ABNORMAL POLLEN TUBE GUIDANCE1, an Endoplasmic Reticulum-Localized Mannosyltransferase Homolog of GLYCOSYLPHOSPHATIDYLINOSITOL10 in Yeast and PHOSPHATIDYLINOSITOL GLYCAN ANCHOR BIOSYNTHESIS B in Human, Is Required for Arabidopsis Pollen Tube Micropylar Guidance and Embryo Development1\[W]\[OPEN]\[2\]

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The perception and response of pollen tubes to the female guidance signals are crucial for directional pollen tube growth inside female tissues, which leads to successful reproduction. In pursuing the mechanisms underlying this biological process, we identified the Arabidopsis (Arabidopsis thaliana) abnormal pollen tube guidance1 (aptg1) mutant, whose pollen tubes showed compromised micropylar guidance. In addition to its male defect, the aptg1 mutant showed embryo lethality. APTG1 encodes a putative mannosyltransferase homolog to human PHOSPHATIDYLINOSITOL GLYCAN ANCHOR BIOSYNTHESIS B and yeast (Saccharomyces cerevisiae) GLYCOSYLPHOSPHATIDYLINOSITOL10 (GPI10), both of which are involved in the biosynthesis of GPI anchors. We found that APTG1 was expressed in most plant tissues, including mature pollen, pollen tubes, mature embryo sacs, and developing embryos. By fluorescence colabeling, we showed that APTG1 was localized in the endoplasmic reticulum, where GPI anchors are synthesized. Disruption of APTG1 affected the localization of COBRA-LIKE10, a GPI-anchored protein important for pollen tube growth and guidance. The results shown here demonstrate that APTG1 is involved in both vegetative and reproductive development in Arabidopsis, likely through processing and proper targeting of GPI-anchored proteins.

Double fertilization is the biological basis for seed propagation and plant reproduction in angiosperms. Pollen tubes grow through maternal tissue to deliver the immobile sperm cells into the female gametophyte (embryo sac). During this process, pollen tube guidance into the micropyle is a critical step and is precisely regulated (Dresselhaus and Franklin-Tong, 2013). Female guidance signals are generated by both sporophytic and gametophytic tissues and operate at different stages during pollen tube growth. The sporophytic signal directs the growth of pollen tubes in the stigma, style, and transmitting tract. The signal that induces pollen tubes to turn to the funiculus and grow into the micropyle is termed gametophytic guidance (Shimizu and Okada, 2000; Higashiyama et al., 2003). Extensive cellular and genetic studies have demonstrated that female gametophytes play key roles in the micropylar guidance of pollen tubes (Kasahara et al., 2005; Márton et al., 2005; Chen et al., 2007; Alandete-Saez et al., 2008; Okuda et al., 2009; Kessler and Grossniklaus, 2011; Takeuchi and Higashiyama, 2011). The molecular natures of such guidance signals have been gradually revealed in recent years (i.e. small peptides secreted by the female gametophyte, egg apparatus, or synergid cells; Márton et al., 2005; Jones-Rhoades et al., 2007; Okuda et al., 2009).

Pollen tubes need to perceive the female guidance signals at the cell surface to initiate intracellular responses for directional growth. However, the mechanisms of pollen tube perception are still obscure. A few male factors involved in signal perception during pollen tube growth into ovules have been identified. For example, the Arabidopsis (Arabidopsis thaliana) sperm cell-specific protein HAPLESS2/GENERATIVE CELL-SPECIFIC1 was necessary for pollen tubes to target the micropyle (von Besser et al., 2006). Arabidopsis
PHATIDYLINOSITOL GLYCAN ANCHOR BIOSYNTHESIS (PIG) A, B, C, F, G, H, L, M, N, O, P, Q, V, W, X, Y (Maeda and Kinoshita, 2011). The core structure of the GPI anchor contains three Man residues donated by the substrate dolichol-phosphate-Man. GPI mannosyltransferases were required for adding the three Man residues of the GPI anchor in the ER lumen (Maeda and Kinoshita, 2011). Arabidopsis PEANUT1 (PNT1) is a homolog of the mammalian GPI mannosyltransferase PIG-M, involved in the addition of the first Man during the biosynthesis of the GPI anchor. The pnt1 mutant showed the defect of pollen viability and embryo development (Gillmor et al., 2005). PIG-B of human and GPI10 of yeast (Saccharomyces cerevisiae) encode GLYCOSYLPHOSPHATIDYLINOSITOL MANNOSETRANSFERASE3, involved in the addition of the third Man during the biosynthesis of the GPI anchor (Takahashi et al., 1996; Sütterlin et al., 1998). Mutation of PIG-B and GPI10 resulted in the accumulation of the GPI intermediate Man2-glucosamine-(acyl) phosphatidylinositol and led to cell death in yeast.

