

VANGUARD1 Encodes a Pectin Methylesterase That Enhances Pollen Tube Growth in the Arabidopsis Style and Transmitting Tract

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In flowering plants, penetration of the pollen tube through stigma, style, and transmitting tract is essential for delivery of sperm nuclei to the egg cells embedded deeply within female tissues. Despite its importance in plant reproduction, little is known about the underlying molecular mechanisms that regulate the navigation of the pollen tube through the stigma, style, and transmitting tract. Here, we report the identification and characterization of an *Arabidopsis thaliana* gene, *VANGUARD1* (*VGD1*) that encodes a pectin methylesterase (PME)-homologous protein of 595 amino acids and is required for enhancing the growth of pollen tubes in the style and transmitting tract tissues. *VGD1* was expressed specifically in pollen grain and the pollen tube. The *VGD1* protein was distributed throughout the pollen grain and pollen tube, including the plasma membrane and cell wall. Functional interruption of *VGD1* reduced PME activity in the pollen to 82% of the wild type and greatly retarded the growth of the pollen tube in the style and transmitting tract, resulting in a significant reduction of male fertility. In addition, the *vgd1* pollen tubes were unstable and burst more frequently when germinated and grown on in vitro culture medium, compared with wild-type pollen tubes. Our study suggests that the *VGD1* product is required for growth of the pollen tube, possibly via modifying the cell wall and enhancing the interaction of the pollen tube with the female style and transmitting tract tissues.

INTRODUCTION

The fertilization of flowering plants requires delivery of the sperm to the egg. The process begins with deposition of pollen grains on the stigmatic tissue. The compatible interaction between pollen and stigmatic cells triggers the hydration and germination of the pollen. The resulting pollen tube invades the stigmatic tissue, penetrates the style, navigates through the transmitting tract, and then is directed to the egg embedded in the ovule (Hülkamp et al., 1995b; Wilhelmi and Preuss, 1996; Ray et al., 1997; Lennon and Lord, 2000; Palanivelu and Preuss, 2000; Johnson and Preuss, 2002; Lord and Russell, 2002; Preuss, 2002; Kim et al., 2003; Palanivelu et al., 2003). Several studies have revealed that components such as lipids on pollen and

stigma are important for transportation of water from stigmatic cells to pollen (Elleman et al., 1992; Preuss et al., 1993; Hülkamp et al., 1995a; Wolters-Arts et al., 1998; Zinkl et al., 1999; Fiebig et al., 2000). The morphological studies also show that a compatible interaction between pollen tubes and stigmatic cells is required for triggering degradation of the stigmatic and stylar cell walls (Atkinson et al., 1993; Hiscock et al., 1994; Johnson and Preuss, 2002; Lord and Russell, 2002). However, the genetic and molecular mechanisms that control the penetration of pollen tubes through stigmatic and stylar tissues are poorly understood.

In plant species such as lily (*Lilium longiflorum*) with a hollow stigma and style, adhesion between the pollen tube and transmitting tract epidermis is required for growth of the pollen tube toward ovule. The stylar matrix secreted by the transmitting tract epidermis also has been suggested to play an important role in such adhesion (Jauh et al., 1997; Lord, 2000, 2001; Lord and Russell, 2002; Kim et al., 2003; Park and Lord, 2003). Similarly, in species such as *Arabidopsis thaliana* with closed style, the intercellular space of the transmitting tract is filled completely with the nutrient-rich extracellular matrix (ECM). The pollen tube grows faster, longer, and more precisely through ECM than in vitro, indicating that the interaction between pollen tube and the transmitting tract ECM is important for the mobilization of pollen tubes in the transmitting tract (Lennon et al., 1998; Lennon and Lord, 2000; Lord, 2000). Nevertheless, despite their importance

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for the delivery of the sperm to the egg, little is known about the underlying molecular mechanisms that regulate the interaction of pollen tubes with female floral tissues.

Pectin methylesterases (PMEs) have been implicated to play roles in different physiological processes of plant development via modification of the cell wall (Bordenave and Goldberg, 1993; Kagan-Zur et al., 1995; Guglielmino et al., 1997; Wakeley et al., 1998; Futamura et al., 2000; Micheli et al., 2000; Ren and Kermodé, 2000; Li et al., 2002). The plant cell wall is a polymeric network of crystalline cellulose microfibrils embedded in a hydrophilic matrix of hemicelluloses and pectin (Denés et al., 2000). PMEs contribute to cell development by regulating the mechanical and chemical properties of plant cell walls via demethylesterification of cell wall pectin. Changes in the mechanical strength and rigidity of the pollen tube wall also have been proposed to be important for the process of pollen tube elongation and its interaction with female floral tissues (Franklin-Tong et al., 1996; Holdaway-Clarke et al., 1997; Franklin-Tong, 1999). Furthermore, several studies have led to the identification of pollen tube-specific PMEs, indicating that plant PMEs may be involved in the processes of pollen tube development and its interaction with female floral tissues (Wakeley et al., 1998; Futamura et al., 2000; Li et al., 2002). However, the actual biological functions of the pollen tube-specific PMEs have not been elucidated. Here, we report the identification and characterization of a novel *Arabidopsis* PME mutation, *vanguard1* (*vgd1*), that significantly retards growth of pollen tubes in the style and transmitting tract. The *VGD1* gene encodes a PME-homologous protein of 595 amino acids and is expressed specifically in the pollen grain and pollen tube. This study suggests that *VGD1* plays an important role in the growth of pollen tubes in the female floral tissues, possibly via modifying the cell wall and thus enhancing the interaction of the pollen tube with the female style and transmitting tract.

RESULTS

Isolation of the *vgd1* Mutant

The *vgd1* mutant was identified by its reduced fertility in a phenotypic screen of the enhancer-trap *dissociation* (*Ds*) insertion lines in *Arabidopsis* ecotype *Landsberg erecta* (Sundaresan et al., 1995). The homozygous *vgd1* plant produced smaller and shorter siliques with fewer seeds (Figures 1A and 1B) compared wild-type plants (Figures 1C and 1D). No morphological abnormality in other floral and vegetative parts was observed (Figures 1E and 1F).

vgd1 Was a Male Gametophytic Mutation That Cosegregated with a Single *Ds* Insertion

To investigate if the *vgd1* mutation affected male or female function, the *vgd1* mutant plant was used as male or female to cross with wild-type plants. When a homozygous *vgd1* plant was used as a female in a cross with a wild-type plant, nearly all ovules (449 out of 455) were able to produce viable seeds as those in the wild type, indicating that the *vgd1* mutation did not affect female

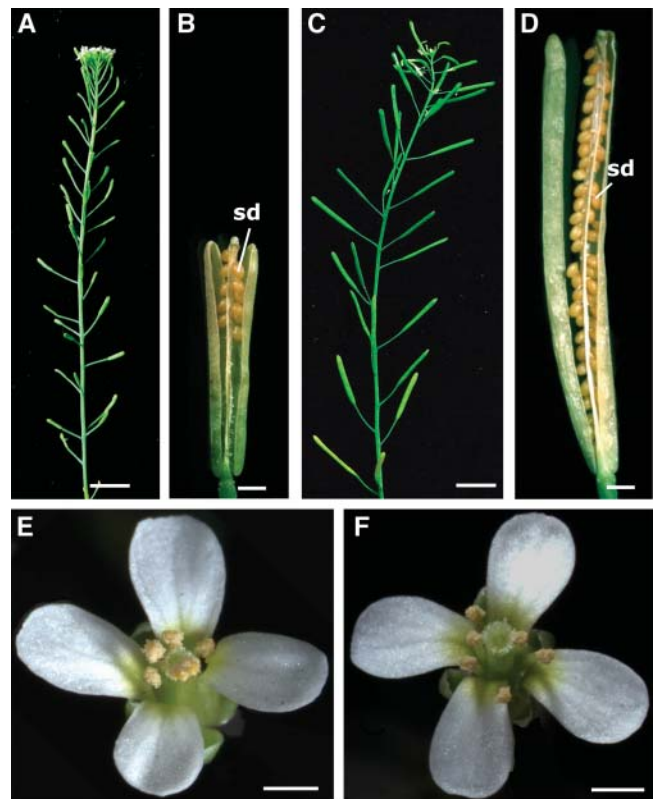


Figure 1. Phenotype of the *vgd1* Mutant Compared with the Wild Type.

