RUPTURED POLLEN GRAIN1, a Member of the MtN3/saliva Gene Family, Is Crucial for Exine Pattern Formation and Cell Integrity of Microspores in Arabidopsis1[C][W][OA]

Yue-Feng Guan, Xue-Yong Huang, Jun Zhu, Ju-Fang Gao, Hong-Xia Zhang, and Zhong-Nan Yang*

National Key Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai 200032, China (Y.-F.G., H.-X.-Z.); and College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, China (X.-Y.H., J.Z., J.-F.G., Z.-N.Y.)

During microsporogenesis, the microsporocyte (or microspore) plasma membrane plays multiple roles in pollen wall development, including callose secretion, primexine deposition, and exine pattern determination. However, plasma membrane proteins that participate in these processes are still not well known. Here, we report that a new gene, RUPTURED POLLEN GRAIN1 (RPG1), encodes a plasma membrane protein and is required for exine pattern formation of microspores in Arabidopsis (Arabidopsis thaliana). The rpg1 mutant exhibits severely reduced male fertility with an otherwise normal phenotype, which is largely due to the postmeiotic abortion of microspores. Scanning electron microscopy examination showed that exine pattern formation in the mutant is impaired, as sporopollenin is randomly deposited on the pollen surface. Transmission electron microscopy examination further revealed that the primexine formation of mutant microspores is aberrant at the tetrad stage, which leads to defective sporopollenin deposition on microspores and the locule wall. In addition, microspore rupture and cytoplasmic leakage were evident in the rpg1 mutant, which indicates impaired cell integrity of the mutant microspores. RPG1 encodes an MtN3/saliva family protein that is integral to the plasma membrane. In situ hybridization analysis revealed that RPG1 is strongly expressed in microsporocyte (or microspores) and tapetum during male meiosis. The possible role of RPG1 in microsporogenesis is discussed.

In angiosperms, the pollen wall usually consists of an outer exine layer and an inner intine layer (Heslop-Harrison, 1971; Piffanelli et al., 1998). The exine layer includes an outer sculptured sexine and a simpler foot layer, the nexine (Heslop-Harrison, 1971). The sexine can be further divided into the rod-shaped bacula and the tectum. The exine wall plays important roles in protecting the pollen from various environmental stresses and in the species-specific adhesion of pollen grains to the stigma (Zinkl et al., 1999). The intine, secreted by the microspore, is the innermost layer and is located between plasma membrane and nexine (Heslop-Harrison, 1971). In addition, a trypiline layer (or pollen coat), which is derived from the remnant of tapetum, fills the gaps within the exine layer and covers the pollen grain.

Successful pollen wall development, especially exine wall formation, requires precise coordination of the microspore and tapetum (Paxson-Sowders et al., 1997; Piffanelli et al., 1998). During meiosis, microsporocytes secrete β-1,3-glucan (callose) between the plasma membrane and the primary cell wall. Multiple roles of the callose wall have been proposed, including preventing microspores from fusion (Waterkeyn and Bienfait, 1970), serving as a molecular filter, and as a mold for primexine patterning (Heslop-Harrison, 1971). At the tetrad stage, primexine matrix, the foot-print of the exine layer, is deposited on microspores based on the regular undulation of the plasma membrane (Fitzgerald and Knox, 1995; Paxson-Sowders et al., 1997). After the release of microspores, the main component of exine, sporopollenin, is secreted by the tapetum and deposited on the microspores following the patterning of the primexine (Paxson-Sowders et al., 1997; Piffanelli et al., 1998).

In Arabidopsis (Arabidopsis thaliana), several male sterile mutants with pollen wall formation defects have been reported. In the male sterility1 (ms1) mutant, the microspore cytoplasm and tapetum become abnormally granular and vacuolated with microspore degeneration (Wilson et al., 2001). Primexine formation is aberrant on the microspore at the tetrad stage, which results in random deposition of sporopollenin (Vizcay-Barrena and Wilson, 2006). Further studies showed that MS1 is a PHD-finger family transcription...
RESULTS

Phenotypical Identification of the rpg1 Mutant

To identify new genes essential for male fertility, T-DNA-tagged lines from a pool of Arabidopsis were screened for mutants that showed a reduced seed set (Qin et al., 2003). One of the tagged lines exhibited very few seed sets upon self-pollination. Based on the observation of postmeiotic rupture of microspores in this mutant (see below), it was designated rpg1. During the vegetative growth phase, rpg1 was indistinguishable from wild-type plants. However, the fertility of rpg1 was dramatically reduced, as indicated by their short siliques (Fig. 1A).

We further compared the seed yield of wild-type and rpg1 plants by examining the number of seeds per siliques. Wild-type plants produced about 25 siliques on the inflorescence axis and approximately 50 seeds per siliques during reproductive development (n = 3), whereas the average seed yield per siliques was reduced by 90% in rpg1 plants (n = 15). Moreover, the fertility of rpg1 varied depending on the reproductive development stage. In the early formed siliques, only one or no seed was produced per siliques. Seed yield of the mutant gradually recovered as the inflorescence development progressed. In the siliques generated from the 25th to 30th flowers on the inflorescence of rpg1, an average of 11 seeds were produced in each siliques (n = 15). Thus, the rpg1 mutation could be propagated in a homozygous state, with all progeny exhibiting a mutant phenotype. To investigate the possible mechanism of this phenomenon, we examined the fertility of the mutant plants in different environments, including under drought, high humidity, and long-day or short-day illumination conditions. The variation of fertility in rpg1 appeared to be consistent under different conditions (data not shown).

