A Mutation in the Catalytic Subunit of the Glycosylphosphatidylinositol Transamidase Disrupts Growth, Fertility, and Stomata Formation[OPEN]

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GPI-anchored proteins (GPI-APs) are essential for plant growth and development; knockout mutations in enzymes responsible for anchor biosynthesis or attachment are gametophyte or embryo lethal. In a genetic screen targeted to identify genes regulating stomata formation, we discovered a missense mutation in the Arabidopsis (Arabidopsis thaliana) homolog of GPI8/PIG-K, a Cys protease that transfers an assembled GPI anchor to proteins. The Arabidopsis genome has a single copy of AtGPI8, and the atgpi8-1 mutation reduces the efficiency of this enzyme, leading to reduced accumulation of GPI-anchored proteins. While the atgpi8-1 mutation strongly disrupts plant growth, it is not lethal. Phenotypic analysis of atgpi8-1 mutants suggests that GPI-APs are important for root and shoot growth, stomata formation, apical dominance, transition to flowering, and male gametophyte viability. In addition, atgpi8-1 mutants accumulate higher levels of callose and have reduced plasmodesmata permeability. Genetic interactions of atgpi8-1 with mutations in ERECTA family (ERf) genes suggest the existence of a GPI-AP in a branch of the ERf signaling pathway that regulates stomata formation. Activation of the ERf signal transduction cascade by constitutively active YODA rescues stomata clustering in atgpi8-1, indicating that a GPI-AP functions upstream of the MAP kinase cascade. TOO MANY MOUTHS (TMM) is a receptor-like protein that is able to form heterodimers with ERfs. Our analysis demonstrates that tmm-1 is epistatic to atgpi8-1, indicating that either TMM is a GPI-AP or there is another GPI-AP regulating stomata development whose function is dependent upon TMM.

A protein can be integrated into a membrane through a polypeptide sequence or via a lipid anchor. While the modification of proteins with lipids is universal, the structure of the lipid anchors and the mechanism of their attachment differ in prokaryotes and eukaryotes. Several different types of lipids attach eukaryotic proteins to the inner leaflet of the plasma membrane. The attachment of eukaryotic proteins to the outer leaflet occurs only via GPI anchors. The biosynthesis of GPI glycolipid is a multistep process that relies on more than 20 proteins (Maeda and Kinoshita, 2011). It begins on the cytoplasmic side of the endoplasmic reticulum (ER) and continues to the luminal side where the anchor is attached to the C terminus of a protein. The GPI is transferred en bloc by the GPI transamidase (GPI-T) complex consisting of five proteins (Kinoshita, 2014). From the ER, GPI-anchored proteins (GPI-APs) are transported to the Golgi where the anchor undergoes remodeling. The backbone structure of a GPI anchor is highly conserved, but it can be remodeled in a variety of ways (Fujita and Kinoshita, 2012).

Proteins destined to acquire a GPI anchor have two signal peptides. On the N terminus there is an ER targeting signal, and on the C terminus there is a signal directing the attachment of the GPI anchor. The latter is usually 17 to 31 amino acids long; it has no consensus sequence but has four specific regions: an unstructured, approximately 10 amino acid-long linker region; small amino acids at the site of GPI anchor addition (the ω site) and the ω+2 site; a hydrophilic spacer region of 5 to 10 amino acids; and a hydrophobic tail of 15 to 20 amino acids. Efficient cleavage and addition of a GPI anchor depends on the marginal hydrophobicity of the C-terminal region when proprotein possibly transitions from a transmembrane form to a soluble form.
within the ER (Galian et al., 2012). Gpi8p/PIG-K and GPA1/GAA1 are the catalytic subunits of GPI-T (Kinoshita, 2014). Gpi8p/PIG-K is a Cys protease that hydrolyzes a peptide bond between residues ω and ω+1. GPA1/GAA1 catalyzes the next step, which is the formation of a bond between the ω site amino acid and the terminal ethanolamine phosphate of GPI (Eisenhaber et al., 2014). Failure to cleave the GPI attachment signal from precursor proteins prevents their O-glycosylation and leads to the retention of corresponding proteins in the ER, where they are subsequently degraded (Field et al., 1994).

The intracellular transport and localization of GPI-APs differs from that of proteins with transmembrane domains (Muñiz and Zurzolo, 2014). Both types of proteins are transported from the ER to the Golgi by COPII vesicles, but transmembrane proteins are directly recruited on the cytoplasmic side by Sec23/24 proteins while GPI-APs have to be recognized by p24 transmembrane cargo receptors (Muniz and Riezman, 2015). In mammalian epithelial cells, GPI anchors promote polar transport of GPI-APs to the apical membrane (Lisanti et al., 1989; Brown and Rose, 1992). Multiple lines of evidence suggest that GPI-AP association with lipid rafts, sphingolipid and cholesterol-rich nano-scale domains in the membrane, is critical for their trafficking and function (Maeda and Kinoshita, 2011).