In this study, we identified the ER-localized ABNORMAL POLLEN TUBE GUIDANCE1 (APTG1), an Arabidopsis homolog of PIG-B and GPI10. Pollen tubes of the aptg1 mutant showed compromised directional growth to the micropyle and lost the apical PM localization of COB10. Besides the male defect, the mutant showed embryo lethality. In addition, reducing the expression of APTG1 resulted in defective seedling growth, indicating that APTG1 plays important roles in both reproductive and vegetative development.

RESULTS

aptg1 Showed Reduced Male Transmission

To understand the mechanism of pollen tube guidance, we tried to isolate Arabidopsis transfer DNA (T-DNA) insertion mutants showing abnormal pollen tube guidance from the Arabidopsis T-DNA insertion collection of the Arabidopsis Biological Resource Center. A pollen tube guidance mutant (SALK_080854) was identified, and it was named aptg1. In this mutant, the T-DNA was inserted in the fifth intron of the genomic sequence of AT5G14850 (1,930 bp downstream from the initiation codon ATG). The progeny genotypes of the hetrozygous plants (aptg1+/+) were identified by a PCR-based method using gene-specific primers and the T-DNA primer (Fig. 1A). However, we did not find homozygous plants in these offspring (Table I). Next, we analyzed the phenotypes of hetrozygous plants and did not observe any difference from the wild-type plants. For example, the seed number and the length of a silique of hetrozygous plants are nearly equal to those of the wild type (Supplemental Fig. S1). The genotypic ratio between the wild-type and heterozygous plants of the offspring from the heterozygous plant was about 1:0.9 (207:185; Table I), implying that the mutation at APTG1 resulted in defective pollen transmission (Table I). This result implies that APTG1 is necessary for male gametophyte function.

Pollon Germination and Tube Growth Are Not Affected by the Mutation at APTG1

To dissect whether the dysfunctional male gametophyte of the aptg1/+ mutant was due to defective pollen, we examined the viability, nuclei, and morphology...
of mature pollen grains by Alexander staining, 4,6-diamidino-2-phenylindole (DAPI) staining, and scanning electron microscopy (SEM), respectively. No difference in viability or morphology was detected between the aptg1/+ mutant and the wild-type mature pollen grains (Fig. 1, B, C, F, and G). Most pollen grains contained two generative nuclei and one vegetative nucleus in both the wild type and the aptg1/+ mutant (Fig. 1, D and E). Then, we examined the germination and tube growth of pollen in vitro to determine why the mutant allele was not transmitted via the male gametophyte. We found that the pollen germination ratio and pollen tube morphology of the aptg1/+ mutant were comparable with those of the wild type in vitro (Fig. 1, H–J). Furthermore, we measured the lengths of pollen tubes germinated in vitro for 4 h but did not find any significant difference between the aptg1/+ mutant and the wild type (Fig. 1K), indicating that the APTG1 mutation does not result in abnormal pollen germination or pollen tube growth in vitro.
Pollen Tubes of aptg1 Were Compromised in Micropylar Guidance

To investigate the defective transmission of aptg1 through male gametophytes, we calculated the transmission efficiency of the aptg1 allele by applying a limited amount (less than 40 grains) of heterozygous aptg1/+ mutant pollen to the emasculated wild-type pistil. We found that the transmission efficiency (31.8%) with limited pollination was much higher than with excessive pollen grains, but it was still less than the expected 100% (Table I), indicating that pollen with the mutant aptg1 allele had a competitive disadvantage compared with the wild type for fertilization. To further dissect why the mutant pollen was less competitive, we observed the growth of pollen tubes in pistils at 24 h after limited pollination using Aniline Blue staining and SEM. When aptg1/+ pollen was used, no abnormalities were observed during pollen tube growth in the style or transmitting tract (Supplemental Fig. S2). Then, we observed pollen tube micropylar guidance. We found that about 52.3% (n = 512) of pollen tubes grew normally and entered the micropyle (Fig. 2, A and F), but many pollen tubes showed abnormal guidance near the micropyle. These pollen tubes showing abnormal guidance can be further broken down into two categories: growth on the surfaces of the ovules or the funiculus but missing the micropyle (nontargeting; 27.4% of pollen tubes [n = 512]; Fig. 2, B, C, and F) and twisted growth around the micropylles before finally entering them (targeting; 20.3% of pollen tubes [n = 512]; Fig. 2, D and F). Additionally, two pollen tubes targeting one ovule were observed, but only one tube could enter the micropyle (Fig. 2E). Similar abnormal guidance of the aptg1/+ pollen tube was observed by SEM (Supplemental Fig. S3, A–D). These abnormal growth patterns might result from the aptg1 mutation, because theoretically, about 50% of pollen grains harbor the aptg1 allele in the aptg1/+ heterozygote. In contrast, abnormal growth of pollen tubes around the micropyle was rarely observed (0.3%; n = 450) when wild-type pollen was used for limited pollination. These results suggest that the micropylar response of the aptg1 pollen tube is compromised.