Siliques with normal seed set were produced by cross-pollination of the mutant stigmas with wild-type pollen (data not shown), indicating that female reproductive development is not affected. Progeny of heterozygous mutant plants segregated fertile and mutant plants with an approximate 3:1 ratio (320:104), indicating that rpg1 is caused by a single, recessive, nuclear mutation.

Microsporogenesis of the rpg1 Mutant Is Aberrant after Meiosis

Detailed histological observations were performed to analyze the male reproductive developmental defects of rpg1. In rpg1 mutant plants, stamen development was impaired and few pollen grains were observed on the stigmas (Fig. 1, B and C). To examine the vitality of rpg1 pollen grains, we used Alexander’s stain, which distinguishes aborted pollen grains from normal ones. Most grains in the rpg1 flowers showed a very faint pink coloration (Fig. 1, D and E), indicating that they were not viable. Some grains showed a more intense pink coloration (Fig. 1, F), suggesting that they were viable but had not yet undergone tetrad formation.

The variation of fertility in rpg1 appeared to be consistent under different conditions (data not shown).
mature pollen grains (Alexander, 1969). Wild-type pollen grains were stained with purple color (Fig. 1D), suggesting that they had successfully completed early development (well-developed protoplasm of pollen grains). In contrast, we observed that green-stained pollen remnants filled the anther in rpg1 (Fig. 1E), which indicated that most of the pollen grains were aborted during early development (did not develop protoplasm). Consistent with the restoration of fertility, pollen grains from early initiated flowers were mostly stained green, whereas purple-stained pollen grains appeared to be increased in the anthers of later flowers along the axis (data not shown). To examine the male meiosis of rpg1, we performed chromosome spreading with 4'6-diamidino-2-phenylindole (DAPI) staining. The tetrads of rpg1 plants are indistinguishable from those of the wild type (Fig. 1, F–I), which suggested that nuclear division of male meiosis is not affected in the mutant.

We also generated anther cross sections to compare anther development of wild-type and rpg1 plants (Fig. 2). In Arabidopsis, anther development can be divided

![Figure 1. Characterization of the rpg1 mutant. A, Comparison of vegetative and reproductive development of 40-d-old wild-type (WT) and rpg1 mutant plants. B and C, Comparison of wild-type (B) and rpg1 (C) flowers. Note that very few pollen grains were observed on rpg1 stigma. D and E, Alexander's staining of wild-type (D) and rpg1 (E) anthers. The green-stained remnants in the rpg1 anther indicate pollen grain abortion. Bars = 20 μm. F and G, DAPI staining of wild-type (F) and rpg1 (G) meiocytes at the telophase I stage. Both show two chromosome clusters with five chromosomes. H and I, DAPI staining of wild-type (H) and rpg1 (I) meiocytes at the telophase II stage. Both show four decondensed chromosome clusters. In F to I, bars = 10 μm.](https://academic.oup.com/plphys/article/147/2/852/6107522)

![Figure 2. Anther development of wild-type and rpg1 plants. Locules from anther sections of wild-type (A, C, E, G, I, and K) and rpg1 (B, D, F, H, J, and L) plants are shown. A and B, Anthers at stage 7. Aniline blue staining of the callose wall is shown. C and D, Anthers at stage 8. rpg1 microspores were vacuolated and turgid compared with wild-type microspores. E and F, Anthers at stage 9. Note that rpg1 microspores were aborted with bumpy surfaces. G and H, Anthers at stage 10. The cytoplasm of most rpg1 microspores was shrunken and disintegrated. Arrows indicate granules in the rpg1 anther locule. I and J, Anthers at stage 11. Degenerated rpg1 microspores were seen. K and L, Anthers at stage 12. Malformed pollen grains and cell remnants were observed in rpg1 anther locule. DPG, Defective pollen grain; E, epidermis; En, endothecium; MSP, microspore; PG, pollen grain; RM, remnants of microspores; T, tapetum; Tds, tetrads. Bars = 10 μm. [See online article for color version of this figure.]](https://academic.oup.com/plphys/article/147/2/852/6107522)
into 14 well-ordered stages by morphological characteristics (Sanders et al., 1999). Until anther development stage 7, when tetrads of haploid microspores are formed, the mutant appeared to be comparable with wild-type plants in tetrad formation and callose secretion (Fig. 2, A and B). However, when the callose wall of tetrads degenerated, released microspores of \textit{rpg1} were distinguishable from those of the wild type. At stage 8, microspores were angular in shape in wild-type plants (Fig. 2C), whereas microspores of \textit{rpg1} plants appeared to be turgid and bumpy (Fig. 2D). At stage 9, microspores of wild-type plants generated the basic exine wall and became vacuolated (Fig. 2E). In contrast, in \textit{rpg1} plants, the cytoplasm of microspores was shrunken and disintegrated (Fig. 2F). Furthermore, granules were observed in the locule of the mutant (Fig. 2F). During stages 10 and 11 in the wild type, anther microspores underwent asymmetric mitotic divisions and generated a significant pollen wall (Fig. 2, G and I). In contrast, in \textit{rpg1}, most of the microspores were degenerated and the granules became more evident (Fig. 2, H and J). Eventually, pollen grains of the wild type were released following anther dehiscence (Fig. 2K), whereas most of the mutant microspores were aborted (Fig. 2L). Some of the microspores in the mutant managed to develop further; nevertheless, they were usually irregular in shape (Fig. 2L). The cell fate of the tapetum in \textit{RPG1} plants appeared to be unaffected, as tapetum differentiation and degeneration occurred in the same manner as in wild-type plants.