According to the UniProt database, a eukaryotic organism can contain anywhere from 60 to 160 different GPI-APs, including receptors, adhesion molecules, proteoglycans, and various enzymes. This is an approximate number, since computational predictions are imprecise and proteomic approaches can underestimate the number of GPI-APs due to some having a low abundance. GPI-APs are essential for the viability of yeast and protozoa (Leidich et al., 1994; Nagamune et al., 2000). The loss of GPI anchoring is embryonic lethal in mammals and plants (Nozaki et al., 1999; Lalanne et al., 2004; Gillmor et al., 2005). In plants, GPI-APs are involved in various metabolic and developmental processes, including cellulose biosynthesis, callose metabolism, morphogenesis, and different aspects of reproduction (Schindelman et al., 2001; Lalanne et al., 2004; Gillmor et al., 2005; Levy et al., 2007; Capron et al., 2008; Simpson et al., 2009; Harpaz-Saad et al., 2011; Li et al., 2013; Cheung et al., 2014). Here, for the first time to our knowledge, we investigate the role of GPI-APs in stomata formation.

Stomata complexes are formed by a series of cell divisions and cell fate transitions. The pathway is initiated by asymmetric division in a subset of protodermal cells called meristemoid mother cells. A meristemoid mother cell gives rise to a smaller triangular meristemoid cell and a larger stomatal lineage ground cell. In Arabidopsis (Arabidopsis thaliana), meristemoids typically undergo one to three rounds of asymmetric division, after which they differentiate into an oval guard mother cell, which then divides symmetrically to generate a pair of guard cells. In the wild type, two stomata do not form directly adjacent to each other; they are separated by at least one pavement cell (Sachs, 1991). Stomata formation is regulated by both positive and negative regulators. The negative regulators are Leu-rich repeat receptor-like kinases from the ERECTA family (ERFs; Shpak et al., 2005). In Arabidopsis, this family consists of three genes: ERECTA, ERL1, and ERL2. The activity of these receptors is regulated by a family of secreted Cys-rich peptides from the EPF/EPFL family (Shimada et al., 2011). The agonist and antagonist ligands compete for binding to the EPFL receptor complexes fine-tuning stomata patterning in an organ specific manner (Lee et al., 2015). TOO MANY MOUTHS (TMM) is a receptor-like protein also involved in regulation of stomata development (Nadeau and Sack, 2002). It can form heterodimers with ERECTA and ERL1 in vivo and has been shown to directly bind EPF2 and STOMAGEN but not EPF1 in vitro (Lee et al., 2012, 2015). While TMM was the first gene linked with regulation of stomata development (Yang and Sack, 1995), its precise molecular function is still unclear. The phenotype of tmm mutants is organ specific (Geisler et al., 1998). In cotyledons and on the abaxial side of sepals and rosette leaves, this mutation increases the number of stomata and leads to their clustering. In stems, there are no stomata, and meristemoids differentiate into pavement cells instead of guard mother cells. Based on these mutant phenotypes, TMM seems to both inhibit and promote stomata development in an organ-specific manner. Recently, it was hypothesized that these seemingly opposing functions of TMM in leaves and stems might be related to the different availability of ligands in those organs rather than differences in TMM function (Abrash et al., 2011; Shpak, 2013).

To further our understanding of the mechanisms regulating stomata development, we have analyzed a novel mutant exhibiting increased stomata clustering. This mutant was found in an enhancer genetic screen that used Arabidopsis erl1 erl2 mutations as a background. Positional cloning determined that the mutation had occurred in a gene homologous to yeast GPI8, a gene encoding one of the catalytic subunits of an enzymatic complex responsible for the attachment of GPI anchors to selected proteins. We named the mutant atgpi8-1 and examined it for its potential to illuminate the importance of GPI anchoring for plant growth and stomata formation. Our analyses suggest that one or more GPI-anchored protein(s) functions in the ERf/TMM signaling pathway.

RESULTS

Positional Cloning of atgpi8-1

A search was performed in a population of ethyl methanesulfonic acid-mutagenized M2 erl1 erl2 seedlings for mutants containing stomata clusters. The screen led to the identification of the 2094 mutant. Through map-based cloning the mutation was determined to be located on the long arm of chromosome 1 between bp
A sequence analysis of genes in this region uncovered a mutation in At1g08750; a G to A substitution at base pair 125 that resulted in replacement of Arg42 to Gln42 (Fig. 1B). Homology analysis of At1g08750 revealed that the protein has high amino acid sequence similarity to *Saccharomyces cerevisiae* GPI8 (77%) and *Homo sapiens* PIG-K (69%; Fig. 1C). GPI8 and PIG-K encode one of the catalytic subunits of the GPI-T. Genome data suggest that the overall mechanism of GPI anchoring is conserved between plants, yeast, and animals, since homologs for most essential genes are present (Eisenhaber et al., 2001, 2003a).

Since Arabidopsis contains only one gene similar to GPI8/PIG-K, At1g08750 is most likely a catalytic subunit of plant GPI-T; we therefore named the gene *AtGPI8* and the mutation *atgpi8-1*. Although knockout of GPI8 is lethal in *S. cerevisiae*, a mutation in the first conserved His produces a partially functional GPI8 (Benghezal et al., 1996; Meyer et al., 2000). The Arg42 to Gln42 mutation is one amino acid ahead of this residue. The *atgpi8-1* mutation does not affect expression of the gene at the transcriptional level (Supplemental Fig. S1).

