast, all germinating tetrads from the double mutant showed germination of only one or two pollen grains (Fig. 5*C*). These results, together with our inability to obtain homozygous mutant and kanamycin-resistant plants from a cross between a male sterile mutant (*cer6-2*) and an *NPG1/npg1* mutant, strongly suggest that pollen carrying the *npg1* allele does not germinate, and *NPG1* is a regulator of pollen germination.

Many sporophytic mutants that affect microsporogenesis have been isolated by screening for male sterility (8, 45). In contrast, isolation of gametophytic mutants affecting pollen development and/or function has been difficult for obvious reasons. Such mutants as heterozygotes produce 50% normal pollen and thus show no easily observable affect on fertility. Further, it would not be possible to obtain homozygous mutant plants. So far, a few male gametophytic mutants that affect pollen development and/or function have been identified by screening T-DNA insertion lines for distorted segregation ratios of antibiotic resistance or by screening mutant populations for abnormal pollen (47–50). To our knowledge, *npg1* is the only gametophytic mutant for which the gene is cloned and the encoded protein is known to be required for pollen germination.

Although numerous reports suggest a critical role for calcium in pollen germination and pollen tube growth, nothing is known

about the CaM target proteins and their function in pollen (15, 21, 29–31). Here, our data clearly indicate that NPG1, a calcium-dependent CaM-binding protein, is required for pollen germination and is likely to be a key protein in the calcium signaling pathway in mediating calcium changes during pollen germination and tip growth. At present, no other male gametophytic gene necessary for pollen germination has been identified, although a sporophytic mutation that affects pollen hydration and *in vivo* germination due to a deficiency in lipoidic tryphine has been reported (8). Because the *NPG1* promoter was found to be active in growing pollen tubes as well as in mature pollen (Fig. 2 *C–E*), it is possible that NPG1 is required for pollen tube growth as well as germination. Our mutant does not permit the analysis of its role in pollen tube growth, because pollen germination is arrested, but blocking the expression of *NPG1* in growing pollen tubes by using antisense oligonucleotides should help reveal the role of *NPG1* in pollen tube growth. In the NPG1, there are six TPR motifs in addition to the CaM-binding domain (Fig. 6). TPR-containing proteins function through protein–protein interaction and modulate diverse cellular processes (42, 51, 52). Mutations in the TPRs have been shown to result in loss of function (42, 52). Hence, it is likely that NPG1 interacts with other proteins. Further studies on identification of proteins that interact with NPG1 should provide insights into its mode of action in controlling pollen germination.