\textit{rpg1} Microspores Are Defective in Exine Pattern Formation and Cell Integrity

To further elucidate the mechanism of pollen degeneration of \textit{rpg1}, we compared the ultrastructure of microspore development in both wild-type and \textit{rpg1} plants by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examinations. Unlike the wild-type pollen grains (Fig. 3A), significant aberration of pollen grain and exine pattern formation was observed in \textit{rpg1} by SEM examination. Consistent with the description above, most of the pollen grains were aborted and collapsed in \textit{rpg1} plants (Fig. 3B). Moreover, in contrast to the reticulate exine pattern that was observed in wild-type pollen grains (Fig. 3C), those aborted pollen grains exhibited a spotted exine pattern (Fig. 3D). This indicated that the sporopollenin deposition and exine patterning were defective in the \textit{rpg1} mutant. Although a small portion of pollen grains appeared to be olive-shaped, as observed for wild-type plants, their exine patterning was often incomplete or flawed (Fig. 3E and F). In addition, fragments of membrane-like material, which usually encases the pollen grains, were observed to surround the anther locule (Fig. 3B). We also examined the pollen grains of heterozygous plants by SEM. The anther development and pollen grains appeared to be indistinguishable from those of the wild type (data not shown), which suggested that the abortion of pollen grains in \textit{rpg1} was caused by the defects in somatic development but not in male gametophytic development.

TEM demonstrated abnormal primexine deposition and rupture of microspores in \textit{rpg1} plants (Fig. 4). At the tetrad stage in wild-type plants, following the regular undulation of microspore plasma membrane, primexine matrix was deposited directly outside the microspore plasma membrane (Fig. 4A). In \textit{rpg1}, primexine was also deposited within the callose wall (Fig. 4B). However, the microspore was irregular in primexine deposition (Fig. 4B). At the released microspore stage, microspores of the wild type presented basic exine wall formation with bacula and tectum (Fig. 4C). In contrast, the tectum was absent and the bacula was malformed in \textit{rpg1} microspores (Fig. 4D).
Moreover, the microspore plasma membrane and pollen wall were bumpy and wavy in \textit{rpg1} (Fig. 4F), which led to breakage of microspores at a later stage (Fig. 4F). Following the rupture of microspores, cytoplasmic leakage of microspores was observed, and the intine layer was not formed (Fig. 4F). When pollen wall development was complete, a wild-type pollen grain was surrounded by an intine layer, an exine layer, and a pollen coat (Fig. 4G). However, pollen grains of the mutant were mostly collapsed and emptied, with no intine layer and an aberrant exine layer. While a small portion of microspores appeared to complete microsporogenesis and microgametogenesis, the exine pattern formation was usually abnormal, with irregular bacula and absence of tectum (Fig. 4H).

Tapetum development of the wild type and mutant was comparable (data not shown), whereas lipid accumulation was aberrant in \textit{rpg1}. Unlike the wild type (Fig. 4I), electron-dense granules that aggregated onto the locule wall were also observed in \textit{rpg1} (Fig. 4J), which may be responsible for the membrane-like material in SEM examination (Fig. 3B).

\textbf{RPG1 Encodes an MtN3/saliva Family Protein That Localizes to the Plasma Membrane}

To identify the corresponding \textit{RPG1} gene, a genomic DNA fragment that flanked the left border of T-DNA was recovered by thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995). Sequencing of the TAIL-PCR products suggested that the T-DNA was inserted in the last intron of a predicted open reading frame (At5g40260; Fig. 5A). PCR analysis with T-DNA and genome-specific primers indicated that all mutant plants analyzed were homozygous for the insertion (data not shown). Because the T-DNA insertion appeared to only affect the last intron and exon of \textit{RPG1}, we performed reverse transcription (RT)-PCR with different primer sets to examine the expression of At5g40260 in \textit{rpg1} plants. The result showed that the expression of the 5' genomic region of the gene was not affected, while no expression of the 3' region was detected (Fig. 5C). Genetic complementation was then performed to validate the results. A 3.6-kb DNA fragment, which included the genomic sequences of At5g40260 and 1.6-kb sequences upstream from the initiation codon, was cloned from wild-type Arabidopsis and introduced into the homozygous mutant plants. Twenty of 21 transgenic plants showed normal fertility. PCR analysis verified that these plants were homozygous for the \textit{rpg1} mutation with the transformed At5g40260 DNA fragment (data not shown). These results verified that At5g40260 is \textit{RPG1} and that the 3.6-kb genomic region is sufficient for \textit{RPG1} function.