To confirm that the observed phenotype is due to the mutation in *AtGPI8*, we performed allelic analysis using an available T-DNA insertion line, CS853564 or *atgpi8-2*. The *atgpi8-2* mutant is distributed as a heterozygous line, and it does not segregate out homozygous plants. Genotyping of 64 *atgpi8-2/+* plant offspring identified 36% of *atgpi8-2/+* and 66% of the wild type. For an allelism test, we crossed *atgpi8-1* with *atgpi8-2/+* and genotyped the F1 progeny. The identified *atgpi8-1/atgpi8-2* seedlings displayed strong stomata clustering in cotyledons (Fig. 2). In addition, *atgpi8-1/atgpi8-2* plants were severely dwarfed, never flowered, and did not survive into maturity (Supplemental Fig. S2).

To test if other phenotypes were due to the *AtGPI8* mutation, we expressed *AtGPI8-EGFP* under its endogenous promoter in *atgpi8-1* mutants, which led to rescue of the majority of the observed phenotypes (Supplemental Fig. S3). These results confirmed that the positional cloning identified the mutation responsible
for the phenotypes observed in the 2094 mutant. Unfortunately, the AtGPI8-EGFP construct is not useful for monitoring expression of AtGPI8, since no fluorescence was detected.

To determine whether the atgpi8-1 mutation alters the metabolism of GPI-APs, we outcrossed the GPI-anchored protein GFP-SKU5 into the mutant (Sedbrook et al., 2002). We observed a decreased accumulation of GFP-SKU5 in the membrane protein fraction compared with the transmembrane receptor-like protein BAK1 (Fig. 3). When a GPI anchor cannot be transferred to the corresponding protein, it is retained in the ER and degraded (Field et al., 1994). For example, in plt mutants deficient in biosynthesis of GPI anchors, the GFP-SKU5 protein and several other GPI-APs could not be detected (Gillmor et al., 2005). Therefore, the reduced accumulation of GPI-APs in atgpi8-1 suggests that the mutation leads to a partial loss of enzyme function.

The atgpi8 Mutations Affect Many Developmental Processes

Studies of mutations that disrupt the GPI anchoring process suggest that it is essential during the early stages of organism development, since mutants rarely survive past embryogenesis (Leidich et al., 1994; Nozaki et al., 1999; Lalanne et al., 2004; Gillmor et al., 2005). The identification of the nonlethal atgpi8-1 mutation affords us a unique opportunity to explore the importance of GPI anchoring during later stages of plant development. In atgpi8-1, the early postgermination growth of above-ground organs is very minimally affected, with 15-d-old cotyledons and first rosette leaves being of similar size in the mutant and the wild type (Fig. 4, A–C). However, root growth and hypocotyl elongation are significantly reduced in mutant seedlings of the same age (Fig. 4, A, H, and I). Leaves formed later in development are smaller in atgpi8-1, and at day 30 the wild-type and atgpi8-1 plants noticeably differ in size (Fig. 4D). The atgpi8-1 mutation also leads to reduced internode and pedicel elongation that results in formation of more compact inflorescence apices (Fig. 4, F, G, L, and N). The final height of atgpi8-1 plants is only moderately reduced since the number of internodes is increased (Fig. 4, K and O). An increased number of cauline branches in atgpi8-1 suggests that GPI anchoring is important for axillary shoot formation (Fig. 4M). The transition to flowering in atgpi8-1 is delayed, with the mutant plants bolting at 39.5 ± 3.3 d (±sd here and below) versus at 21.1 ± 1.1 d in the wild type (Fig. 4, E and J). All these phenotypes are rescued by expression of AtGPI8-EGFP in atgpi8-1 (Supplemental Fig. S3). However, the root elongation defects are only partially rescued, which could be due to additional mutations retained after three backcrosses or because the selected promoter region did not contain all of the necessary elements for the correct expression of the gene.

Since the progyn of atgpi8-2/+ plants contained only heterozygotes and no homozygotes, we investigated gametophyte viability using reciprocal crosses between the wild type and atgpi8-2/+ . The cross with atgpi8-2/+ as a male produced F1 progeny that were all wild type (20 plants genotyped), suggesting that this mutation leads to male gametophyte lethality. The cross with atgpi8-2/+ as a female produced F1 that was 43% atgpi8-2/+ and 57% wild type (37 plants genotyped), suggesting that the atgpi8-2 mutation does not have a strong impact on female gametophyte viability. The T-DNA insertion of atgpi8-2 includes a BASTA resistance gene. The progeny of atgpi8-2/+ contained 54.5% BASTA sensitive seedlings that were not statistically different from the 50% expected from plants with nonfunctional male gametophyte and viable female gametophyte (n = 87; χ² = 0.87). As only 16.1% of atgpi8-1/+ progeny were mutants, which is significantly less than the expected 25% (n = 371; χ² = 3.89, P = .0485), male gametophyte viability is likely reduced in atgpi8-1 as well.

Our analysis of atgpi8 mutants implies that GPI-APs play important roles in multiple developmental processes, including lateral organ growth, axillary shoot formation, and transition to flowering. In addition, GPI-APs are essential for male gametophyte viability but do not have a strong effect on female gametophytes.