To confirm the function of APTG1, we amplified the predicted promoter (1,799 bp) and coding sequence (CDS) of APTG1, subcloned them into pCAMBIA1300 vector (conferring hygromycin resistance to transgenic plants), and introduced them into the aptg1/+ heterozygote through Agrobacterium tumefaciens-mediated infiltration. The T-DNA sequence in the aptg1/+ mutant contains a kanamycin resistance gene (Kanr). We found that the Kanr:Kans ratio was nearly 1:1, and the Kanr plants were all aptg1/+ mutants identified by the PCR-based method. Thirty independent transformants with the aptg1/+ background were obtained using kanamycin and hygromycin selection and named aptg1/+ APTG1_CDS (T1 generation). Then, we performed kanamycin resistance segregation analysis by plating the selfed progeny seeds (T2 generation) of several randomly selected lines, as described by Li et al. (2011). The Kanr:Kans ratio rose to 1.8:1 to 2.67:1 of several randomly selected lines, as described by Schwab et al. (2006). Fourteen transgenic lines expressing Late anther tomato gene52 (Lat52):amiRNA-APTG1 were obtained, and the pollen tube guidance in T2 generation transgenic plants was examined following the limited pollination transgenic pollen onto the wild-type emasculated pistils. Most of the pollen tubes showed misguidance for the micropyle under limited pollination (Supplemental Fig. S5). Therefore, the pollen tube misguidance in our study might be involved in the aptg1 mutation.

Table I. Segregation of selfed and crossed progeny of the aptg1/+ mutant and the genetic transmission of the aptg1 allele

<table>
<thead>
<tr>
<th>Progeny</th>
<th>Genotype/a</th>
<th>Ratio</th>
<th>Expected Ratio</th>
<th>Transmission Efficiencyb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APTG1/+</td>
<td>aptg1/+</td>
<td>aptg1/-</td>
<td></td>
</tr>
<tr>
<td>Self-pollination</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>♀ aptg1/+ × ♀ aptg1/+</td>
<td>207</td>
<td>185</td>
<td>0</td>
<td>1:0.89:0</td>
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<tr>
<td>♀ aptg1/+ × ♀ Wild type</td>
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<td>93</td>
<td>0</td>
<td>1:0.97:0</td>
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<tr>
<td>♀ Wild type × ♀ aptg1/+</td>
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<td>9</td>
<td>0</td>
<td>1:0.08:0</td>
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<tr>
<td>Limited pollination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♀ Wild type × ♀ aptg1/+</td>
<td>88</td>
<td>28</td>
<td>0</td>
<td>1:0.32:0</td>
</tr>
</tbody>
</table>

aThe genotypes of progeny were analyzed by a PCR-based method as described in “Materials and Methods.” bThe genetic transmission of aptg1 was calculated according to Howden et al. (1998). The transmission efficiency equals aptg1/+ APTG1/+ × 100%.

There are significant differences of transmission efficiency between them (Student’s t test, P < 0.01). cThe number of pollen grains pollinated on one stigma is less than 40.

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Functional Loss of APTG1 Affected Early Embryo Development

Our analysis demonstrated that about 20.3% of pollen tubes showed abnormal guidance but still entered the micropyle, and the transmission efficiency of the aptg1 allele through male gametophytes increased with limited pollination (Table I). However, we did not obtain any homozygous aptg1 mutants from the selfed progeny of the aptg1/+ plants. This suggested that development of the aptg1/− embryo is defective. To test this hypothesis, we observed embryo development in aptg1/+ ovaries at 3 and 4 d after limited pollination with aptg1/+ pollen. We found that most of the early embryos (88.8%) reached the globular and heart-shaped embryo stages at 3 and 4 d after pollination (Fig. 3, A and G). The development of about 11.2% of embryos was retarded at the globular embryo or earlier stages (Fig. 3, B–E and H–J), which was about half of the percentage (20.3%) of pollen tubes showing twisted growth around the micropyle, but entered the micropyle finally under limited pollination conditions. It was suggested that these retarded developmental embryos were the homozygotes of aptg1.

Furthermore, we observed that cell division was retarded after the first division of zygotes in some abnormal embryos (Fig. 3, B, C, and H). In other abnormal embryos, cell division in the embryo proper was inhibited (Fig. 3D) or the suspensor cell showed lateral overexpansion (Fig. 3E). The transverse multiple divisions and abnormal oblique division of embryo-proper cells were also observed in the homozygotic embryo of the aptg1/− line.