In addition, we obtained three individual \textit{rpg1} alleles from the SIGnAL collection at the Arabidopsis
Biological Resource Center: \( rpg1-1 \) (SALK-142803), \( rpg1-2 \) (SALK-092239), and \( rpg1-3 \) (SALK-062567; Fig. 5A). The three T-DNA-tagged lines were verified by PCR. Both \( rpg1-1 \) and \( rpg1-2 \) showed a similar phenotype with that of \( rpg1 \) (Supplemental Fig. S1). In \( rpg1-3 \), the fertility of mutant plants was moderately affected. SEM analysis showed that pollen grains of \( rpg1-3 \) were shrunched, but exine formation of the pollen grains resembled that of the wild-type pollen grains (Supplemental Fig. S1H). RT-PCR analysis revealed that the \( RPG1 \) gene was moderately knocked down in \( rpg1-3 \), which may have resulted in the partial function of \( RPG1 \) (data not shown).

To verify the primary structure of the \( RPG1 \) gene, its full-length genomic DNA and cDNA were cloned and sequenced. Genomic sequences of the \( RPG1 \) gene are 1,686 bp in length and comprise six exons and five introns, which is consistent with the gene predictions in the National Center for Biotechnology Information (NCBI) database (Gi: 22328163). There are two putative gene models of At5g40260 in the NCBI database that differ in the predicted splice site of the last intron (At5g40260.1, Gi: 26451731; At5g40260.2, Gi: 79329352). The cDNA we cloned from inflorescences is identical to At5g40260.1. Furthermore, RT-PCR with At5g40260.1- and At5g40260.2-specific primers showed that only At5g40260.1 cDNA could be amplified from inflorescence cDNA (data not shown). Therefore, we chose At5g40260.1 mRNA and protein sequences for further analyses.

The \( RPG1 \) mRNA encodes an unknown protein of 209 amino acids with a putative molecular mass of 27 kD. Domain analysis showed that \( RPG1 \) protein is an integral membrane protein with seven putative transmembrane helices (Fig. 5B) and contains two copies of the MtN3/saliva domain (Fig. 6C). This domain was originally identified in root nodulin-related proteins of the legume \textit{Medicago truncatula} (Gamas et al., 1996).
and in the saliva protein of *Drosophila melanogaster* (Artero et al., 1998). MtN3/saliva family proteins are prevalent in eukaryotes, including yeast, mammals, and plants (data from the Pfam database, http://pfam.sanger.ac.uk/).

Topology prediction indicated that RPG1 is an integral membrane protein (Fig. 5B), whereas the exact subcellular localization was unclear. Therefore, we constructed a protein fusion in which the GFP was fused to the C terminus of RPG1. The chimeric protein was then introduced into wild-type plants under the control of the cauliflower mosaic virus 35S promoter. GFP fluorescence of transgenic plants was observed under confocal microscopy. The fluorescence of the RPG1:GFP protein was located strictly in the plasma membrane region of epidermal cells (Fig. 5D) and protoplast cells of transgenic plants (Fig. 5E) and was separated from the cell wall when the epidermal cells were plasmolysed by 0.8 M mannitol (Fig. 5D). These results demonstrated that RPG1 is a plasma membrane-localized protein.

According to the Pfam database (http://pfam.sanger.ac.uk/Software/Pfam/), there are 18 putative MtN3/saliva family genes in Arabidopsis. Phylogenetic analysis showed that RPG1 solely forms a distinct clade in the phylogenetic tree of the AtMtN3/saliva family, which indicates that RPG1 may play a distinct role in Arabidopsis (Fig. 6A). Furthermore, the homologues of RPG1 protein have also been identified in various plant species by BLASTp or tBLASTn search in the NCBI database and The Institute for Genomic Research functional genome database, including rice (*Oryza sativa*), grape (*Vitis vinifera*), poplar (*Populus trichocarpa*), maize (*Zea mays*), alfalfa (*Medicago sativa*), tomato (*Solanum lycopersicum*), lily (*Lilium longiflorum*), petunia (*Petunia hybrida*), and castor bean (*Ricinus communis*). For example, the homolog from rice, OsL_002726, shared highest identity (50%) and similarity (68%) with RPG1. In petunia, NEC1, an anther dehiscence-associated protein (Ge et al., 2000, 2001), shared identity of 34% and similarity of 58% with RPG1. In lily, LIM7, which was previously reported to be induced in meiotic prophase in microsporocytes (Kobayashi et al., 1994), showed identity of 45% and similarity of 63% with RPG1 over a 159-amino acid region. An alignment with these protein sequences was performed by ClustalW (Fig. 6, B and C). Residues in the predicted MtN3/saliva domains are conserved among these proteins. Moreover, the predicted intracellular residues (TKSVYMFPF) in the second MtN3/saliva domain of RPG1 are highly conserved. Furthermore, when we modeled the putative functional motifs of the RPG1 protein with a motif scan tool (http://scansite.mit.edu/motifscan_seq.phtml), the two conserved intracellular regions in each MtN3/saliva domain exhibited potential motifs, each of which includes a Ser residue (Fig. 6C). Unexpectedly, the C-terminal intracellular tail, which appeared to be essential for RPG1 function, as T-DNA ablation of this region leads to a mutant phenotype, is highly variable among the proteins.