Figure 3. Expression of GFP-SKU5 is decreased in atgpi8-1. A, Protein gel detection of GFP-SKU5 and BAK1 in the membrane fractions of 8-d-old seedlings. B, A Coomassie-stained gel shows the total proteins in the membrane fraction.
The \textit{atgpi8-1} Mutant Has Decreased Plasmodesmata Conductivity

\textit{Atgpi8-1} was isolated as a mutant by its substantial stomata clustering (Fig. 2B). One of the potential causes of stomata cluster formation is an increase in plasmodesmata conductivity as in \textit{chorus} and \textit{kobito1-3}, two mutants with stomata clustering and multiple other developmental defects (Guseman et al., 2010; Kong et al., 2012). The accumulation of callose at the neck regions of plasmodesmata has a strong impact on conductivity; a decrease in callose deposition leads to plasmodesma opening (Iglesias and Meins, 2000; Levy et al., 2007; Guseman et al., 2010). Aniline blue staining detected increased callose accumulation in \textit{atgpi8-1}, which was particularly evident in the vasculature and thick inner walls of guard cells (Fig. 5, A and B). To determine if the \textit{atgpi8-1} mutation also has an impact on the proper size exclusion limit of plasmodesmata, we performed a cell to cell mobility assay. Two plasmids, one carrying a gene encoding GFP and the other a gene encoding ER-localized RFP, both under control of the 35S cauliflower mosaic virus (CaMV) promoter, were co-bombarded into the abaxial epidermis of 7-d-old seedlings. During bombardment, the particle gun usually transforms individual epidermal cells that can be confirmed by analysis of RFP expression, as this protein cannot move to neighboring cells due to its ER retention. If plasmodesmata are open, GFP can be detected in the surrounding cells due to diffusion. In wild-type seedlings, we observed that in 85% of transformation events, GFP was able to move to the neighboring cells while no GFP movement was observed in \textit{atgpi8-1} seedlings (Fig. 5, C and D). The average cluster size of cells expressing GFP was 4.0 ± 2.1 (±SD) for the wild type and 1.0 ± 0 for \textit{atgpi8-1}. These data strongly suggest that plasmodesmata conductivity in \textit{atgpi8-1} is significantly decreased, and therefore formation of stomata clusters cannot be caused by changes in the plasmodesmata structure.

Synergistic Interactions of ERfs and AtGPI8 during Stomata Formation

Since GPI anchoring has not previously been associated with stomata formation, we were especially interested to investigate the impact of the \textit{atgpi8-1} mutation...
Role of GPI Anchoring in Plant Development

To investigate whether a potential GPI-AP functions in the ER signaling pathway, we analyzed genetic interactions between mutants of ERf genes and atgpi8-1. In cotyledons and rosette leaves, stomata formation is not altered by addition of the er or erl1 erl2 mutations to atgpi8-1, possibly because the stomatal phenotype of atgpi8-1 is already very strong in those organs (Fig. 6, A and B). Thus, in cotyledons and rosette leaves, the SI and the percent of stomata in clusters are identical in atgpi8-1 and er erl1 erl2 (Fig. 6, A and B). However, in stems and pedicels where the atgpi8-1 phenotype is not so strong, we observed synergistic interactions between atgpi8-1 and er and between atgpi8-1 and erl1 erl2 (Fig. 6). For example, in stems the atgpi8-1, er, and erl1 erl2 mutations do not increase SI on their own, but the combination of er or erl1 erl2 with atgpi8-1 increases SI to 21.5 ± 0.9% and 32.3 ± 5.3%, respectively, from 14.3 ± 2.8% (±SD here and below) in the wild type (Fig. 6A). In pedicels of atgpi8-1, only 3.2 ± 4.9% of stomata are in clusters and no stomata clusters were detected in wild type, er, or erl1 erl2 pedicels. However, pedicels of atgpi8-1 er and atgpi8-1 erl1 erl2 contained 24.3 ± 7.1% and 39.4 ± 9.2% of stomata in clusters, respectively. At the same time, in stems and pedicels the synergistic interaction between erl1 erl2 and atgpi8-1 does not increase the SI or the percent of stomata in clusters to the same level as in er erl1 erl2 (Fig. 6, A and B).

To further investigate the connection between GPI anchoring and the ER signaling pathway, we analyzed genetic interactions of AtGPI8 with YODA (YDA), a MAPKK kinase that functions downstream of TMM and the ERfs (Bergmann et al., 2004; Meng et al., 2012). The N terminus of YDA contains a negative regulatory domain, and its deletion produces a constitutively active YDA (CA-YDA) that inhibits stomata development and promotes stem and pedicel elongation when expressed in the wild type (Bergmann et al., 2004). We transformed the CA-YDA construct into atgpi8/+ and then analyzed stomata development in F2 progeny of a transgenic line selected based on increased length of pedicels and stems. The F2 progeny segregated CA-YDA in wild-type and atgpi8-1/+ backgrounds as well as CA-YDA atgpi8-1 plants. While in this particular line CA-YDA did not statistically significantly change development of stomata on its own (P < 0.04), it was able to partially rescue atgpi8-1 plants, decreasing both SI and stomata clustering in the mutant (Fig. 7, A and B). Thus, the GPI-anchored protein that functions in stomata formation is likely to be upstream of YDA. It has been previously reported that a GSK3 kinase regulates stomata development downstream of TMM and the ERfs and upstream of YDA (Kim et al., 2012). To further validate the existence of a GPI-anchored protein in the ER signaling pathway, we examined whether bikinin, a GSK3 kinase inhibitor, could rescue the stomata phenotype of atgpi8-1. Similar to its effect on stomata development in tmm and er erl1 erl2, bikinin decreased the stomata index and stomata clustering in atgpi8-1 seedlings (Fig. 7, C–H). Growth in the presence of bikinin