Table II. Segregation analysis of the selfed progeny of aptg1/+ mutant and aptg1/− APTG1_CDS lines

<table>
<thead>
<tr>
<th>Progeny</th>
<th>Kan'</th>
<th>Kan′</th>
<th>Kan'/ Kan′</th>
</tr>
</thead>
<tbody>
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<td>207</td>
<td>0.995:1</td>
</tr>
<tr>
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<td>61</td>
<td>2.03:1</td>
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<tr>
<td>aptg1/+ APTG1_CDS line 9</td>
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<td>57</td>
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<tr>
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<td>251</td>
<td>139</td>
<td>1.81:1</td>
</tr>
<tr>
<td>aptg1/+ APTG1_CDS line 18</td>
<td>147</td>
<td>55</td>
<td>2.67:1</td>
</tr>
<tr>
<td>aptg1/+ APTG1_CDS line 26</td>
<td>169</td>
<td>92</td>
<td>1.84:1</td>
</tr>
</tbody>
</table>

*aThe Kan’/Kan′ ratio of the selfed progeny of the aptg1/+ mutant was nearly 1:1, and the genotype of Kan′ plants was all aptg1/+ identified by a PCR-based method.
aptg1 mutant (Fig. 3, I and J). These retarded embryos started degenerating at the globular embryo stage (Fig. 3, F and K). In contrast, in the wild-type siliques pollinated with limited aptg1/+ pollen, the development of embryo reached the globular and heart-shaped embryo stages at 3 and 4 d after pollination, and no (n = 189) abnormal developing embryo was observed. The difference of the embryo defect percentage between the aptg1/+ and the wild-type siliques is statistically significant (Student’s t test, P < 0.01). These results imply that APTG1 is important for the vegetative development of Arabidopsis. To confirm this hypothesis, we made an amiRNA construct (35S::amiRNA-APTG1) and transformed it into Arabidopsis to decrease the expression level of APTG1. The transgenic seedling growth was inhibited markedly, indicating that APTG1 is required for seedling growth (Supplemental Fig. S6).

**APTG1 Encodes a Mannosyltransferase**

APTG1 encodes a putative protein of 548 amino acids that belongs to the Alg9-like mannosyltransferase family. Some members of this family are localized in the ER and are involved in GPI anchor biosynthesis. The mannosyltransferase domain of APTG1 contains eight transmembrane helices, indicating that APTG1 is a membrane-anchored protein (Fig. 4A). Phylogenetic analysis indicated that APTG1 shared high homology with several proteins from dicotyledons, such as Arabidopsis lyrata, Vitis vinifera, Ricinus communis, Medicago truncatula, and Glycine max (Fig. 4B). The HKEXRF motif is a conserved signature sequence for mannosyltransferase PIG-B homologs of various species (Oriol et al., 2002). Protein sequence alignments revealed an HKEXRF motif near the C terminus of APTG1 (Fig. 4C). APTG1 shares 24.8% and 34.9% identities with the yeast GPI10 and human PIG-B proteins, respectively (Fig. 4D), and both mannosyltransferases are involved in the addition of the third Man of the GPI anchor (Takahashi et al., 1996; Sütterlin et al., 1998). To determine whether APTG1 can functionally substitute for the yeast GPI10, the full-length CDS of APTG1 was cloned behind the pGAL1 promoter and transformed into a yeast gpi10 mutant YGL142c thermosensitive strain that could not grow at 37°C. The transfected yeast clone expressing the CDS of APTG1 eliminated the thermosensitivity of the gpi10 mutant. In contrast, the empty vector could not complement the phenotype of the gpi10 mutant. Thus, APTG1 might be a functional homolog of yeast GPI10 (Fig. 4E).

**Figure 3.** Early embryo development in the aptg1/+ pistil. A to F, Embryo development 3 d after limited pollination using aptg1/+ pollen and emasculated aptg1/+ pistils. A, Globular embryo showing normal development. B to F, Putative homozygous mutant embryos showing abnormal development at different stages, including retarded development at the two-cell stage (B), amorphous zygotic tissue (C), retarded apical cell division (arrow; D), abnormal suspensor cell (arrow) at the globular stage (E), and a degenerating globular embryo (F). G to K, Embryo development 4 d after limited pollination using aptg1/+ pollen and emasculated aptg1/+ pistils. G, Heart-shaped embryo showing normal development. H to K, Putative homozygous mutant embryos showing abnormal development at different stages, including a retarded and degenerating two-cell embryo (arrow; H), abnormal transverse division (arrows) of the apical cell (I), abnormal oblique division (arrows) of the apical cell (J), and a degenerating globular embryo (arrow; K). Pseudocolor was used to depict the young embryos in A to E and G. Bars = 20 μm.
APTG1 Is Localized at the ER