**Expression Pattern of RPG1**

To determine the expression pattern of RPG1, we performed semiquantitative RT-PCR to analyze its expression levels in the root, stem, rosette leaf, inflorescence, and 7-d-old seedling. RPG1 is preferentially expressed in inflorescence and slightly expressed in stem. In contrast, RPG1 mRNA was barely detectable in other organs (Fig. 7A). This result is consistent with a previous study, in which RPG1 (At5g40260) was reported to be expressed preferentially in male and female gametophytes (Yu et al., 2005). Therefore, we performed RNA in situ hybridization experiments to study the precise expression pattern during anther development. RPG1 transcript was initially detected at anther development stage 4 (Fig. 7B), and the signal was increased significantly in tapetal cells and microsporocytes (or meiocytes) during meiosis (Fig. 7, C and D). After stage 7, the expression of RPG1 was still detectable in tapetum and microspores, but it became less than that at the earlier stages (Fig. 7E). When the tapetum completely degenerated at stage 12, RPG1 transcript was detectable in pollen grains only (Fig. 7F).

**DISCUSSION**

**RPG1 Is Crucial for Exine Pattern Formation and Microspore Cell Integrity in Arabidopsis**

We have characterized an MtN3/saliva family gene, RPG1, that is essential for microspore cell integrity and primexine pattern formation in Arabidopsis. In the *rpg1* mutant, the formation of the microspore plasma membrane is affected. As the primexine deposition is abnormal at the tetrad stage, exine pattern formation of the microspores is severely impaired. Moreover, microspores are mostly ruptured and aborted during postmeiotic development. RPG1 exhibits its strongest expression in microsporocytes (or meiocytes) and tapetum during microsporogenesis, which confirms the important role of this gene in the early determination of microspore formation and exine pattern formation.

It has been well documented that *nef1*, *dex1*, and *cals5* mutants are also defective in primexine patterning during the tetrad stage. Primexine was completely absent in *nef1*, and sporopollenin failed to deposit on the microspore wall (Arizumi et al., 2004), whereas the microspore plasma membrane was barely undulated and primexine deposition was affected at the tetrad stage in *dex1* (Paxson-Sowders et al., 1997, 2001). Aberrant lipid accumulation on the locule wall, which was observed in *rpg1*, was also observed in *dex1* and *nef1* (Paxson-Sowders et al., 1997; Arizumi et al., 2004). In *cals5*, primexine pattern defects were caused by the insufficient callose synthesis, and the lipid accumulation defect was not observed in the mutant (Dong et al., 2005; Nishikawa et al., 2005). In *rpg1*, abnormal primexine development indicates that the mutant is defective in the early determination of exine...
pattern formation, which is consistent with the expression pattern of the RPG1 gene.

Another common characteristic of rpg1 and other exine pattern mutants (e.g. ms1, ms2, nef1, dex1, and cals5) is the postmeiotic degeneration of microspores (Aarts et al., 1997; Paxson-Sowders et al., 1997; Wilson et al., 2001; Ariizumi et al., 2004; Dong et al., 2005). Our study demonstrated that microspore abortion in rpg1

**Figure 6.** Phylogenetic analysis of MtN3/saliva family and RPG1 homologous proteins. A, Unrooted phylogenetic tree of the MtN3/saliva family in Arabidopsis. Amino acid sequences of MtN3/saliva family proteins were analyzed by the neighbor-joining method with genetic distance calculated by MEGA3.1. The numbers at the nodes represent percentage bootstrap values based on 1,000 replications. The length of the branches is proportional to the expected numbers of amino acid substitutions per site, with a scale provided at the bottom of the tree. The arrow indicates RPG1 (At5g40260). B, Unrooted phylogenetic tree of RPG1 and homologous proteins. Protein sequence files are as follows: Pt, *Populus trichocarpa*, LG_I2590; Rc, *Ricinus communis*, 29822.m003348; Os, *Oryza sativa*, Os01g0605700; LIM7, *Lilium longiflorum*, BAA04837; Sl, *Solanum lycopersicum*, BAA04837; Vv, *Vitis vinifera*, CAN64755; Zm, *Zea mays*, AZM5_12004; MtN3, *Medicago truncatula*, CAA69976; NEC1, *Petunia hybrida*, AAG34696. C, Multiple alignments of RPG1 and homologous proteins. Boxes, Similar to MtN3/saliva domain; black bars, putative transmembrane regions; gray bars, conserved intracellular regions of each domain; black dots, conserved Ser that was predicted to be a phosphorylation site. Sequences were aligned using ClustalW and displayed using BOXSHADE (www.ch.embnet.org/software/BOXform.html).
is apparently due to the rupture of microspores. This indicated that RPG1 is required for the cell integrity of microspores. Generally, plant cells are bound by a rigid cell wall that prevents cellular migration and maintains cellular integrity (Martin et al., 2001). Previous studies have shown that reduced cell wall strength leads to altered cell formation and integrity. For example, a cellulose synthase-like gene, AICS LD3 (KOJAK), is required for root hair formation. Mutations of KOJAK/AICS LD3 result in root hairs that initiate bulge formation but then rupture at their tips (Favery et al., 2001; Wang et al., 2001). In microsporogenesis, a common cell wall is replaced by callose in microspores during meiosis, and exine and intine layers are subsequently formed as the pollen cell wall to protect pollen grains (Heslop-Harrison, 1971). In the rpg1 mutant, plasma membrane formation and pollen wall development of microspores are severely impaired beginning at the tetrad stage. Therefore, the rupture of microspores may be due to the reduced integrity of the plasma membrane and pollen wall. In the weak mutant allele rpg1-3, pollen grains are usually irregular in formation, whereas exine patterning is not severely affected (Supplemental Fig. S1H). This indicates that microspore cell integrity, rather than pollen wall formation, tends to be affected by RPG1 lesions. Our results indicate that proper development of the plasma membrane and pollen wall may be required for the maintenance of microspore cell integrity.