Figure 5. Increased callose accumulation and decreased plasmodesmata conductivity of atgpi8-1. A and B, The accumulation of callose in 17-d-old cotyledons of atgpi8-1 (B) is increased compared to wild type (A) as determined by aniline blue staining. C and D, Analysis of GFP movement in the epidermis of 7-d-old seedlings suggests decreased plasmodesmata conductivity in atgpi8-1. C, Representative images of the abaxial side of the epidermis expressing cobombarded ER-localized RFP (left), GFP (center), and both merged (right). D, Distribution analysis of the number of cells in clusters expressing GFP from a single transformation event based on RFP expression; n = 40, P < 0.0001.

on epidermis differentiation. Analysis of the epidermis in cotyledons, rosette leaves, stems, and pedicels demonstrated an increase in both the stomatal index (SI) and stomata clustering in atgpi8-1 versus the wild type (Fig. 6). The change in epidermis development is especially dramatic on the abaxial side of cotyledons and leaves, with SI being increased 2.5 times in atgpi8-1 cotyledons and 2.3 times in atgpi8-1 leaves. While <1% of stomata are in clusters in the wild type, 72.2 ± 6.3% and 33.9 ± 8.9% of stomata are in clusters on the abaxial side of atgpi8-1 cotyledons and rosette leaves, respectively. These data suggest the existence of a GPI-anchored protein that works to inhibit stomata formation.

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decreased SI on the abaxial side of atgpi8-1 cotyledons from 73 ± 12% to 33 ± 8% and stomata clustering from 70 ± 14.8% to 16 ± 12%. Thus, a GPI-anchored protein is likely to function upstream of a GSK3 kinase. Taken together, these data strongly suggest that a GPI-AP regulating formation of stomata functions in the ERf signaling pathway upstream of the MAP kinase cascade.

The tmm-1 Mutation Is Epistatic to the atgpi8-1 Mutation

The receptor-like protein TMM forms heterodimers with ERfs (Lee et al., 2012). In contrast to ERfs that always inhibit stomata development, TMM inhibits stomata development in cotyledons and leaves but promotes it in stems and pedicels (Geisler et al., 1998). To understand the genetic interactions between TMM and GPI-AP involved in stomata formation, we outcrossed atgpi8-1 into the tmm-1 mutant and analyzed SI and stomata clustering. Cotyledons and leaves of tmm-1 and atgpi8-1 have very similar phenotypes: increased SI and massive stomata clustering (Fig. 8, A and B). The only difference observed was slightly weaker clustering of stomata in atgpi8-1 (Fig. 8, A and B). No additive effects of the tmm-1 and atgpi8-1 mutations were observed in leaves and cotyledons, and the phenotype of tmm-1 atgpi8-1 was identical to the phenotype of tmm-1 (Fig. 8, A, B, and E), suggesting that tmm-1 is epistatic to atgpi8-1.

DISCUSSION

The GPI-anchor biosynthesis pathway has been studied intensively in yeast and in humans, but less is known about this pathway in plants. In Arabidopsis, homologous genes exist for almost all the known components of the GPI anchoring pathway, but currently the only genes that have been studied are SETH1, SETH2, PEANUT1 (PNT1), and APTG1 (Lalanne et al., 2004; Gillmor et al., 2005; Dai et al., 2014). These genes encode enzymes that catalyze specific steps in the biosynthesis of the anchor. In this paper, we describe a mutation in a protein that is required for attachment of the anchor to the designated proteins. The yeast and mammal GPI transamidase complexes consist of five subunits, two of which have enzymatic activity (Kinoshita, 2014). Arabidopsis has homologs of all five GPI-T subunits GPI8p/PIG-K/AtGPI8 is the most conserved. Amino acid sequence similarity of AtGPI8 is 77% with S. cerevisiae Gpi8p and 69% with H. sapiens PIG-K. The similarity of other Arabidopsis GPI-T subunits with their human and yeast
homologs is <50%. In a genetic screen, we identified a point mutation in At1g08750 that results in replacement of Arg42 to Gln42. Gpi8p/PIG-K/AtGPI8 is a Cys protease with a Cys/His catalytic dyad that hydrolyzes a peptide bond in proteins containing a signal for GPI anchoring. Mutation of either Cys or His in the active site of Gpi8p abolishes activity of the enzyme (Meyer et al., 2000). The mutation of another His (in yeast His-54) leads to partial loss of Gpi8p function (Meyer et al., 2000). The amino acid substitution in atgpi8-1 is directly adjacent to His-43 that is homologous to yeast His-54. Accumulation of the GPI-AP SK5 is reduced in the atgpi8-1 mutant, suggesting a partial loss of function due to the Arg-42 mutation. Thus, our results corroborate the importance of this region for Gpi8p/PIG-K/AtGPI8 subunit function.