- (A) A *vgd1* mutant plant with reduced fertility indicated by the smaller siliques with a few seeds.
 (B) A *vgd1* silique showing that the seeds were produced only in the upper part of the silique.
 (C) A wild-type plant with full fertility indicated by the siliques with a full seed set.
 (D) A wild-type silique showing the full seed set.
 (E) A *vgd1* flower with normal floral organs and pollen grains.
 (F) A wild-type flower with normal floral organs and pollen grains.
 sd, seeds. Bars = 1 cm in (A) and (C) and 1 mm in (B) and (D) to (F).

fertility. In a reciprocal cross, however, only a few ovules in the upper part of the silique were fertilized and developed into seeds. When a heterozygous *vgd1* plant was used as a male in a cross with wild-type plants, the F1 seedlings segregated 1:40.8 (80:3261) kanamycin resistant to kanamycin sensitive instead of the expected 1:1 segregation. This result indicated that the *vgd1* mutation led to a male gametophytic defect. In summary, the *vgd1* mutation resulted in defects of male gametophytic function but did not affect female gametophytic function.

When a heterozygous *vgd1* plant was used as a female in a cross with a wild-type plant, the F1 seedlings showed an average segregation ratio of approximately 1:1 (321:316) kanamycin resistant (*Kan^r*) to kanamycin sensitive (*Kan^s*). All *Kan^r* F1 seedlings gave rise to fully fertile plants. All F2 families resulting from the selfing of *Kan^r* F1 plants showed segregation of the *vgd1* mutant phenotype. When a homozygous *vgd1* plant was used as a female in a cross with a wild-type plant, all F1 seedlings were

resistant to kanamycin (Kan^r) upon germination and fully fertile when matured. All resulting F2 families also showed segregation of the *vgd1* mutant phenotype. These results showed tight linkage of a single recessive *vgd1* mutation to the kanamycin selection marker carried by a single *Ds* element in the *vgd1* genome. We further confirmed a single *Ds* insertion with DNA gel blotting hybridization using a 755-bp *Ds* 5'-end fragment as a probe (data not shown).

The *vgd1* Mutation Retarded Growth of the Pollen Tube in the Style and Transmitting Tract

Genetic studies showed that only a few seeds were produced in the upper part of wild-type or *vgd1* siliques when pollinated with

the *vgd1* pollen. To investigate the cause of the abnormal seed set, we compared the growth rate of *vgd1* pollen tubes in the style and transmitting tract to that of wild-type pollen tubes using aniline blue staining and scanning electron microscopy techniques. As shown in Figure 2A, the *vgd1* pollen tubes were able to germinate and grow on stigmatic cells similar to the wild type (Figure 2B). Therefore, it is unlikely that the *vgd1* mutation affects the ability of pollen germination on female stigmatic tissue. Transmitting electronic microscopic (TEM) observations showed that the *vgd1* pollen tubes also grew within stigmatic cell walls and intercellular spaces in the style and transmitting tract like wild-type pollen tubes (Figures 2C to 2F). This result indicated that the *vgd1* mutant pollen tube still was able to burrow into the stigmatic cell wall as wild-type pollen tubes. However, the

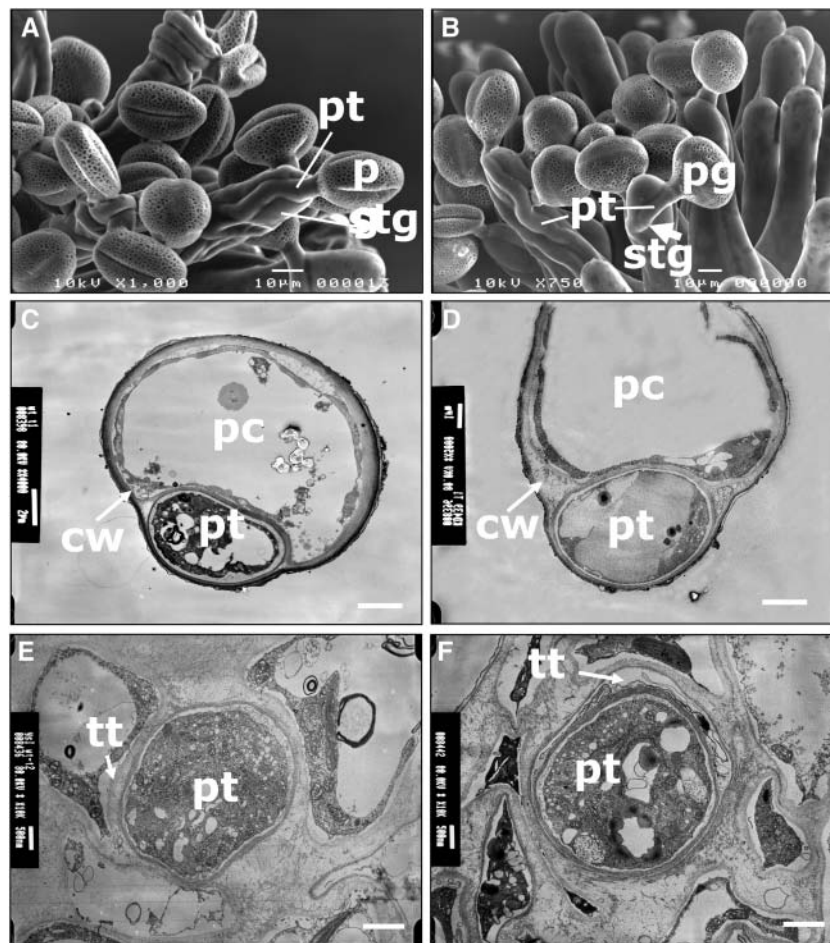


Figure 2. Electronic Microscopic Observation of *vgd1* Pollen Tubes Compared with the Wild-Type Pollen Tubes.

- (A) Scanning electron microscopy of *vgd1* pollen tubes on wild-type stigma.
 (B) Scanning electron microscopy of wild-type pollen tubes on wild-type stigma. The arrow indicates the invasion of a pollen tube into a stigmatic cell.
 (C) TEM of a papillary cell transversal section, showing a *vgd1* pollen tube inside the cell wall of the papillary cell.
 (D) TEM of a papillary cell transversal section, showing a wild-type pollen tube inside the cell wall of the papillary cell.
 (E) TEM of a style transversal section, showing a *vgd1* pollen tube inside the transmitting tract.
 (F) TEM of a style transversal section, showing a wild-type pollen tube inside the transmitting tract.
 cw, cell wall; pc, papillary cell; pg, pollen grain; pt, pollen tube; stg, stigmatic cell; tt, transmitting tract. Bars = 10 μm in (A) and (B), 2 μm in (C) and (D), and 1 μm in (E) and (F).

penetration rate of *vgd1* pollen tubes was reduced compared with the wild type. Wild-type pollen tubes were able to pass through the style in 4 h after pollination (hap) (Figure 3A) and reached the base of the transmitting tract by 12 hap (Figure 3C). By contrast, the *vgd1* pollen tubes were restricted within the stigmatic tissue at 4 hap (Figure 3B) and took ~24 h to pass through the style (Figure 3D); they only reached approximately half a transmitting tract length at 48 hap (Figure 3E). This result was consistent with the fact that seeds were produced only in the

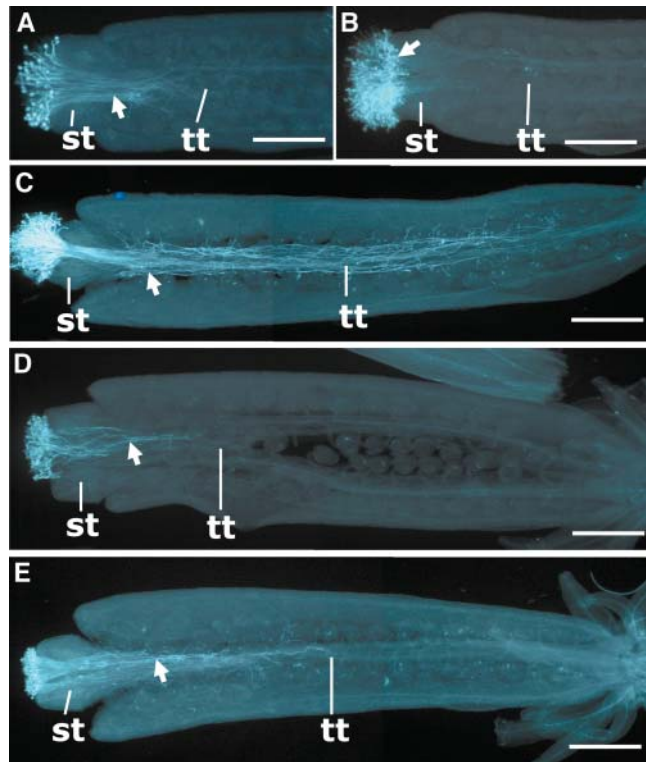


Figure 3. Growth Patterns of the *vgd1* Pollen Tubes in Female Floral Tissues Compared with Those of Wild-Type Pollen Tubes.