rpg1 is partially fertile later in reproductive development, and apparently pollen development is gradually rescued. A similar phenotype was also reported in ms2 and atgpat1 mutants, although the precise mechanism was unclear (Aarts et al., 1997; Zheng et al., 2003). To investigate the possible mechanism of this phenomenon, we examined the fertility of the mutant plants in different environments, including under drought, high humidity, and different illumination conditions. Unexpectedly, the recovery of fertility in the mutant plants appeared to be consistent (data not shown), which indicated that the rescued pollen development might be caused by some endogenous factors. We speculate that some genes might be expressed in late development (e.g. senescence-induced genes) and could partially complement the function of RPG1.

A previous report demonstrated that RPG1 (At5g40260) is also highly expressed in the embryo sac of the ovule, but an examination of two T-DNA insertion mutant lines, SALK_137176 and SALK_092239 (rpg1-2), suggested no observable phenotype in the embryo sac (Yu et al., 2005). Our results showed that the female fertility of rpg1 and allele mutant lines is indistinguishable from that of the wild type (data not shown). In addition, RPG1 expression in the SALK_137176 line is not significantly affected (data not shown). Therefore, the functional role of RPG1 in female gametophytes may be unnecessary or redundant with other proteins, as proposed previously (Yu et al., 2005).

**RPG1 Encodes an MtN3/saliva Family Protein**

RPG1 is a member of the MtN3/saliva gene family, which exists in a wide variety of eukaryotes. Members of this family usually contain two copies of an MtN3/saliva domain (Gamas et al., 1996; Artero et al., 1998). The MtN3/saliva domain was originally found in the legume *M. truncatula* and the saliva protein of *D. melanogaster* (Gamas et al., 1996; Artero et al., 1998). In humans, a member of the MtN3/saliva family, Rga (for Recombination-activating gene1 gene activation), was found to facilitate the gene activation of *Recombination-activating gene1* (Tagoh et al., 1996). In *Ciona intestinalis*, knockdown of CiRga, an ortholog of mouse Rga, resulted in abnormal embryos in which the cleavage pattern became atypical and the expression of marker genes was suppressed at the tailbud stage (Hamada et al., 2005). In plants, reported MtN3 family genes are usually related to reproductive development. In petunia, the NEC1 gene is expressed preferentially in nectaries and stamens (Ge et al., 2000).
Partial silencing of NEC1 resulted in premature dehiscence of anthers and reduced fertility (Ge et al., 2001). In rice, an MtN3/saliva gene, Os8N3, was characterized to be a host susceptibility gene for bacterial blight targeted by the type III effector. Also, Os8N3 appeared to be required by inflorescence development, as loss of function of this gene affected pollen development and other floral tissues (Yang et al., 2006).

Recently, the Rga protein was revealed to be associated with the ion channel protein TRPV2 in a rat mast cell line (Barnhill et al., 2004; Stokes et al., 2005). TRPV2 is a member of the TRPV (vanilloid receptor-related) subfamily of the TRP (for Transient Receptor Potential) family. Ion channels of the TRP family respond to diverse cellular stimuli, including variations across a physiological and pathophysiological temperature range (Jin et al., 2006). The Rga protein may be localized to a vesicular subcompartment of the endoplasmic reticulum/Golgi apparatus and interact with TRPV2 intracellularly. The interaction depends on a cellular glycosylation event, suggesting that Rga may play a chaperone or targeting role for TRPV2 during the maturation of the ion channel protein (Barnhill et al., 2004; Stokes et al., 2005). The function of MtN3/saliva proteins in plants may be different from that in mammals, as the TRP family does not exist in plants. Moreover, the conserved Asn residues in mammalian MtN3/saliva proteins, suggested to be possible N-glycosylation sites, are absent in characterized plant MtN3/saliva proteins (data not shown). Nevertheless, this discovery provides us a clue that MtN3/saliva proteins may function through interaction with other membrane proteins.

**Putative Role of RPG1 in Microsporogenesis**

Our results indicated that the predicted RPG1 intracellular regions are essential for its function. By phylogenetic analysis, we found that the putative intracellular region of the MtN3/saliva domain is highly conserved in RPG1 and other MtN3/saliva proteins (Fig. 6C). In addition, it is worth noting that the highly conserved intracellular region in the MtN3/saliva domain presents putative phosphorylation motifs in RPG1 (Fig. 6C), which indicates a potential role of RPG1 in protein regulatory networks. The C-terminal tail of RPG1, which is also predicted to be intracellular, appears not to be conserved in our analysis. However, ablation effect of T-DNA insertion in the RPG1 gene indicated that this region is essential for RPG1 function. In the two strong mutant alleles, rpg1 and rpg1-2, T-DNA insertion sites are in the last intron and fifth exon, respectively. RT-PCR showed that in the rpg1 mutant the T-DNA insertion mainly affects the transcription of the last exon of RPG1, which corresponds to the C-terminal tail of RPG1 function. There are two possible reasons why the important C-terminal region is not conserved in our phylogenetic analysis. First, this may be due to the incomplete genome information for other species. The importance of the C terminus may be specific to RPG1 in Arabidopsis and closely related species (e.g. in Brassica napus), and the absence of orthologous proteins in the phylogenetic analysis may lead to false-negative results. Alternatively, the conservation of the C terminus may not be present at the sequence level but rather at the topological level.