Studies of mutants with damaged biosynthesis and attachment of the GPI anchor reveal functions of GPI-APs during plant growth and development. Previous analysis of mutations in PNT1, SETH1, SETH2, and APTG1 demonstrated that GPI-APs are essential for male fertility. In seth1 and seth2, pollen grains develop normally but pollen tube germination and growth are drastically reduced, and pnt1 is characterized by reduced pollen transmission (Lalanne et al., 2004; Gillmor et al., 2005). In the aptg1 mutant, pollen tubes are able to elongate but suffer a reduced ability to find microspores (Dai et al., 2014). Unsurprisingly, the atgpi8-2 knockout mutation cannot be transmitted through the male gametophyte.

GPI-APs play important roles during embryogenesis. Aptg1 embryos are arrested at a globular stage and pnt1 embryos undergo delayed morphogenesis (Gillmor et al., 2005; Dai et al., 2014). The pnt1 mutants are seedling lethal and can proliferate only as callus, indicating that GPI-APs are required for coordinated multicellular growth (Gillmor et al., 2005). As a consequence, the role of GPI-APs in the later stages of plant development has been investigated only on the level of individual proteins. This study of the atgpi8-1 mutant allowed us to explore the role of GPI-APs on a more global scale at the later developmental stages. First, we noticed that root growth is sensitive to interference with GPI-AP biosynthesis. Since GPI-APs promote the accumulation of crystalline cellulose and decrease the accumulation of xylloglucans, pectins, and callose in the cell wall, they are expected to have a strong impact on cell elongation (Gillmor et al., 2005). Likewise, specific GPI-APs such as COBRA, SOS5, and SKU5 have been linked with cell elongation, including expansion of cells in roots (Schindelman et al., 2001; Sedbrook et al., 2002; Shi et al., 2003; Roudier et al., 2005; Xu et al., 2008). Our analysis of atgpi8-1 suggests that GPI-APs promote plasmodesmata permeability and are essential for reduced callose accumulation. This is not surprising, as β-1,3-glucanases, enzymes that degrade callose, are GPI-APs (Elortza et al., 2003; Elortza et al., 2006), and mutation of AtBG_ppap, a plasmodesmata-localized β-1,3-glucan synthase, increases callose accumulation and reduces plasmodesmata conductivity (Levy et al., 2007).

We speculate that increased callose accumulation in atgpi8-1 might disrupt proper root development due to impaired cell-to-cell communication as previously observed in gain-of-function mutants of the callose synthase CALS3 (Vatén et al., 2011). Our other findings concern the importance of GPI-APs for apical dominance
and transition to flowering. Whether those developmental processes are regulated by specific GPI-APs through changes in callose deposition is a question for the future. The callose lining in sieve plate pores is essential for normal phloem transport and as a result for the movement of FLOWERING LOCUS T (Barratt et al., 2011; Rinne et al., 2011).

We also find that GPI-APs are important for stomata formation. Analysis of genetic interactions uncovered a synergy between AtGPI8 and the ERf genes implying the existence of a GPI-AP in the ERf signaling pathway. The ability of constitutively active YODA to rescue stomata clustering in atgpi8-1 corroborates this hypothesis and suggests that a GPI-AP functions upstream of the MAP kinase cascade. TMM is a receptor-like protein that can potentially be a GPI-AP. TMM was previously computationally identified as a putative GPI-AP by two independent methods (Borner et al., 2002; Eisenhaber et al., 2003b). However, analysis of plants expressing TMM fused with GFP at the C terminus that are expected to prevent addition of the GPI anchor implied that TMM can function as a transmembrane protein (Nadeau and Sack, 2002). Analysis of genetic interactions demonstrated that tmm-1 is epistatic to atgpi8-1, indicating that either TMM is a GPI-AP or there is another GPI-AP whose function in stomata formation is entirely dependent on TMM. Future analysis of TMM attachment to the plasma membrane should distinguish between these two possibilities. In mammalian cells, the majority of GPI-APs are polar localized and are associated with lipid rafts (Lisanti et al., 1989; Brown and Rose, 1992; Maeda and Kinoshita, 2011). The presence of a GPI-AP in the ERf signaling pathway suggests the possibility that the ERf receptors directly interact with that protein and the receptor complexes might be targeted to lipid rafts. Lipid rafts have been proposed to act as signaling platforms where receptor complexes are stabilized (van Zanten et al., 2009). Alternatively, a GPI-AP might facilitate transport of ERfs to the plasma membrane or promote polar localization of ERf complexes in epidermal cells. The GPI-APs LORELEI and LRE-like GPI-AP function as chaperones of the receptor-like kinase FERONIA delivering it to the plasma membrane (Li et al., 2015). A GPI-linked lipid transfer protein has recently been shown to be dynamically targeted to specific regions of epidermal cells (Ambrose et al., 2013). Finally, a GPI-AP might contribute to specificity of ligand binding. In mammalian cells, the GPI-APs CRIPTO and GFRα function as coreceptors of corresponding transmembrane receptor kinases and define the specificity of ligand binding (Yeo and Whitman, 2001; Klein et al., 1997). Thus, the existence of a GPI-AP in the ERf signaling pathway poses new questions about the mechanism of ERf receptor function.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

The Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col) was used as the wild type. The mutant atgpi8-1 was obtained from an ethyl methanesulfonic acid-mutagenized (0.3% for 14 h) screen in an erf1-2 erf2-1 population (Shpak et al., 2004). Individual M2 seed lines were grown on modified Murashige and Skoog media plates supplemented with 1× Gamborg B5 vitamins and 1% w/v Suc and screened for stomata patterning defects in cotyledons.