(A) Growth pattern of the wild-type pollen tubes in the wild-type female organs 4 hap, showing that the pollen tubes had penetrated through the style tissue and reached the upper end of transmitting tract.

(B) Growth pattern of the *vgd1* pollen tubes in the wild-type female organs 4 hap, showing that the pollen tubes had not been able to penetrate through the style tissue.

(C) Growth pattern of the wild-type pollen tubes in the wild-type female organs 12 hap, showing that the pollen tubes almost had reached the base of the transmitting tract.

(D) Growth pattern of the *vgd1* pollen tubes in the wild-type female organs 24 hap, showing that the pollen tubes had reached only approximately one-fifth of the transmitting tract length.

(E) Growth pattern of the *vgd1* pollen tubes in the wild-type female organs 48 hap, showing that the pollen tubes had reached approximately half of the transmitting tract length.

st, style; tt, transmitting tract. The arrows indicate the pollen tubes. Bars = 200 μ m.

upper part of a wild-type or *vgd1* mutant silique when pollinated with the *vgd1* pollens.

The *vgd1* Pollen Tube Was Unstable in Vitro

In vitro germination showed that the *vgd1* pollen tubes were unstable when cultured in vitro. Eighty-four percent of wild-type pollen could germinate in a wide range of in vitro conditions. Wild-type pollen tubes grew straight on the medium surface and were able to elongate up to an average length of 350 μ m after an overnight incubation (Figure 4D). By contrast, although ~83% of *vgd1* pollens could germinate in an optimized in vitro condition (see Methods), they were shorter, grew more slowly, and had an unusual shape (Figure 4C) compared with the wild-type pollen tubes germinated in the same conditions (Figure 4D). In all, 93.8% (334 out of 356) of germinating *vgd1* pollen tubes burst (Figure 4E), indicating that the *vgd1* pollen tubes were structurally unstable. By contrast, bursting of wild-type pollen tubes was only occasionally observed, at a very low rate of 3.7% (12 out of 328). These results suggested that *VGD1* might be involved in stiffening the pollen tube wall and enhancing its interaction with the medium surface.

The *vgd1* Pollen Tube Is Morphologically Normal When Germinated on Stigma and Is Not Defective in Guidance

The scanning electronic microscopic observations showed that the *vgd1* pollen appeared morphologically normal (Figures 4A and 4B) and had the same germination rate (>95%) on stigmatic cells as wild-type pollen. There were no morphological differences between the *vgd1* and wild-type pollen tubes when germinated on stigmatic cells of both *vgd1* and wild-type stigmas (Figures 2A and 2B). In addition, the scanning electron microscopy observations showed that the *vgd1* pollen tubes were directed to ovules normally as wild-type pollen tubes (data not shown), indicating that the *vgd1* mutation did not affect pollen tube guidance.

Molecular Cloning of the *VGD1* Gene

To identify the *VGD1* gene, we used thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995; Grossniklaus et al., 1998) to obtain the genomic flanking sequences adjacent to both ends of the *Ds* element in *vgd1*. Sequencing the TAIL-PCR products revealed that a *Ds* element had inserted into the second exon of a predicted gene *At2g47040* on chromosome II (BAC F14M4, AC004411) (Figure 5A) in the *vgd1* genome.

To confirm the identification of *VGD1*, a 5.131-kb genomic DNA fragment, including the predicted promoter, transcribed region, and 3'-end nontranscribed region, was amplified by high-fidelity PCR and introduced into the *vgd1* homozygous plants through *Agrobacterium tumefaciens*-mediated infiltration. Thirty-six independent transformants were obtained in a screen. Eighteen of the thirty-six T1 seedlings gave rise to fully fertile plants. The other transformants exhibited variable restorations

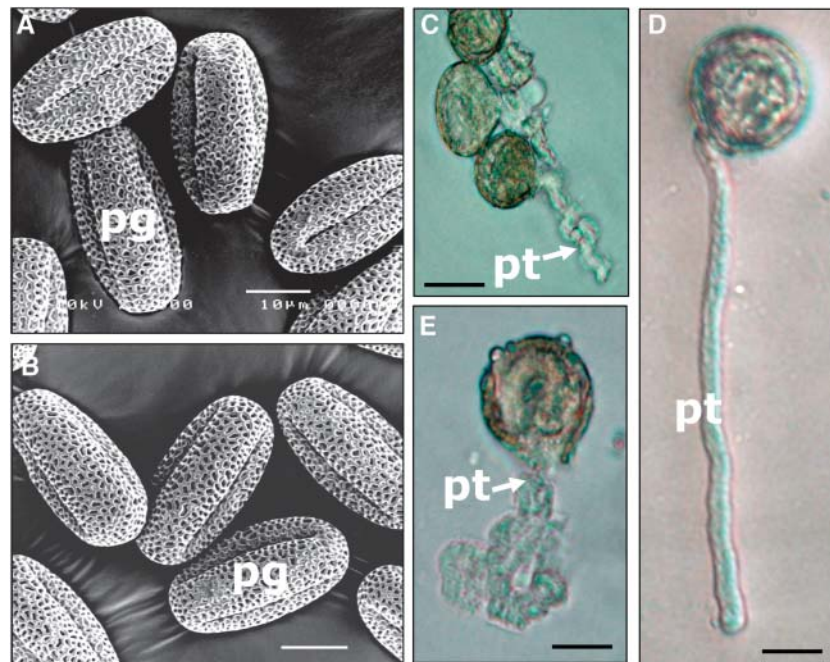


Figure 4. Morphology and in Vitro Germination of *vgd1* Pollen Compared with Wild-Type Pollen.

(A) Scanning electron microscopy of wild-type pollen grains.

(B) Scanning electron microscopy of *vgd1* pollen grains.

(C) to (E) Growing *vgd1* and wild-type pollen tubes.

(C) The unusual shape of the *vgd1* pollen tube.

(D) A wild-type pollen tube.

(E) A burst of a *vgd1* pollen tube.

pt, pollen tube; pg, pollen grain. Bars = 10 μm in (A) and (B) and 20 μm in (C) to (E).

of male fertility. In the T2 generation, all hygromycin-selected seedlings from the 18 fully fertile families gave rise to normal fully fertile plants, whereas nonselected T2 plants showed the segregation of the *vgd1* phenotype with concordance of hygromycin-sensitive segregation. The results show that the 5.131-kb genomic fragment from chromosome II contained all the genetic information required for normal functioning of *VGD1*. A BLASTN search of the databases showed that an 1823-bp cDNA sequence (AJ250430) in the GenBank database matched the predicted 1952-bp mRNA sequence (AY091768) from gene *At2g47040*. We used RT-PCR to clone the full-length cDNA. The cloned cDNA (AY830948) was 1952 nucleotides in length, which was consistent in size to a band shown by RNA gel blot hybridization (Figure 7A). To confirm the genetic function of cloned *VGD1* cDNA, the 1952-bp cDNA fragment was fused to the predicted *VGD1* promoter (a 1513-bp fragment flanking the 5'-end of the *VGD1* coding region) and *VGD1* terminator (a 1786-bp fragment flanking the 3'-end of the *VGD1* coding region). The resulting construct [P_{VGD1} -*VGD1*(cDNA)- T_{VGD1}] was subcloned into pCAMBIA1300 Ti-plasmid vector and introduced into the homozygous *vgd1* mutant plants. Twenty-four independent transformants were obtained in a screen. Eight of the twenty-four T1 seedlings gave rise to fully fertile plants. Others exhibited

a variable restoration of male fertility. In the T2 generation, all hygromycin-selected seedlings from the eight families gave rise to fully fertile plants, whereas nonselected T2 plants showed segregation of the *vgd1* phenotype. These results showed that the 1952-bp mRNA sequence was sufficient to encode the functional *VGD1* protein.