In conclusion, our results demonstrate that (1) ablation of RPG1 causes aberrant cell integrity and exine patterning of microspores; (2) RPG1 is localized to the plasma membrane in microspores and tapetum; and (3) the predicted intracellular regions are essential for RPG1 function. Based on these results, we can infer two possible roles of RPG1 in microsporogenesis. First, RPG1 may play a role in maintaining the plasma membrane integrity of microspores by regulating membrane traffic, such as protein maturation or activation. Ablation of RPG1 results in disrupted integrity and abnormal invagination of the microspore plasma membrane, which in turn leads to the rupture of microspores and impaired exine pattern formation. Alternatively, RPG1 may regulate the timely undulation of the microspore plasma membrane, which is required for primexine patterning. Lesions of RPG1 affect the proper undulation of the plasma membrane and result in severely disrupted primexine patterning. Without the protection of a basic exine layer, the developing microspore with a bumpy plasma membrane tends to burst in the stressful condition of the pollen sac.

Although the characterization of the rpg1 mutant provides insights into the role of the plasma membrane protein in pollen wall development, even more questions arise about the RPG1 protein. Which proteins do RPG1 interact with on the plasma membrane? Why is RPG1 expressed highly in both tapetum and microspores? What are the roles of other MtN3/saliva proteins in Arabidopsis? Investigation of these questions will provide further insights into the molecular basis of microsporogenesis and the roles of MtN3/saliva proteins in plant development.

**MATERIALS AND METHODS**

**Plant Growth and Mutant Isolation**

Arabidopsis (Arabidopsis thaliana) plants used in this study are in the Columbia-0 background. Seeds were sown on vermiculite and allowed to imbibe for 3 d at 4°C. Plants were grown under long-day conditions (16 h of light/8 h of dark) in an approximately 22°C growth room. The rpg1 mutant was characterized from the pSK115 activation-tagging T-DNA mutant pools (Qin et al., 2003).

**Phenotype Characterization and Microscopy**

Plants were photographed with a Canon digital camera (Powershot-A710IS). Flower images were taken using an Olympus dissection microscope with an Olympus digital camera. Alexander solution and DAPI staining were performed as described (Alexander, 1969; Ross et al., 1996). Cross section and callose staining were performed as described previously (Zhang et al., 2007). Photography was performed with an Olympus BX-51 microscope. For SEM examination, fresh stamens and pollen grains were coated with 8 nm of gold and observed on a JSM-840 microscope (JEOL). For TEM...
examination, Arabidopsis buds from the inflorescence were fixed and embedded as described (Zhang et al., 2007). Ultrathin sections (90–100 nm thick) were observed with a JEM-1230 transmission electron microscope (JEOL).

**TAIL-PCR and Molecular Cloning of the RPG1 Gene**

The presence of T-DNA insertion in the mutant was validated using primers that specifically amplify the BAR gene of T-DNA (Bar-F, 5'-CGAACCTATCCGCTGAACTTCT-3' and Bar-R, 5'-TTTTACGGTCTCCCTGCTG3'); TAIL-PCR, T-DNA left border primers (AtLB1, 5'-AGATCTGAACCCTCTCCGTAGCAGAATCTCAAC-3' and AtLB2, 5'-TAATAACGCTGCGGACATCTAC-3') and genomic DNA of mutant plants were used. The TAIL-PCR procedure and arbitrary degenerate primers were as described (Liu et al., 1995). Cosegregation of the T-DNA insertion site and mutant phenotype were analyzed with AtLB3 and plant-specific primers (LP, 5'-CTCCGTAACGAGAATCTCAAC-3' and RP, 5'-TTTTACGGTCTCCCTGCTG3'). For mutant plants, only PCR with AtLB3 and RP primers could amplify a DNA fragment of about 700 bp. For wild-type plants, only PCR with LP and RP primers could amplify a DNA fragment of 760 bp. For heterozygous mutant plants, PCR with both primer pairs showed positive results.

For complementation, a DNA fragment of 3.6 kb including 1.6 kb upstream and 1 kb downstream sequences was amplified using LA-Taq polymerase (Takara Biotechnology; CMP-F, 5'-ATACGACGGATCGGCTGCGAATACTCCTGCATG-3' and CMP-R, 5'-CGAACCTATCCGCTGAACTTCT-3'). For TAIL-PCR, T-DNA left border primers (AtLB1, 5'-ATACGACGGATCGGCTGCGAATACTCCTGCATG-3' and AtLB2, 5'-TAATAACGCTGCGGACATCTAC-3') and genomic DNA of mutant plants were used. The TAIL-PCR procedure and arbitrary degenerate primers were as described (Liu et al., 1995). Cosegregation of the T-DNA insertion site and mutant phenotype were analyzed with AtLB3 and plant-specific primers (LP, 5'-CTCCGTAACGAGAATCTCAAC-3' and RP, 5'-TTTTACGGTCTCCCTGCTG3'). For mutant plants, only PCR with AtLB3 and RP primers could amplify a DNA fragment of about 700 bp. For wild-type plants, only PCR with LP and RP primers could amplify a DNA fragment of 760 bp. For heterozygous mutant plants, PCR with both primer pairs showed positive results.