**Figure 8.** TMM is epistatic to AtGPI8. A and B, Analysis of stomata formation in different organs of wild type, atgpi8-1, tmm, and atgpi8-1 tmm. SI (A) and percent of stomata in clusters (B) were measured in the abaxial epidermis of mature cotyledons and first rosette leaf as well as in the epidermis of stems and pedicels. Values are means ± sd, n = 6. Values in the table represent the mean.

C to E, Representative images of stem epidermis in atgpi8-1, tmm, and tmm atgpi8-1. All images are under the same magnification.
(C58S35364) and tmm-1 (CS6140) were obtained from the Arabidopsis Biological Resource Center. The er-105 and er-105 elr-2 elr-2 mutants have been described previously (Torii et al., 1996; Shpak et al., 2004). Plants were grown on a soil mixture of a 1:1 ratio of Promix PGX (Premier Horticulture) and Vermiculite (Pametto Vermiculite) and were supplemented with Miracle-Gro (Scotts) and approximately 3.5 mg/cm² of Osmocote 15-9-12 (Scotts). All plants were grown at 20°C under long-day conditions (18 h light/6 h dark).

Map-Based Cloning of *atgpi8*-1

A mapping population was created by crossing *atgpi8*-1 elr1 elr2 to the Landsberg erecta and Landsberg ers-105 background (Landsberg erecta and Landsberg ers-105) as well as the Arabidopsis Mapping Platform (http://www.Arabidopsis.org/Cereon/index.jsp) as well as the Arabidopsis Mapping Platform (http://amp.genomics.org.cn/). For primer sequences and amplified fragment sizes in both Col and Landsberg erecta, see Supplementary Table S1.

Sequence Alignment

Full-length amino acid sequences of AtGPI8 homologs from Saccharomyces cerevisiae (GPI8; accession NP_010618) and Homo sapiens (PIG-K; accession CA21820) were retrieved from the NCBI database and aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Genes and Genotyping

To obtain the *atgpi8*-1 mutant, *atgpi8*-1 elr1 elr2 plants were crossed with Col. The *atgpi8*-1 plants were identified both by the short root phenotype and by genotyping. The primers AtGPI8 F1 (5’-GACTGGATCTCCATCGTG-3’) and AtGPI8 R1 (5’-CGTGACTCTGGATCTCTCCTG-3’) were used to amplify a 1.5-kb fragment containing a BsrFI restriction site in the wild type but not in *atgpi8*-1. In the wild type, restriction digestion of this fragment results in two 750-bp fragments. Genotyping of *elr1* and *elr2* was performed as described by Shpak et al. (2004). For the allelism test, the *atgpi8*-1 mutant was crossed with *atgpi8*-2. The *atgpi8*-1/*atgpi8*-2 plants were identified in the F1 generation. The presence of the *atgpi8*-1 mutation was confirmed by genotyping described above. The presence of *atgpi8*-2 was determined through PCR using AtGPI8 1780 (5’-GCTGTACTCCAGAACGCTG-3’) and AtGPI8 2171 (5’-TCATCTTCGGAACTGCTCCTC-3’) primers and the left border primer p745 (5’-AACGGTGCCCAATGGTTAAAATCCTC-3’). The size of the PCR product is 400 bp in the wild type and 200 bp in *atgpi8*-2.

To construct a plasmid carrying cAtGPI8-EGFP (pEISH 504), the cDNA sequence of AtGPI8 was cloned behind the 2.1-kb AtGPI8 promoter sequence and the 3’ untranslated region of the AtGPI8 cDNA. The cDNA sequence was amplified from the cDNA of AtGPI8 using the following primers: AtGPI8 1780 (5’-GCTGTACTCCAGAACGCTG-3’) and AtGPI8 2171 (5’-TCATCTTCGGAACTGCTCCTC-3’). The sequence of the PCR product is 180 bp. The primers 5’-TCATCTTCGGAACTGCTCCTC-3’ and 5’-CGTGACTCTCCATCGTG-3’ were used to amplify *atgpi8*-1 mutants by the thermal cycle method. While *atgpi8*-1 plants produced very few T1 seeds, we still were able to identify multiple transgenic lines after selection on gentamycin. The *atgpi8*-1 genotype of the selected T1 lines was confirmed as described above. In a separate experiment, *atgpi8*-1/+, plants were transformed with a plasmid containing CA-YDA (Lukowitz et al., 2004). Transgenic plants carrying CA-YDA were selected on soil using 0.02% Fluo (Bayer CropScience) with 0.005% of Silwet t-77 (Lehle Seeds) and then genotyped for *atgpi8*-1.