***VGD1* Encodes a PME-Homologous Protein**

The *VGD1* mRNA encodes a PME-homologous protein of 595 amino acids, which consists of a secretion-related transmembrane domain, a PME inhibitor (PMEI)-homologous domain, and a pectinesterase-homologous domain (Figure 5B). A BLASTP search was performed using the entire amino acid sequence of the predicted *VGD1* protein and showed that there was a group of PME proteins in the Arabidopsis genome with >50% amino acid sequence identity to *VGD1*. Specifically, *VGD1* had 85% (514/598) identity and 91% (550/598) similarity to the predicted *At2g47030* gene product (T02184; T52330) and 69% (426/611) identity and 81% (503/611) similarity to the predicted *At3g62170* gene product (Figure 6). In addition, *VGD1* is located in a genomic locus consisting of three homologous genes, *At2g47030*, *VGD1*

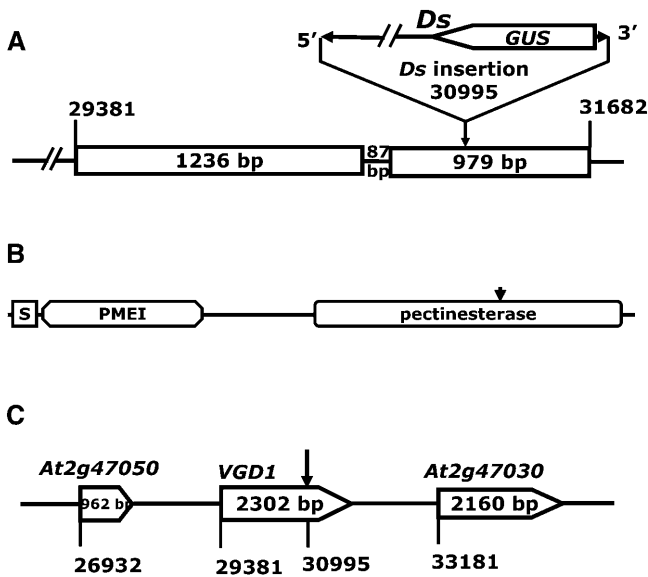


Figure 5. The Genomic Organizations of *VGD1* and the Structure of *VGD1* Protein.

(A) Genomic organization of the *VGD1* gene. The numbers over the vertical lines indicate the positions of the start and end nucleotides of the fragment in the BAC clone. The boxes indicate the positions of exons, and the intervening lines indicate the positions of introns. The numbers in the boxes indicate the sizes of the fragments. The arrow points out the *Ds* insertion site.

(B) The structure of *VGD1*, showing the positions of the secretive signal peptide (S), PME1, and PME domains. The arrow indicates the *Ds* insertion site.

(C) Genomic organization of the *VGD1* locus and the *Ds* insertion. The numbers under the vertical lines indicate the positions of start nucleotides of the fragment in BAC clone F14M4. The numbers in the boxes indicate the sizes of the transcribed regions.

(*At2g47040*), and *At2g47050* (Figure 5C). The predicted *At2g47050* protein (T02182) has only 216 amino acids and contains only one PME1 domain, which is likely to be a unique PME1 gene sharing a lower homology (27% identity) with the PME1 domain of *VGD1*.

The *vgd1* Mutation Reduced the Total Pectin Demethylesterification Activity of PMEs in Pollen Grain

Molecular cloning shows that the *VGD1* gene encoded a PME-homologous protein. PMEs belong to a family of cell wall enzyme proteins that act in the modification of cell walls via demethylesterification of cell wall pectin. To investigate whether the *vgd1* mutation affected the PME activity in pollen and the pollen tube, we compared the total pectin demethylesterification activity of PMEs in the *vgd1* pollen grain to that in wild-type pollen grain. The result showed that the total pectin demethylesterification activity of PMEs in the *vgd1* pollen grain decreased $\sim 18\%$ compared with that of wild-type pollen grain. This result in-

dicated that *VGD1* encoded a PME enzyme and that the retardation of pollen tube growth was caused by the loss of *VGD1* PME activity in the pollen tubes.

VGD1 and Its Homologs, *At2g47030* and *At3g62170*, All Were Expressed Specifically in the Pollen Grain and Pollen Tube

RNA gel blot hybridization showed that *VGD1*, *At2g47030*, and *At3g62170* were expressed specifically in flowers (Figure 7A). We further investigated their expression in pollen and elongating pollen tubes using transgenic plants containing the *P_{VGD1}- β -glucuronidase (GUS)* transcriptional fusion genes. Histochemical staining for GUS activity was used to monitor the activity of different promoters. The GUS staining patterns showed that all three genes were expressed specifically in pollen grains and pollen tubes (Figures 7B to 7G). Because they were expressed in pollen grains before pollination, the expressions of *VGD1*, *At2g47030*, and *At3g62170* were unlikely to be induced by pollination. This finding showed that the expression of *VGD1* is consistent with a function in pollen tube development.

Comparing the densities of GUS stains revealed that the expressional levels of three genes were very different. The expressional signal of *VGD1* was the strongest, and that of *At2g47030* was the weakest. We further used quantitative real-time PCR to compare the expression levels of *VGD1* and its homologs, *At2g47030* and *At3g62170*, in wild-type plants. The results showed that the level of *VGD1* mRNA transcription was ~ 2.7 times higher than that of *At3g62170* and 21 times higher than that of *At2g47030*. These results were very suggestive that *VGD1* may be functionally redundant to its homologs, *At2g47030* and *At3g62170*. If so, *VGD1* must be a main contributor to the process.

The *At2g47030* Product Has the Same Biological Function as *VGD1* Protein

As shown above, the *VGD1* protein shares a very high sequence identity to *At2g47030* and *At3g62170* predicted proteins. To study whether these three genes are functionally redundant to each other, we overexpressed *At2g47030* and *At3g62170* in pollen and pollen tubes of homozygous *vgd1* plants under the control of the *VGD1* promoter. More than 40 independent transformants were obtained from each transformation. In the *At2g47030* transgenic T1 population, three plants were fully fertile and the others exhibited variable restorations of the *vgd1* phenotype. Further genetic and molecular analysis confirmed the complementation of *vgd1* phenotype by overexpression of the *At2g47030* coding region (data not shown). This result indicated that *At2g47030* might have the same genetic function as *VGD1*. However, in the *At3g62170* transgenic T1 population, all plants exhibited the typical *vgd1* phenotype, indicating that *At3g62170* may not have the same genetic function as *VGD1*.