For complementation, a DNA fragment of 3.6 kb including 1.6 kb upstream and 1 kb downstream sequences was amplified using LA-Taq polymerase (Takara Biotechnology; CMP-F, 5'-ATACGACGGATCGGCTGCGAATACTCCTGCATG-3' and CMP-R, 5'-CGAACCTATCCGCTGAACTTCT-3'). For TAIL-PCR, T-DNA left border primers (AtLB1, 5'-ATACGACGGATCGGCTGCGAATACTCCTGCATG-3' and AtLB2, 5'-TAATAACGCTGCGGACATCTAC-3') and genomic DNA of mutant plants were used. The TAIL-PCR procedure and arbitrary degenerate primers were as described (Liu et al., 1995). Cosegregation of the T-DNA insertion site and mutant phenotype were analyzed with AtLB3 and plant-specific primers (LP, 5'-CTCCGTAACGAGAATCTCAAC-3' and RP, 5'-TTTTACGGTCTCCCTGCTG3'). For mutant plants, only PCR with AtLB3 and RP primers could amplify a DNA fragment of about 700 bp. For wild-type plants, only PCR with LP and RP primers could amplify a DNA fragment of 760 bp. For heterozygous mutant plants, PCR with both primer pairs showed positive results.

For complementation, a DNA fragment of 3.6 kb including 1.6 kb upstream and 1 kb downstream sequences was amplified using LA-Taq polymerase (Takara Biotechnology; CMP-F, 5'-ATACGACGGATCGGCTGCGAATACTCCTGCATG-3' and CMP-R, 5'-CGAACCTATCCGCTGAACTTCT-3'). For TAIL-PCR, T-DNA left border primers (AtLB1, 5'-ATACGACGGATCGGCTGCGAATACTCCTGCATG-3' and AtLB2, 5'-TAATAACGCTGCGGACATCTAC-3') and genomic DNA of mutant plants were used. The TAIL-PCR procedure and arbitrary degenerate primers were as described (Liu et al., 1995). Cosegregation of the T-DNA insertion site and mutant phenotype were analyzed with AtLB3 and plant-specific primers (LP, 5'-CTCCGTAACGAGAATCTCAAC-3' and RP, 5'-TTTTACGGTCTCCCTGCTG3'). For mutant plants, only PCR with AtLB3 and RP primers could amplify a DNA fragment of about 700 bp. For wild-type plants, only PCR with LP and RP primers could amplify a DNA fragment of 760 bp. For heterozygous mutant plants, PCR with both primer pairs showed positive results.

**Phylogenetic Analysis**

The multiple sequence alignment of full-length protein sequences was performed using the ClustalW tool online (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed and performed using the ClustalW tool online (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed and performed using the ClustalW tool online (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed and performed using the ClustalW tool online (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed and performed using the ClustalW tool online (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed and tested by MEGA3.1 based on the neighbor-joining method.

**Expression Analysis**

For expression analysis of RPG1 in mutant, RNA was extracted from inflorescences of mutant and wild-type plants using TRizol (Invitrogen). Semi-quantitative RT-PCR for 30 cycles was used to assess the levels of expression of RPG1 and alleles, using primer sets as follows: RPG1-F (5'-ATGGTTGATGCAAAACAAGTTCTG-3') and RPG1-R (5'-AACCACAG-GAGACCGTTA-3') were used to examine 5' region expression (exons 1–3), and LP and RP (see above) were used for 3' region expression analysis (exons 3–5).

For RT-PCR, RNA was extracted from root, rosette leaves, 14-d-old seedlings, and inflorescences. PCR was performed by LP/RP primer set. In situ hybridization was performed with the DIG (digoxigenin) RNA Labeling Kit (Roche) and the DIG Probe Synthesis Kit (Roche). An RPG1-specific cDNA fragment of 458 bp was amplified and cloned into the pSK vector. Antisense and sense digoxigenin-labeled probes were prepared with EcoRI or BamHI digestion and in vitro transcription using T3 or T7 RNA polymerase, respectively.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP_588579, BAA05436, CAE47577, CAN64755, AZM3_12004, CA609976, and AAC54696.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Phenotype of rpg1 mutant alleles.

**ACKNOWLEDGMENTS**

We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants. We thank Xiao-yan Gao from Shanghai Institute of Plant Physiology and Ecology and Hui-qi Zhang from Shanghai Normal University for their help with SEM and TEM. We are grateful to Dr. Gareth H Jones from the University of Birmingham for kindly suggestions on DAPI staining.

Received February 21, 2008; accepted April 16, 2008; published April 23, 2008.

**LITERATURE CITED**


RPG1 Is Required for Pollen Wall Development in Arabidopsis