The expression of APTG1 in various organs was determined by quantitative real-time (qRT)-PCR. APTG1 showed higher expression in leaves, roots, stems, flowers, siliques, and pollen than in seedlings (Fig. 5A). To examine the expression patterns in detail, we detected both GUS and GFP signals in transgenic Arabidopsis harboring ProAPTG1::GUS and ProAPTG1::GFP, respectively. APTG1 was expressed in vascular tissue of seedlings, leaves, root tips, in fluorescence stems, and flowers (Fig. 5, B–E). In pistils, APTG1 expression was
detected in the ovary walls, styles, mature embryo sacs, and developing embryos (Fig. 5, F–I). In mature pollen grains at anthesis and in germinated pollen tubes, APTG1 was much more highly expressed than in young pollen grains (Fig. 5, J–M). Because both PIG-B and GPI10 are ER membrane proteins (Maeda and Kinoshita, 2011), we tried to confirm whether APTG1 was an ER localization protein. The 35S::APTG1-GFP construct was introduced into Arabidopsis plants expressing the ER marker ER-rb (Nelson et al., 2007) by A. tumefaciens-mediated infiltration and examined by confocal microscopy. We found that APTG1 showed ER localization (Fig. 6A). Furthermore, to test whether APTG1 was localized in pollen tubes, we made a ProAPTG1::APTG1-GFP construct, which was then transformed into the aptg1/+ mutant. Indeed, ProAPTG1::APTG1-GFP significantly

Figure 5. Expression pattern of APTG1. A, qRT-PCR analysis of APTG1 expression in seedlings, leaves, roots, stems, flowers, siliques, and mature pollen grains. B to F, J, and K, GUS staining analysis in transgenic plants of ProAPTG1::GUS showing the expression of APTG1 in the seedling (B), leaf (C), root tip (D), inflorescence (E), pistil (F), immature pollen (J), and mature pollen (K). The inset in K is a magnified mature pollen grain. G, H, I, L, and M, GFP fluorescence analysis in transgenic plants of ProAPTG1::APTG1-GFP showing the expression of APTG1 in the embryo sac (G), torpedo embryo (I), and pollen tube (L). H and M are bright-field images of G and L, respectively. Bars = 10 μm (K inset), 50 μm (D, G, H, J–L, and M), 100 μm (F and I), 1 mm (B and E), and 5 mm (C).
increased the transmission of the mutant aptg1 allele by introducing Pro\textsubscript{APTG1}\_GFP into the aptg1/+ mutant, suggesting the APTG1-GFP is functional in plants (Supplemental Table S1). The APTG1-GFP signal was also colocalized with the ER marker in a growing pollen tube expressing Pro\textsubscript{APTG1}\_GFP (Fig. 6B), suggesting that APTG1 is an ER-localized protein in the pollen tube.

Functional Loss of APTG1 Impairs the Apical PM Localization of COBL10 in Pollen Tubes

Our previous study showed that Arabidopsis COBL10, a GPI-AP, was important for the growth of pollen tubes in the transmitting tract of pistil (Li et al., 2013). The cobl10 pollen tubes showed the compromised guidance growth toward micropyles similar to that in the aptg1 mutant. COBL10 is a GPI-AP localized in the apical PM and cytoplasm in growing pollen tubes (Supplemental Fig. S7), and its PM localization depends on the GPI anchor (Li et al., 2013). We wondered whether the PM localization of COBL10 was altered in the aptg1 mutant, because APTG1 encodes a mannosyltransferase functioning in GPI anchor biosynthesis. Thus, we observed the localization of COBL10-citrine in the pollen tubes of the aptg1/+ mutant by introducing Pro\textsubscript{COBL10}\_SP\_citrine-COB10 into the aptg1/+ mutant (Li et al., 2013). About 54.8% of aptg1/+ pollen tubes (n = 453) showed normal COBL10-citrine localization in the apical PM and the cytoplasm (Fig. 7A, A and C), which is similar to that observed in the wild-type pollen tubes (Li et al., 2013). In contrast, some tubes (45.2%; n = 453) lost the apical PM localization of COBL10-citrine (Fig. 7B, B and C). In the control, only one pollen tube (1.1%; n = 92) showed abnormal COBL10 localization in the 92 observed pollen tubes from the wild-type plants with Pro\textsubscript{COBL10}\_SP\_citrine-COB10 (Fig. 7C). The fluorescence intensity of COBL10-citrine was very low in the apical region cytoplasm in the normal COBL10 localization of the pollen tube (Fig. 7, A and D), but the intensity was higher in the apical cytoplasm in the abnormal tube (Fig. 7, B and D). Additionally, the fluorescence intensity of COBL10-citrine in the cytoplasm of the subapical and shank regions of the aptg1 mutant pollen tube did not show any difference from that of wild-type pollen tubes (Fig. 7D). A previous study showed that the Arabidopsis receptor-like kinases LIP1 and LIP2 are PM localized in growing pollen tubes and function in guiding pollen tube growth to the micropyle (Liu et al., 2013). Because these two LIPs are not GPI-APs, we used them as the control by investigating whether their localization was also changed in aptg1 mutant pollen tubes. We found that the PM localization of LIP2-mRFP (for monomeric red fluorescent protein) did not show any changes in the aptg1/+ mutant pollen tubes expressing Pro\textsubscript{LIP2}\_LIP2\_mRFP compared with the wild-type tubes expressing Pro\textsubscript{LIP2}\_LIP2\_mRFP (Supplemental Fig. S8). These results suggest that the aptg1 mutation disrupts the localization of GPI-APs in the PM of the pollen tube.