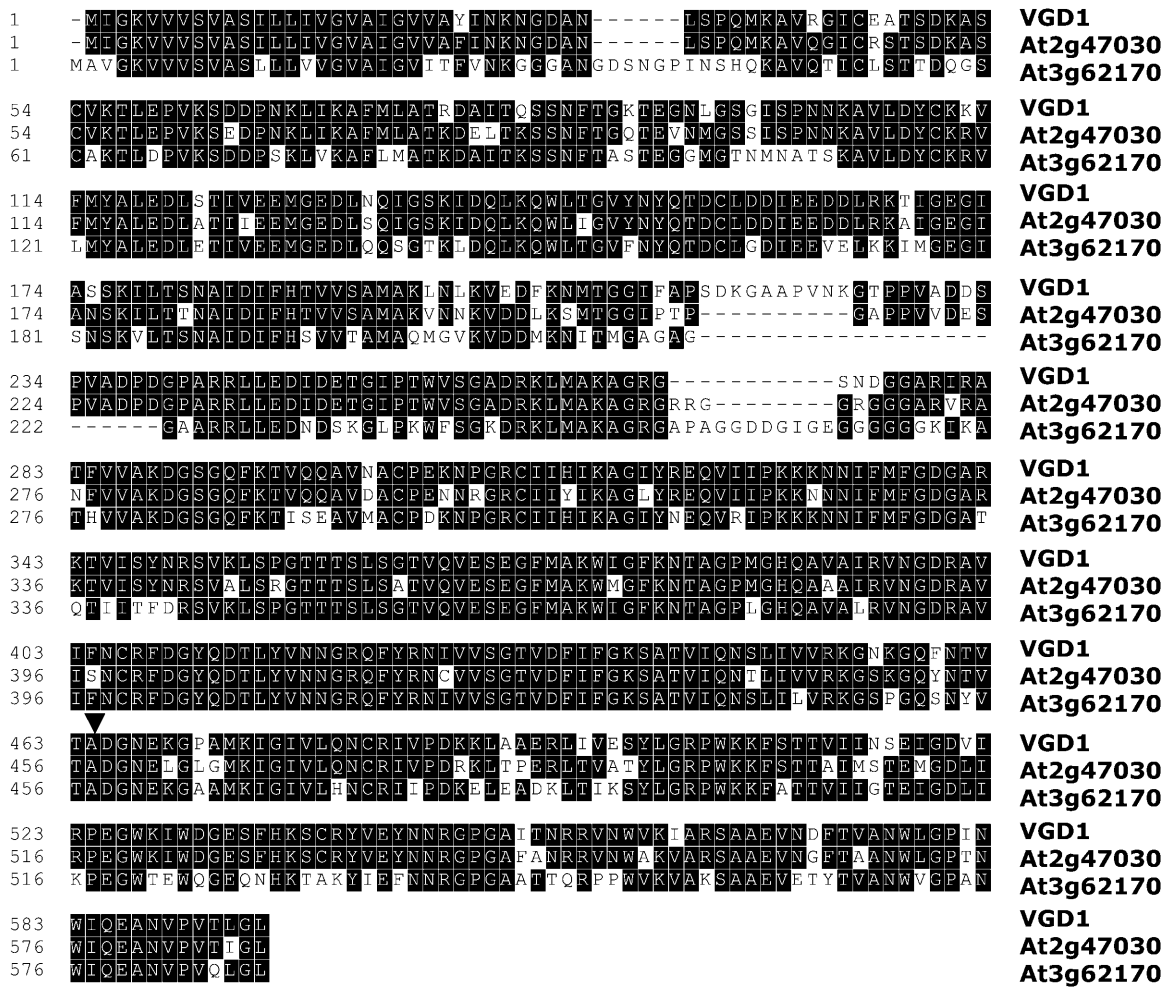


Figure 6. The Alignment of VGD1, At2g47030, and At3g62170 Proteins. The black boxes indicate the identical amino acids. The arrowhead points out the *Ds* insertion site in VGD1.

The VGD1–Green Fluorescent Protein Fusion Was Distributed in the Whole Pollen Tube, Including the Plasma Membrane and Pollen Tube Wall

If *VGD1* encodes a PME enzyme protein that acts in cell wall modification, it should be localized to the cell wall of the pollen tube. To investigate whether the subcellular localization of VGD1 protein was associated with the pollen tube wall, a green fluorescent protein (GFP) reporter protein was fused to the C terminus of VGD1 and expressed specifically in pollen and the pollen tube under the control of the *VGD1* promoter in the wild-type background plants. In the transgenic plant, the GFP signal was first detected in the mature pollen (data not shown). After germination, the GFP signal was detected in whole pollen tube (Figure 7H). To investigate whether the GFP signal was located in the pollen tube wall, the germinating transgenic pollen tubes were treated with 40% sucrose buffer (see Methods) to separate the cell wall from the cytoplasm. The resulting plasmolysis image

showed that the GFP signal was detected in the cell wall region of the pollen tube (Figure 7I). All these data suggest that VGD1 PME protein may be required all the time during growth of the pollen tube, and its subcellular localization is consistent with a function on the pollen tube wall.

DISCUSSION

***VGD1* Is Important for Growth of the Pollen Tube in Female Floral Tissue**

The *vgd1* mutation we report here is a novel mutation that affects growth of pollen tubes in the Arabidopsis style and transmitting tract. Genetic studies indicated that the *vgd1* mutation was a male gametophytic mutation and affected only the growth of pollen tubes. The *vgd1* pollen tubes grew much more slowly than

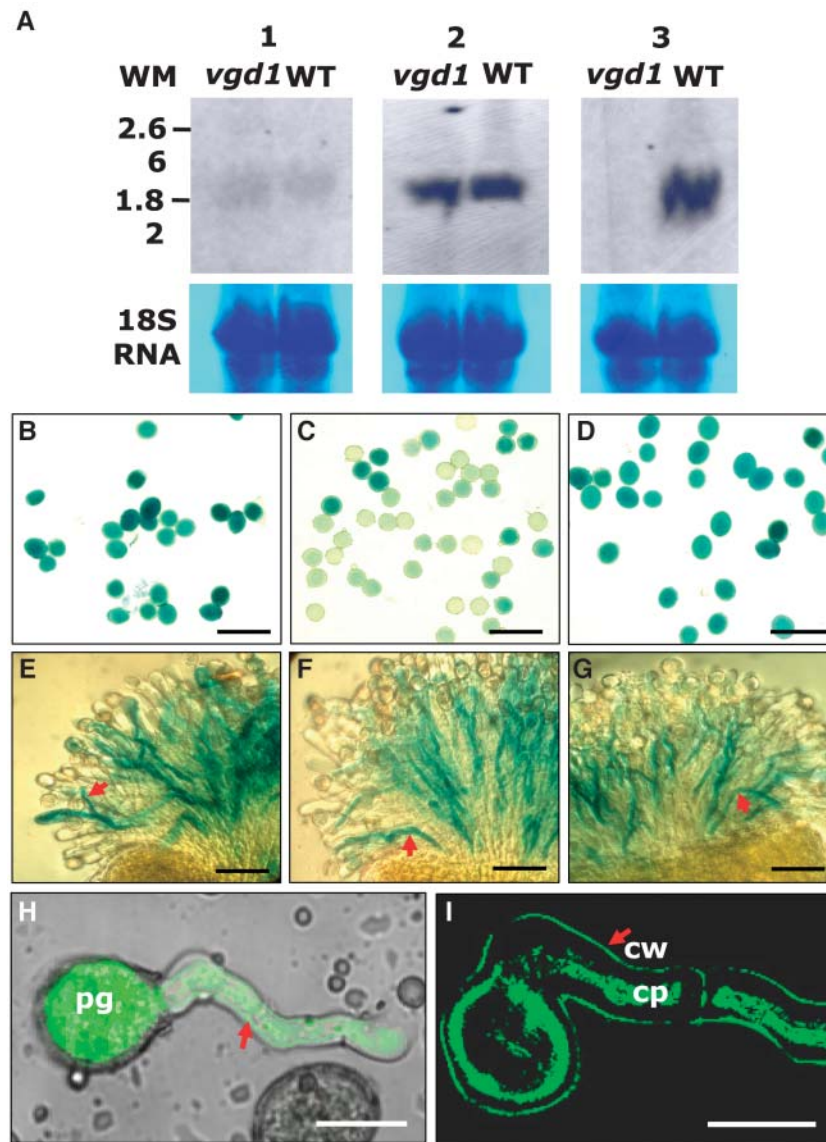


Figure 7. Promoter Activities of *VGD1*, *At2g47030*, and *At3g62170* Monitored by GUS Activity and Subcellular Localization of *VGD1*-GFP.

(A) Expression of *VGD1*, *At2g47030*, and *At3g62170* in the wild-type and *vgd1* flowers revealed by RNA gel blot hybridization, showing that *VGD1* was expressed only in wild-type floral tissue (3), whereas *At2g47030* and *At3g62170* were expressed in both *vgd1* and wild-type floral tissues (1 and 2), respectively. Thirty micrograms of total RNA was loaded in each lane. The bottom panels are the loading controls showing the 18S RNA. WT, wild-type flower total RNAs; *vgd1*, *vgd1* flower total RNA; WM, RNA molecular weight markers; 1, hybridized with *At2g47030*-specific probe; 2, hybridized with *At3g62170*-specific probe; 3, hybridized with *VGD1* (*At2g47040*)-specific probe.

(B) The *P_{VGD1}-GUS-T_{NOS}* transgenic pollen grains, showing the GUS activity in the pollen grains.