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1. Mascarenhas, J. P. (1993) *Plant Cell* **5,** 1303–1314.
2. Bedinger, P., Hardeman, K. J. & Loukides, C. A. (1994) *Trends Cell Biol.* **4,** 132–138.
3. Hepler, P. K. (1997) *Trends Plant Sci.* **2,** 79–80.
4. Lord, E. M. & Russell, S. D. (2002) *Annu. Rev. Cell Dev. Biol.* **18,** 81–105.
5. Pruitt, R. E. (1999) *Curr. Opin. Plant Biol.* **2,** 419–422.
6. Johnson, M. A. & Preuss, D. (2002) *Dev. Cell* **2,** 273–281.
7. Lord, E. (2000) *Trends Plant Sci.* **5,** 368–373.
8. Preuss, D., Lemieux, B., Yen, G. & Davis, R. W. (1993) *Genes Dev.* **7,** 974–985.
9. Mo, Y., Nagel, C. & Taylor, L. P. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7213–7217.
10. Ylstra, B., Muskens, M. & Van Tunen, A. J. (1996) *Plant Mol. Biol.* **32,** 1155–1158.
11. Mascarenhas, J. P. & Machlis, L. (1964) *Plant Physiol.* **39,** 70–77.
12. Franklin-Tong, V. E. (1999) *Plant Cell* **11,** 727–738.
13. Palanivelu, R. & Preuss, D. (2000) *Trends Cell Biol.* **10,** 517–524.
14. Trewavas, A. (2000) in *Biochemistry and Molecular Biology of Plants*, eds. Buchanan, B., Gruissem, W. & Jones, R. l. (Am. Soc. Plant Physiologists, Rockville, MD), pp. 930–987.
15. Hepler, P. K., Vidali, L. & Cheung, A. Y. (2001) *Annu. Rev. Cell Dev. Biol.* **17,** 159–187.
16. Tirlapur, U. K. & Cresti, M. (1992) *Ann. Bot.* **69,** 503–508.
17. Taylor, L. P. & Hepler, P. K. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 461–491.
18. Pierson, E. S., Miller, D. D., Callaham, D. A., Shipley, A. M., Rivers, B. A., Cresti, M. & Hepler, P. K. (1994) *Plant Cell* **6,** 1815–1828.
19. Pierson, E. S., Miller, D. D., Callaham, D. A., van Aken, J., Hackett, G. & Hepler, P. K. (1996) *Dev. Biol.* **174,** 160–173.
20. Malho, R., Read, N., Pais, M. S. & Trewavas, A. J. (1994) *Plant J.* **5,** 331–341.
21. Malho, R. & Trewavas, A. J. (1996) *Plant Cell* **8,** 1935–1949.
22. Holdaway-Clarke, T. L., Feijo, J. A., Hackett, G. R., Kunkel, J. G. & Hepler, P. K. (1997) *Plant Cell* **11,** 1999–2010.
23. Yang, Z. (2002) *Plant Cell* **14,** S375–S388.
24. Li, H., Lin, Y., Heath, R. M., Zhu, M. X. & Yang, Z. (1999) *Plant Cell* **11,** 1731–1742.
25. Poovaiah, B. W. (1988) *HortScience* **23,** 267–271.
26. Reddy, A. S. (2001) *Plant Sci.* **160,** 381–404.
27. Snedden, W. A. & Fromm, H. (2001) *New Phytol.* **151,** 35–66.
28. Yang, T. & Poovaiah, B. W. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 4097–4102.
29. Tirlapur, U. K., Scali, M., Moscatelli, A., Del Casino, C., Cai, G., Tiezzi, A. & Cresti, M. (1994) *Zygote* **2,** 63–68.
30. Moutinho, A., Love, J., Trewavas, A. J. & Malho, R. (1998) *Sex. Plant Reprod.* **11,** 131–139.
31. Ma, L., Xu, X., Cui, S. & Sun, D. (1999) *Plant Cell* **11,** 1351–1363.
32. Ma, L. & Sun, D. (1997) *Planta* **202,** 336–340.
33. Golovkin, M. & Reddy, A. S. N. (1996) *Plant Cell* **8,** 1421–1435.
34. Safadi, F., Reddy, V. S. & Reddy, A. S. (2000) *J. Biol. Chem.* **275,** 35457–35470.
35. Huang, S., An, Y. Q., McDowell, J. M., McKinney, E. C. & Meagher, R. B. (1997) *Plant Mol. Biol.* **33,** 125–139.
36. Krysan, P. J., Young, J. C. & Sussman, M. R. (1999) *Plant Cell* **11,** 2283–2290.
37. Preuss, D., Rhee, S. Y. & Davis, R. W. (1994) *Science* **264,** 1458–1460.
38. Ottenschlager, I., Barinova, I., Voronin, V., Dahl, M., Heberle-Bors, E. & Touraev, A. (1999) *Transgenic Res.* **8,** 279–294.
39. Fan, L. M., Wang, Y. F., Wang, H. & Wu, W. H. (2001) *J. Exp. Bot.* **52,** 1603–1614.
40. Smyth, D. R., Bowman, J. L. & Meyerowitz, E. M. (1990) *Plant Cell* **2,** 755–767.
41. Ruzin, S. E. (1999) *Plant Microtechnique and Microscopy* (Oxford Univ. Press, Oxford).
42. Lamb, J. R., Tugendreichs, S. & Hieter, P. (1995) *Trends Biochem. Sci.* **20,** 257–259.
43. Tzamarias, D. & Struhl, K. (1995) *Genes Dev.* **9,** 821–831.
44. Sussman, M. R., Amasino, R. M., Young, J. C., Krysan, P. J. & Austin-Philips, S. (2000) *Plant Physiol.* **124,** 1465–1467.
45. Sanders, P. M., Bui, A. Q., Weterings, K., McIntire, K. N., Hsu, Y.-C., Lee, P. Y., Truong, M. T., Beals, T. P. & Goldberg, R. B. (1999) *Sex. Plant Reprod.* **11,** 297–322.
46. Copenhaver, G. P., Keith, K. C. & Preuss, D. (2000) *Plant Physiol.* **124,** 7–16.
47. Chen, Y. C. & McCormick, S. (1996) *Development (Cambridge, U.K.)* **122,** 3243–3253.
48. Howden, R., Park, S. K., Moore, J. M., Orme, J., Grossniklaus, U. & Twell, D. (1998) *Genetics* **149,** 621–631.
49. Park, S. K., Howden, R. & Twell, D. (1998) *Development (Cambridge, U.K.)* **125,** 3789–3799.
50. Procissi, A., de Laissardiere, S., Ferault, M., Vezon, D., Pelletier, G. & Bonhomme, S. (2001) *Genetics* **158,** 1773–1783.
51. Prodromou, C., Siligardi, G., O'Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Ladbury, J. E., Piper, P. W. & Pearl, L. H. (1999) *EMBO J.* **18,** 754–762.
52. Venolia, L., Ao, W., Kim, S., Kim, C. & Pilgrim, D. (1999) *Cell Motil. Cytoskeleton* **42,** 163–177.