DISCUSSION

Processing of GPI anchors mainly occurs in the ER, and at least 16 PIG proteins participate in this process (Maeda and Kinoshita, 2011). PIG-B is a human functional protein in GPI anchor biosynthesis by adding the third Man to the backbone of GPI anchors (Takahashi et al., 1996). GPT10 is a yeast homolog of PIG-B. Mutation of GPT10 led to cell death in yeast (Sütterlin et al., 1998). In this study, we showed that APTG1 is a functional homolog of PIG-B and GPT10 and is localized in the ER. The aptg1 mutant is defective in pollen tube guidance and embryo development, suggesting that APTG1 functions in GPI anchor biosynthesis. Previous studies demonstrated that defects in GPI anchor biosynthesis led...
to abnormal reproductive development in Arabidopsis. For example, Arabidopsis SETH1 and SETH2 encode homologs of PIG-C and PIG-A, respectively. The disruption of SETH1 and SETH2 in Arabidopsis resulted in reduced pollen germination and tube growth by interfering with pollen tube wall deposition or metabolism (Lalanne et al., 2004). Arabidopsis PEANUT1, encoding a putative PIG-M, is important for pollen viability and embryo development (Gillmor et al., 2005). Additionally, we found that APTG1 plays a role in seedling growth by using an RNA interference approach. These studies indicate that deficiency in GPI biosynthesis leads to defects in the vegetative and reproductive development of plants. Identification of the GPI-APs whose processing and targeting are disturbed in the Arabidopsis PIG-defect mutants would give a deeper insight into the biological roles of GPI anchors and GPI-APs in plant vegetative and reproductive development.

Deficiency in GPI biosynthesis interferes with the correct targeting of GPI-APs to the PM (Fujita and Kinoshita, 2012). However, few GPI-APs have been reported in plants. COBL10 is a GPI-AP localized in the apical PM of growing pollen tubes, and pollen tubes of the cobl10 mutants showed compromised ovule guidance (Li et al., 2013). In pollen tubes of the aptg1 mutant, the apical PM localization of COBL10 was lost. COBL10 also lost its apical membrane localization in the seth1 and seth2 mutants (Li et al., 2013).

We suggest that the defect of GPI anchor biosynthesis caused the mistargeting of COBL10. At least 47 genes expressed in pollen potentially encode GPI-APs, and at least 11 of these proteins are associated with pollen membranes by GPI anchoring (Lalanne et al., 2004). There are a number of Arabidopsis genes encoding predicted GPI-APs with enhanced expression in germinating pollen tubes in vitro or in tubes growing through the stigma and style, such as lipid-transfer protein genes and arabinogalactan protein genes (Wang et al., 2008; Qin et al., 2009). These predicted GPI-APs might be important for pollen tube growth and micropylar guidance; however, whether they are targeted to the PM and whether their localizations are altered in aptg1 mutant pollen tubes remain to be investigated. Additionally, aptg1 showed embryo lethality, which is different from cobl10 (Li et al., 2013). It is possible that the localizations of other substrates of APTG1, functioning in embryo development, were lost in aptg1. Several studies suggested that receptors localized at the apical PM of pollen tubes were candidates for perceive attractive signals from female tissues (Tang et al., 2002, 2004; Chae and Lord, 2011; Michard et al., 2011; Liu et al., 2013). The addition of a GPI anchor provides an alternative strategy for the external localization of receptor proteins on the PM (Fujita and Kinoshita, 2012). Some GPI-anchored receptors were identified in the PM of mammalian cells, such as folate receptor and urokinase-type plasminogen activator receptor (Kinoshita et al., 2008). In aptg1 pollen tubes, some GPI-anchored receptors involved in sensing the signal molecules from the female tissues might lose their membrane localization, which led to the misguidance of aptg1 pollen tubes. The identification of such GPI-anchored receptors in pollen tubes will help us understand the mechanism of pollen tube guidance by female tissues.

cobl10 pollen tubes grew slowly in the transmitting tract, which resulted from changes in the wall structure and the distribution of esterified pectins in the pollen tube (Li et al., 2013). However, the pollen tube growth of aptg1 did not show obvious abnormality, and the distribution of both esterified and deesterified pectins in pollen tubes of the aptg1 mutant was not altered (Supplemental Fig. S9). In SP-citrine-COBL10AC9, the last nine amino acids of COBL10 proteins were replaced by an Asn residue (Li et al., 2013), which may impair its biochemical function in cell wall formation due to mislocalization. In aptg1, COBL10 lost its localization at the apical PM of pollen tubes. Previous studies suggested that GPI-APs could be released from the membrane after cleavage by specific phospholipases and become free proteins that potentially serve as signals, diffusible enzymes, or structural components (Roudier et al., 2002). COBL10 was localized at the apical PM and in the cytoplasm of pollen tubes (Li et al., 2013). Here, we showed that the localization of COBL10 in the cytoplasm of the subapical and shank regions was not changed in the aptg1 pollen tubes compared with that of the wild type, and more COBL10 protein