(C) The *P_{At2g47030}-GUS-T_{NOS}* transgenic pollen grains, showing the GUS stain in pollen grains.

(D) The *P_{At3g62170}-GUS-T_{NOS}* transgenic pollen grains, showing the GUS stain in pollen grains.

(E) The *P_{VGD1}-GUS-T_{NOS}* transgenic pollen tubes, showing the GUS stain in the transgenic pollen tubes germinating in the wild-type stigmatic tissues.

(F) The *P_{At2g47030}-GUS-T_{NOS}* transgenic pollen tubes, showing the GUS stain in the transgenic pollen tubes germinating in the wild-type stigmatic tissues.

(G) The *P_{At3g62170}-GUS-T_{NOS}* transgenic pollen tubes, showing the GUS stain in the transgenic pollen tubes germinating in the wild-type stigmatic tissues.

(H) A transgenic pollen tube (arrow) showing that the *VGD1*-GFP fusion protein is distributed throughout the whole pollen tube. The picture was merged from a GFP signal and an image using transmitted light. pg, pollen grain.

(I) A section of a transgenic pollen tube showing the localization of *VGD1*-GFP fusion protein in the wall of the pollen tube after plasmolysis. The picture was scanned for GFP signal without light transmission. cw, cell wall; cp, cell plasma. The arrow indicates the pollen tube wall.

Bars = 100 μ m in **(B)** to **(G)** and 25 μ m in **(H)** and **(I)**.

Table 1. Primer Pairs Used for the Isolation of P_{VGD1} , T_{VGD1} , and cDNA Fragments

Gene Names	Primer Sequences	
	Forward Primers (Adaptor with Restriction Site)	Reverse Primers (Adaptor with Restriction Site)
<i>VGD1</i> promoter	5'-CTGCAGTGATGCTCCACATTCTGACG-3' (<i>Pst</i> I)	5'-ACTAGTATTTTTGCTCTCCCTCCGGT-3' (<i>Spe</i> I)
<i>VGD1</i> terminator	5'-GCTAGCGGCGTATAAATCAAATCAAAT-3' (<i>Nhe</i> I)	5'-GAATTCCTATTGTGATGGTTACTGGAG-3' (<i>Eco</i> RI)
<i>VGD1</i> cDNA	5'-ACTAGTACTGGCCCAAGTCATTCAACA-3' (<i>Spe</i> I)	5'-GCTAGCGATTTGATTTGATTATACGC-3' (<i>Nhe</i> I)
<i>At2g47030</i> cDNA	5'-ACTAGTATAAAGCCTCTCCCTCTCCGA-3' (<i>Spe</i> I)	5'-GCTAGCTTAAAAAGTTTTTCATAACCA-3' (<i>Nhe</i> I)
<i>At3g62170</i> cDNA	5'-ACTAGTCCACCGGGCTCTGTTTCAATA-3' (<i>Spe</i> I)	5'-GCTAGCGAGACGACGTCGTATCGTTAC-3' (<i>Nhe</i> I)

wild-type pollen in the style and transmitting tract, although they were able to germinate on the surface of the stigmatic cell and invaded into the stigmatic cell. Our observations also indicated that the *vgd1* mutation did not obviously affect the growth rate of the pollen tube on the surface of stigmatic cells, and the retardation of pollen tube extension occurred mainly in the style and transmitting tract. Therefore, we conclude that the *VGD1* product is required for enhancing the growth of the pollen tube in the style and transmitting tract.

The *VGD1* protein was highly homologous to PMEs, a group of cell wall proteins. Consistently, shutting down of *VGD1* function reduced PME activity in the pollen grain and indicated that *VGD1* protein might function as a PME enzyme. In higher plants, different PME isoforms are encoded by multiple gene families (Richard et al., 1996; Micheli et al., 1998) and act in the demethylesterification of cell wall pectin, contributing to different processes of plant development (Micheli, 2001). Therefore, the *VGD1* product may be involved in growth of the pollen tube by modification of the pollen tube wall. *VGD1* was expressed specifically in pollen grains and pollen tubes. If *VGD1* is a PME protein, it is a pollen- and pollen tube-specific PME because it is not expressed elsewhere.

The null *vgd1* mutation did not block the growth of pollen tubes in the style and transmitting tract completely. A possible reason for this finding is that the *VGD1* protein may act redundantly with *At2g47030*. The *At2g47030* protein had a high amino acid sequence identity to *VGD1* and exhibited the same expression pattern as *VGD1*. The complementation experiment also showed that the *At2g47030* gene product had the same biological function as the *VGD1* protein. Disruption of *VGD1* function significantly reduced the growth rate of pollen tubes in the style

and transmitting tract. If these two genes act redundantly, *VGD1* must be the major contributor to the process.

As discussed above, the expression level of *At2g47030* was 21 times lower than that of *VGD1*. Expression of *At2g47030* at the same level as *VGD1* using *VGD1* promoter could complement the *vgd1* mutant phenotype. This result indicates that different PMEs might have the same biological function when they are expressed at the same level and location.

The *At3g62170* gene product also was highly homologous to *VGD1* protein and had the same expression profile as *VGD1*. However, we failed to complement the *vgd1* mutant by overexpression of *At3g62170* protein using the *VGD1* promoter. It is possible that *At3g62170* might not have the same function as *VGD1*. This result showed that homologous genes with high sequence similarity and even the same location of expression might not unconditionally have the same biological function. We still do not know the actual biological function of the *At3g62170* gene product. The *At3g62170* protein also might have lost its function in pollen tube growth during evolution, although it still is expressed actively in the pollen tube.

The *VGD1* Product May Be Involved in Building up the Strength of the Pollen Tube Wall

The in vitro germination study showed that the *vgd1* pollen tube was very unstable when grown in the culture medium. Although it was able to germinate in vitro, the resulting pollen tubes often burst after germination. The result indicated that the *vgd1* mutation altered the mechanical characteristics of the pollen tube wall. In other words, *VGD1* is involved in the pollen tube wall.

Table 2. Primer Pairs Used for the P_{VGD1} -*GUS* Transcriptional Fusion Constructs

Gene Fragment Names	Primer Sequences	
	Forward Primers	Reverse Primers
<i>At2g47030</i> promoter	5'-CTGCAGAAATGTTCCCGTCACGCTTGA-3'	5'-TCTAGATTTCTCTCCGATCCCTCCGGA-3'
<i>At3g62170</i> promoter	5'-CTGCAGGATCGTATTAAGGGATTGGAT-3'	5'-TCTAGATGGTAGAGATTGTGGTGCATT-3'
<i>VGD1</i> promoter	5'-GAATTCGTGATGCTCCACATTCTGACGT-3'	5'-TCTAGATTTTTGCTCTCCCTCCGGT-3'

Table 3. Primer Pairs Used for mRNA Quantification

Gene Names	Primer Sequences	
	Forward Primers	Reverse Primers
<i>VGD1</i> -specific fragment (431 bp)	5'-CAGAGATGCTATAACCCAATCA-3'	5'-AGCTGCTCCTTTGTCTGAAGGA-3'
<i>At2g47030</i> -specific fragment (323 bp)	5'-AATGAGCACCGAGATGGGAGATT-3'	5'-TCATAAACCAATTTTCATACACCA-3'
<i>At3g62170</i> -specific fragment (1077 bp)	5'-CCAGCTGGCGGTGATGATGGT-3'	5'-CCAGAGACGACGTCGTATCGT-3'

Studies have shown that mature PMEs could have different modes of action. They may act either randomly or linearly along the pectin chain (Markovic and Kohn, 1984; Micheli, 2001). It is commonly believed that random demethylesterification of pectin depends on acidic PMEs, whereas linear demethylesterification of pectin requires basic PMEs (Micheli, 2001). The *VGD1* protein exhibited a predicted basic isoelectric point of 8.9. Therefore, *VGD1* may act by linear demethylesterification of pollen wall pectin. The linear demethylesterification on homogalacturonans by PME gives rise to blocks of free carboxyl groups that could interact with Ca^{2+} , creating a pectate gel (Goldberg, 1996). The Ca^{2+} pectate gel is believed to contribute to cell wall stiffening and cell attachment by limitation of the action of endopolygalacturonases and formation of Ca^{2+} pectate gel lawn. In the *vgd1* mutant, therefore, the loss of *VGD1* function could lead to loss or reduction of formation of Ca^{2+} pectate gel lawn on the surface of the pollen tube wall, resulting in loss or reduction of strength of the pollen tube wall. As a result, the *vgd1* pollen tube could have burst easily when grown in vitro.

Interestingly, we did not observe any burst pollen tubes on stigmatic cells. Possibly, the hydration expansion of pollen in vivo is milder or more controlled compared with our in vitro conditions. This finding implies that germination and development of pollen tubes are different in vivo and in vitro, and it is known that growth of pollen tubes in vivo is subject to intricate regulation. The interaction between pollen tubes and stigmatic cells may be important for the stabilization of the pollen tubes. The pollen tube grew within cell walls of stigmatic papillar cells; presumably the surrounding layer of the papillar cell wall also could provide additional physical support to the pollen tube wall.

The *VGD1* Product May Involve the Interaction between the Pollen Tube and Female Floral Tissues via the Modification of Cell Walls

Microscopic observations showed that the *vgd1* mutation did not affect the growth rate of pollen tubes on the surface of stigmatic cells; however, growth of pollen tube was retarded greatly in the style and transmitting tract. This finding indicated that the *VGD1* product might be involved in the interaction between pollen tubes and female floral tissues via the modification of cell walls. Because *VGD1* encodes a PME protein, it may act in the degradation of the papillary cell wall to promote the invasion of the pollen tube into the papillary and stylar cells and modification of the pollen tube wall to enhance its interaction with female floral tissues.

On one hand, the retardation of pollen tubes in the *vgd1* mutant may result from the inefficient degradation of stigmatic and stylar cell walls. Studies have shown that many factors affect the mode of action of PMEs, such as pH, the initial degree of demethylesterification of pectins, and the presence of cations (Catoire et al., 1998; Denés et al., 2000). Some PME isoforms act randomly in one medium, but linearly in other medium. Therefore, *VGD1* may act randomly or linearly depending on the environmental conditions. The *VGD1*-GFP fusion protein was found in the pollen tube wall, indicating that *VGD1* protein might be secreted out of the pollen tube. It might diffuse to the cell walls of the female floral tissues, where the condition is suitable for random demethylesterification. The random demethylesterification of homogalacturonans releases protons, which promotes the action of endopolygalacturonases and contributes to the degradation or loosening of cell walls (Goldberg, 1996). In the *vgd1* mutant, the loss of *VGD1* function reduced significantly the PME activity in pollen, indicating that the *vgd1* pollen tube also may have weaker PME activity. The weaker PME activity in extracellular space would reduce the efficiency of the degradation of the female tissue cell wall, resulting in retardation of pollen tube growth in female tissues.

On the other hand, the retardation of the pollen tube also could result from the inefficient interaction of pollen tubes with the ECM in the style and transmitting tract. As mentioned previously, the *VGD1* protein exhibited a predicted basic isoelectric point of 8.9, which is advantageous to linear demethylesterification. The conditions in the transmitting tract also may be suitable for linear demethylesterification by *VGD1*. The linear demethylesterification of pectin can make a contribution to the formation of the Ca^{2+} pectate gel lawn that benefits the interaction between pollen tubes and ECM in the style and transmitting tract, promoting the elongation of the pollen tube in the style and transmitting tract. Loss of *VGD1* function may reduce the efficiency of interaction of the pollen tube with the ECM in the style and transmitting tract, resulting in the retardation of the pollen tube in the transmitting tract.

In conclusion, we have described a novel *vgd1* mutation that significantly retarded the navigation of the pollen tube through the style and transmitting tract, resulting in a significant reduction of male fertility. The *VGD1* gene encoded a PME-homologous protein and was expressed specifically in pollen grain and the pollen tube. This study suggests that *VGD1* plays an important role in growth of pollen tubes in female floral tissues, possibly via enhancing the interaction between the pollen tube and female floral tissues by modification of the cell walls.

METHODS

Plant Materials and Mutant Isolation

All *Arabidopsis thaliana* plants used in this study were in the Landsberg *erecta* background. The seeds were pregerminated on MS-salt agar plates with or without 50 $\mu\text{g}/\text{mL}$ of kanamycin at 22°C under a light cycle of 16 h light/8 h dark. The plants were grown in soil at 22°C under the same light cycle as for germination. The generation of *Ds* insertion lines and screen of mutants were performed as described by Sundaresan et al. (1995). The selected mutant plants were backcrossed with wild-type plants to purify the *vgd1* mutation. The F3 plants with a single *Ds* insertion linked to the *vgd1* phenotype were selected for further phenotypic characterization.

Characterization of *vgd1* Mutant Phenotype

To assay in vitro pollen germination, wild-type and *vgd1* pollen grains were collected and cultured as described by Fan et al. (2001), with a modification by spreading several drops of stigma water extract onto the surface of agar plates containing different amounts of agar (0.8, 1.0, 1.2, and 1.5%, w/v) and sucrose (12, 16.6, and 20%, w/v). The pollen grains spread on the agar plates were cultured immediately at 23°C, 100% relative humidity, and cool fluorescence light at 30 $\mu\text{mol}/\text{m}^2\text{s}$. The germinating pollen grains were counted under a microscope after overnight incubation. From each culture, at least 300 pollen grains were examined to calculate an average germination rate, and 20 pollen tubes were measured for the average pollen tube length.

Aniline blue staining of pollen tubes in pistils was performed as described by Sumie et al. (2001). The preemasculated mature wild-type flowers were pollinated either with wild-type or *vgd1* pollen. The pollinated pistils were collected 4, 12, 24, and 48 h and briefly fixed in fixing solution of ethanol:acetic acid (3:1) for 2 h at room temperature. The fixed pistils were washed three times with distilled water and treated in softening solution of 8 M NaOH overnight. Then, the pistil tissues were washed in distilled water and stained in aniline blue solution (0.1% aniline blue in 0.1 M K_2HPO_4 -KOH buffer, pH 11) for 3 to 5 h in the dark. The stained pistils were observed and photographed with a Leica DMRA2 fluorescence microscope (Wetzlar, Germany).

The morphological observations of pollen grains and pollen tubes by scanning electronic microscopy and TEM were performed as described by Hülkamp et al. (1995b).

Genetic Analyses of *vgd1*

All crosses of *vgd1* plants with wild-type plants were performed as described previously (Yang et al., 1999, 2003).

Molecular Cloning of the *VGD1* Gene and DNA Sequencing

Isolation of the flanking sequences adjacent to the *Ds* element by TAIL-PCR (Liu et al., 1995; Grossniklaus et al., 1998) was performed as described previously (Yang et al., 1999, 2003) with the *vgd1* genomic DNA and *Ds3/AD2* or *Ds5/AD4* primers. The full-length cDNAs of *VGD1* (AY830948), *At2g47030* (AY830949), and *At3g62170* (AY830950) were amplified using the Access RT-PCR system (Promega, Madison, WI) with the gene-specific primer pairs listed in Table 1. All resulting DNA fragments were cloned into the pGEM-T vector or pGEM-T Easy vector (Promega). DNA Sequencing was performed using ABI PRISM Rhodamine Terminator thermal cycle sequencing ready reaction kit (PE-Applied Biosystems, Foster City, CA) with gene-specific primers or vector-derived M13 or T7 primers.

Complementation Experiments

A 5.131-kb *VGD1* genomic fragment was amplified by the ACCuTaq LA DNA polymerase PCR kit (Sigma-Aldrich, St. Louis, MO) with gene-specific primers 5'-GGATCCTGATGCTCCACATTCTGACGT-3' and 5'-GAATTCTGGGTCAACGAATGGCTGAGA-3' and cloned into the pGEM-T vector. After sequence verifications, the fragment was subcloned into pCAMBIA1300 Ti-derived binary vector (CAMBIA, Canberra, Australia; www.cambia.org.au).