![Image](48x504 to 287x711)

**Figure 7.** Localization of citrine-COBL10 in pollen tubes of the aptg1/+ mutant. A, Citrine-COBL10 showing normal PM localization (arrow) in the tip of the aptg1/+ pollen tube expressing ProCOBL10:SP-citrine-COBL10. B, Citrine-COBL10 showing abnormal PM localization (arrow) in the tip of the aptg1/+ pollen tube expressing ProCOBL10:SP-citrine-COBL10. C, Percentage of pollen tubes showing normal and abnormal PM localization of citrine-COBL10 (n = 453) in the wild type and the aptg1/+ mutant. D, Relative fluorescence intensity through the red lines in the tips of pollen tubes showing normal (A) and abnormal (B) PM localization expressing citrine-COBL10 in aptg1/+.

Bars = 10 μm.
was localized in the apical cytoplasm in the aptg1 mutant (Fig. 7D). COBL10 localized in the cytoplasm of the aptg1 pollen tubes may still function in cell wall construction and pollen tube growth. The different COBL10 distributions between the cobl10 and aptg1 mutants might result in the difference of pollen tube phenotypes in these mutants.

Most PIG proteins involved in GPI biosynthesis are localized in the ER (Maeda and Kinoshita, 2011). In this study, we demonstrated that APTG1 is also an ER-localized protein. Immunoblot analysis showed that APTG1 is only detected in the microsomal fraction, indicating that APTG1 is an integral membrane protein (Supplemental Fig. S10). Thus, the localization of APTG1 is similar to that of yeast PGI10. To date, several male factors that are endomembrane localized have been shown to be involved in the perception of female cues during pollen tube growth. For example, the Arabidopsis sperm cell-specific protein HAPLESS2/GENERATIVE CELL-SPECIFIC1, which is involved in pollen tube micropylar targeting, is mainly localized in the ER membrane (von Besser et al., 2006), whereas the Arabidopsis Rab GTPase RABA4D is localized in the ER membrane (von Besser et al., 2006), whereas pollen tube micropylar targeting, is mainly localized in the ER membrane (von Besser et al., 2006), whereas the Arabidopsis sperm cell-specific protein HAPLESS2/GENERATIVE CELL-SPECIFIC1, which is involved in pollen tube micropylar targeting, is mainly localized in the ER membrane (von Besser et al., 2006), whereas the Arabidopsis sperm cell-specific protein HAPLESS2/GENERATIVE CELL-SPECIFIC1, which is involved in pollen tube micropylar targeting, is mainly localized in the ER membrane (von Besser et al., 2006), whereas the Arabidopsis sperm cell-specific protein HAPLESS2/GENERATIVE CELL-SPECIFIC1, which is involved in pollen tube micropylar targeting, is mainly localized in the ER membrane (von Besser et al., 2006), whereas

GENERATIVE CELL-SPECIFIC1, which is involved in the Arabidopsis sperm cell-specific protein HAPLESS2/GENERATIVE CELL-SPECIFIC1, which is involved in pollen tube micropylar targeting, is mainly localized in the ER membrane (von Besser et al., 2006), whereas

was shown to be involved in the perception of male factors that are endomembrane localized proteins involved in pollen tube guidance require as well as receptor maturation during cell signaling in these mutants.

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) SALK_080854 seeds were obtained from The Arabidopsis Information Resource seed stock center. The genotypes of SALK_080854 mutant plants were determined by a PCR-based method (primers L8B.3, LPI, and RPI). Seeds of the mutant and transgenic lines were plated on Murashige and Skoog (MS) medium containing kanamycin (25 mg L⁻¹), hygromycin (150 mg L⁻¹), and 1% (w/v) agarose. The pollen tubes and pistils from transgenic plants expressing PAP1 (Wang et al., 2015) were used for further analysis of the subcellular localization of APTG1. For the subcellular localization of APTG1, the 35S::PAP1-GFP construct was introduced into Arabidopsis plants expressing ER::GFP. The pollen tubes expressing PAP1-GFP were selected with 0.5 mg L⁻¹ kanamycin and 30 mg L⁻¹ hygromycin. After 6 weeks, the transgenic lines were selected on the kanamycin and hygromycin-containing medium. The pollen tubes and pistils from transgenic plants expressing PAP1 (Wang et al., 2015) were used for further analysis of the subcellular localization of APTG1.
in pollen tubes. For the localization analysis of COBL10 and LIP2 in the aptg1/+ mutant, we used pollen tubes expressing Pro_COBL10::SP-citrine-COBL10 in coo10 and Pro_LIP2::mRFP in the aptg1/+ mutant background, respectively. APTG1-GFP was excited at 488 nm, and emissions were collected at 505 to 530 nm. ER-rb and ER-Tracker Red were excited using a 561-nm laser, and the emissions were observed at 600 to 630 nm. COBL10-citrine and LIP2-mRFP were excited at 514 and 561 nm, and the emissions were collected at 525 to 600 nm and 570 to 660 nm, respectively. Immunofluorescence labeling was performed with JIM5 and JIM7 as described (Li et al., 2013). Images were captured with a TCS SP5 II confocal laser scanning microscope (Leica).