For cDNA complementation experiments, the *VGD1* promoter (P_{VGD1}) and *VGD1* terminator (T_{VGD1}) fragments were amplified from wild-type genomic DNA using the primers listed in Table 1. *VGD1*, *At2g47030*, and *At3g62170* cDNAs were amplified from the flower-specific cDNA pool using the gene-specific primers homologous to both end sequences of cDNAs (Table 1). The resulting fragments were cloned into pGEM-T vector and verified by DNA sequencing. Restriction enzyme pairs *Pst*I and *Spe*I, *Spe*I and *Nhe*I, or *Nhe*I and *Eco*RI were used to excise the promoter or cDNAs or terminator from the pGEM-T vector, respectively. They were then subcloned into pCAMBIA1300 vector, resulting in the transcriptional fusion construct P_{VGD1} -cDNA- T_{VGD1} . All constructs in pCAMBIA vector for complementation experiments were introduced into the *vgd1* homozygous plants using the *Agrobacterium tumefaciens*-mediated infiltration method. The transformants were selected using 20 mg/L of hygromycin and 50 mg/L of kanamycin.

Measurement of the Relative PME Activity in *vgd1* Pollen Grain

PME activity assay was performed according to Hou and Lin (1998) and Ren and Kermod (2000). Approximately 50 mg of *vgd1* or wild-type pollen grains were collected in an Eppendorf tube from newly mature anthers and frozen immediately in liquid nitrogen. Then, pollen grains were ground in ~250 μL of PME extraction buffer (0.1 M citrate to 0.2 M Na_2HPO_4 buffer containing 1.0 M NaCl, pH 5.0). The homogenized slurry was centrifuged for 10 min at 14,000g at 2°C. The supernatants were collected and stored in -20°C temporarily. Exude protein was quantified using the Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL) according to the supplier's instructions. Fifty micrograms of exude protein from each sample was added to 1 mL of 0.1% (w/v) 92% esterified pectin (Sigma-Aldrich) in 0.2 M Na_2HPO_4 buffer, pH 6.3. After overnight incubation at 37°C, 0.2 mL of 0.05% ruthenium red (Sigma-Aldrich) was added, mixed, and incubated for 10 min. Then, 0.5 mL of 0.6 M CaCl_2 (Sigma-Aldrich) was added to precipitate the demethylated pectin that bound to ruthenium red. The mixture was centrifuged at 14,000g for 15 min to remove the precipitate. The supernatants of the samples and the control were measured for the absorbance at 534 nm. All measurements were repeated four times to calculate the average values.

Subcellular Localization of VGD1-GFP Fusion Protein in the Pollen Tube

The *VGD1* coding cDNA fragment without the stop codon and *VGD1* promoter fragments were amplified using the ACCuTaq LA DNA polymerase PCR kit (Sigma-Aldrich) with gene-specific primer pairs *VGD1*-cDNA-*xbal*1 (5'-TCTAGAATGATTGGAAAGTTGTGGTC-3')/*VGD1*-cDNA-*Kpn*1 (5'-GGTACCTAATCCAAGCGTGACGGGAAC-3') and *VGD1*-5'prom-*pst*1 (5'-CTGCAGGATGCTCCACATTCTGACGTA-3')/*VGD1*-3'Prom-*xbal*1 (5'-TCTAGAATTTTTTGCTCTCCCTCCGGT-3'), respectively. The resulting DNA fragments were cloned into pGEM-T Easy vector and verified by DNA sequencing. The *VGD1* coding fragment was excised from pGEM-T Easy vector with *Xba*I and *Kpn*I restriction enzymes and subcloned into pGFP-2 vector before the start codon of the *GFP* coding sequence, resulting in a *VGD1*-GFP fusion coding sequence. The *VGD1* promoter fragment was excised with *Pst*I and *Xba*I restriction enzymes. The *VGD1*-GFP fusion coding fragment was

excised from the pGFP-2 vector with *Xba*I and *Sac*I restriction enzymes. Then, both fragments were subcloned before the *NOS* terminator sequence in a modified pCAMBIA-1300 Ti-derived binary vector (CAMBIA), resulting in a *P_{VGD1}-VGD1-GFP-T_{NOS}* construct. The *P_{VGD1}-VGD1-GFP-T_{NOS}* construct was introduced into a wild-type Arabidopsis plant using the Agrobacterium-mediated infiltration method. The transformants were selected using 20 mg/L of hygromycin. For plasmolysis, the germinating pollen tubes on medium were soaked in 40% (w/v) sucrose solution for half an hour and then air-dried for 15 min. The subcellular localization of VGD1-GFP fusion protein in the pollen and pollen tubes was visualized under a confocal microscope (Zeiss, Jena, Germany or Bio-Rad, Hercules, CA).

RNA and DNA Gel Blot Hybridization

The total RNAs were extracted using a TRIzol reagent kit (Gibco BRL, Gaithersburg, MD) as described by the supplier. The extractions of the plant genomic DNAs were performed as described by Yang et al. (1999). The RNA and the restricted DNA samples were fractionated in 1% agarose gel. For the *VGD1* gene-specific probe, a cDNA fragment was amplified by PCR using the primers 5'-GCCATGGAAGAAATTCCTAC-3' and 5'-GGTTTGATTGATTGATTATCAG-3'. For the *At2g47030* gene-specific probe, a cDNA fragment was amplified by PCR using the primers 5'-ACGTTGAGTACAACAACCGTGG-3' and 5'-TCATAAACC AATTCA-TACACCA-3'. For the *At3g62170* gene-specific probe, a cDNA fragment was amplified by PCR using the primers 5'-AAACCAGAAGGATGGACC-GAA-3' and 5'-CTCTTACAATCCTAGTTGGAC-3'. For the *Ds* probe, a 755-bp 5'-terminal fragment of the *Ds* element was amplified from plasmid pWS32 (Sundaresan et al., 1995) using primers 5'-CTCACAG-CACTTAGCAGTACA-3' and 5'-CATACATCCGATGTGCACTTC-3'. The resulting DNA fragments were cloned into pGEM-T vector and verified via sequencing. All RNA and DNA probes were labeled using the DIG RNA labeling kit (Roche, Indianapolis, IN) or the PCR DIG probe synthesis kit (Roche) as described by the supplier. RNA and DNA gel blot hybridizations were performed following the instructions in the DIG system and DIG application manual provided by the supplier.

Analysis of Promoter Activity

The promoters of *VGD1*, *At2g47030*, and *At3g62170* were amplified using the ACCUtaq LA DNA polymerase PCR kit (Sigma-Aldrich) with the gene-specific primer combinations listed in Table 2 and cloned into the pGEM-T vector. After sequence verification, the fragments were subcloned upstream of the GUS reporter gene in pCAMBIA1300 Ti-derived binary vector (CAMBIA). Then, they were introduced into the wild-type Arabidopsis plants. Plant transformation was performed as described above. GUS staining was performed as described previously (Sundaresan et al., 1995; Yang et al., 1999).

Quantification of the mRNAs Transcribed from the *VGD1*, *At2g47030*, and *At3g62170* Genes in Wild-Type Flower Tissue

Real-time PCR technology was used to quantify the mRNAs of *VGD1*, *At2g47030*, and *At3g62170* in wild-type floral tissue. The poly(A)⁺ RNAs were purified using the Oligotex mRNA Midi kit (Qiagen, Valencia, CA) as described by the supplier and converted into single cDNAs with Stratagene cDNA synthesis kit (Stratagene, La Jolla, CA) following the instructions of the supplier. The single-strand cDNA pool was diluted to 50 ng/μL of single strand cDNA. Two microliters of diluted cDNA solution was used for each real-time PCR reaction. To prepare the standard template series, the cDNA fragments of the three genes were amplified by PCR using the gene-specific primer pairs listed in Table 3 and cloned into pGEM-T Easy vector, respectively. The plasmids containing the cDNA fragments were

reproduced in *Escherichia coli* and recovered using the Qiagen plasmid mini preparation kit and then linearized with the restrictive enzyme *Nco*I or *Sal*I (Roche). The linearized plasmid DNAs were purified with Qiagen DNA spin columns and quantified with a UV spectrum photometer (UV-1601; Shimadzu, Columbia, MD). The template standards for each gene were prepared in a concentration series of 10¹⁰, 10⁹, 10⁸, 10⁷, 10⁶, 10⁵, and 10⁴ copies of the target cDNA molecules per microliter. The preparation of standard curves and quantification of each mRNA were performed using the Roche Lightcycler and FastStart DNA Master SYBR Green I kit following the instructions from the supplier (Roche).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY830948 (*VGD1* full-length cDNA), AY830949 (*At2g47030* full-length cDNA), and AY830950 (*At3g62170* full-length cDNA).

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