GUS Assays

GUS staining was performed according to Sieburth and Meyerowitz (1997). Flowers, siliques, and seedlings were incubated in GUS staining solution [1 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Biosynth), 2 mM K₃Fe(CN)₆, 2 mM K₄Fe(CN)₆, and 0.1% (v/v) Triton X-100 in 50 mM sodium phosphate buffer, pH 7] for 2 to 3 d at 37°C. The stained samples were cleared in 70% (v/v) ethanol. Stained specimens were visualized and photographed with an Olympus BX51 microscope equipped with a CCD camera.

Complementation of the Yeast aptg1 Mutant

For complementation of the yeast (Saccharomyces cerevisiae) aptg1 mutant, the CDS regions of APTG1 were amplified with primers APTG1H0-5’ and APTG1H0-A and ligated to yPS2. The genotype for the wild-type strain BY4741 is MATα his3Δ0/MET15 can1Δ0 ura3Δ0. The aptg1 deletion mutant YGL142c thermosensitive strain was derived from the yeast magic marker heterozygous gene deletion collection (MATα/a ura3Δ0/ura3Δ0 leu2Δ1/leu2Δ1 his3Δ1/hi3Δ1 lys2Δ0/lys2Δ0 MTE15 can1Δ1-LEU2-MFA1pr::His3/CAN1 yigΔ1-KanMX/FPC). The haploid YGL142c thermosensitive mutant had the following genotype: MATα ura3Δ0 leu2Δ1 his3Δ1 lys2Δ0 (or lys2Δ0 MTE15 can1Δ1-LEU2-MFA1pr::His3 yigΔ1-KanMX-FPC). The selective plate contained 20 g L⁻¹ Gal, 10 g L⁻¹ yeast extract, and 2 g L⁻¹ peptone. All primer sequences are listed in Supplemental Table S2.

Sequence data from this article can be found in the GenBank/EMBL database under accession numbers NP_568305.1 (APTG1), AAH17731.3 (PGI-B), and DAA07986.1 (GPI10).

Supplemental Data

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

Supplemental Figure S1. The plants and number of seeds of the wild type and aptg1/+ mutant.

Supplemental Figure S2. Growth of pollen tubes of the wild type and aptg1/+ in the style and transmitting tract of the wild type.

Supplemental Figure S3. Analysis of aptg1/+ pollen tube guidance by scanning electron microscopy.

Supplemental Figure S4. Micropylar guidance of pollen tubes of a complementary transgenic line of aptg1/+ APTG1_CDS.

Supplemental Figure S5. Micropylar guidance of pollen tubes of transgenic lines expressing Late52:amiRNA-APTG1.

Supplemental Figure S6. The T3 seedling of 35S:amiRNA-APTG1 transgenic lines.

Supplemental Figure S7. The COBL10 subcellular localization observed in the wild type pollen tube.

Supplemental Figure S8. The plasma membrane-localization of LIP2 in the wild type and aptg1/+ mutant pollen tubes.

Supplemental Figure S9. Immunofluorescence labelling of pectins in semi-in vivo pollen tubes.

Supplemental Figure S10. Immunoblot analysis for the localization of APTG1.

Supplemental Table S1. Segregation analysis of the selected progeny of aptg1/+ mutant and aptg1/+ APTG1_CDS::GFP lines.

Supplemental Table S2. Sequences of the primers.

ACKNOWLEDGMENTS

We thank all persons who provided materials for this study. The origins of the materials were as follows: Pro_COBL10::SP-citrine-COBL10 transgenic plants (Dr. Yan Zhang, Shandong Agricultural University); Pro_LIP2::mRFP seeds (Dr. Li Ge, Peking University); ER-rb seeds (Dr. Lei Ge, Shandong Agricultural University); yeast BY4741 and YGL142c thermosensitive strains (Dr. Jundiao Dai, Tsinghua University); antibodies against PDI11 and CNXI (Dr. Qi Xie, Chinese Academy of Sciences); pRS500 vector (Dr. Detlef Weigel, Max Planck Institute for Developmental Biology); and T-DNA insertion line SALK_080854 (Arabidopsis Biological Resource Center).

Received January 17, 2014; accepted June 23, 2014; published June 24, 2014.

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