To generate *er atgpi8*-1, *atgpi8*-1 was crossed with *er-105. The presence of *er-105* was determined phenotypically in the F2 generation as *er-105* plants are shorter, have clustered inflorescence and short, blunt siliques. To generate *tmm atgpi8*-1, *atgpi8*-1 was crossed to *tmm-1*. Plants homozygous for *tmm-1* were selected based on the absence of stomata in the stems. The presence of the *atgpi8*-1 mutation in both of these crosses was confirmed by genotyping. *AtGPI8*-expressing GFP-SKU5 was generated by crossing the mutant with the transgenic plants (Sedbrook et al., 2002) and selecting for the *atgpi8*-1 short root phenotype and GFP fluorescence in the F2 generation.

Analysis of Plant Development and Growth

The SI and stoma clusters were measured in cotyledons and leaves of 17-d-old seedlings and in stems and pedicels of mature plants using Differential Interference Contrast (DIC) microscopy. To analyze the effects of bikinin on stoma development, seedlings were grown on Murashige and Skoog plates supplemented with 30 μM bikinin for 7 days. For DIC microscopy, plant tissues were fixed overnight in ethanol:acetic acid (9:1) and cleared in a chloral hydrate solution (chloral hydrate:water:glycercol 8:1.1) for approximately 24 h. Structure of epidermis was observed using a Nikon Eclipse 80i microscope with DIC optics, and pictures were obtained with a 12-megapixel cooled color DXM-1200c (Nikon) camera. The number of stomata were counted using NIS-Elements BR 2.30.

Plant height, the number of siliques and cauli branches, and internode and pedicel length were measured at full maturity at 60 d for wild type and at 90 d for *atgpi8*-1. The number of cauli branches was determined by counting the number of lateral branches on the main stem. To analyze callose accumulation, 7-d-old seedlings were fixed overnight in ethanol:acetic acid (9:1), rinsed in 90% ethanol, incubated for 30 min in 0.09 M sodium phosphate buffer (pH 7.4), and finally submersed for 1 h in 0.1% aniline blue dissolved in the indicated buffer. A Nikon Eclipse 80i epifluorescence microscope with a 12-megapixel cooled color camera and a UV-2A filter (Nikon) was used to observe the seedlings immediately after incubation.

Transient Transformation of Seedlings and Cell-to-Cell Mobility Assay

The abaxial epidermis of 7-day-old Arabidopsis seedlings was transformed with the vectors pAVA 321 (CaMV 35S:mgF658T; von Arnim et al., 1998) and pAN1656 (CaMV 35S:RFP with ER retention signal; Nelson et al., 2007). During transformation, 1.1-μm tungsten M17 microcarriers (Bio-Rad) were fired at 400 psi using a PDS-1000/He particle bombardment system (Bio-Rad). The fluorescence was observed 18 h postbombardment using a Zeiss Axio Observer.Z1 microscope, and images were obtained with 1.3-megapixel cooled black and white ORCA-AG (Hamamatsu) camera. The presence of GFP designated transformed cells. Protein mobility was established by analyzing GFP fluorescence.

**Protein Gel-Blot Analysis**

Eight-day-old GFP-SKU5, GFP-SKU5 *atgpi8*-1, and wild-type seedlings were ground in liquid nitrogen to a fine powder, and then 3 volumes of extraction buffer (100 mM Tris-HCl at pH 8.8, 150 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM NaF, 1 mM PMSF, 1:1000 Complete protease inhibitor cocktail [Sigma]) were added to the ground material. The extracts were sonicated and centrifuged at 10,000 g for 10 min at 4°C to remove insoluble debris. The supernatant was ultra-centrifuged at 100,000 g for 1 h at 4°C to obtain the crude membrane fraction as a pellet. Membrane pellets were suspended in 100 mM potassium phosphate with pH 7.4. The protein gel-blot analysis was performed as described previously with minor modifications (Shpak et al., 2003). Proteins were separated by 9% or 10% SDS-PAGE. The primary anti-GFP polyclonal antibody (Life Technology) was used at a dilution of 1:5,000 following by the secondary HRP Conjugated Goat Anti-Rabbit IgG antibody (Pierce) at a dilution of 1:4,500. Primary anti-BAK1 polyclonal antibody (Agrisera) was used at a dilution of 1:5,000 following by the secondary HRP Conjugated Goat Anti-Rabbit IgG antibody (Agrisera) at a dilution of 1:5,000. The detection of GFP-SKU5 and BAK1 was performed with SuperSignal West Pico Rabbit IgG detection kit (Pierce).

**Reverse Transcription-PCR**

Total RNA was isolated from 12-d-old Arabidopsis seedlings using a Spectrum Plant Total RNA Kit (Sigma). First-strand cDNA was synthesized from 785 ng of RNA with a ProtoScript M-MuLV Taq RT-PCR Kit (New England Biolabs) according to the manufacturer’s instructions. PCR was performed with the first-strand synthesized cDNA at 95°C for 2 mins, varying cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s, followed by a final 72°C for 5 min. The primers 5’-GCTGTACTCCAGAACGCTG-3’ and 5’-TCATCTTCGGAACTGCTCCTC-3’ were used to amplify *AtGPI8* and the primers 5’-GCTCACTCAACGCTTCTCCTC-3’ and 5’-GCTCTGATGGCACACGACAA-CAAA-3’ were used to amplify *ACTIN-2* (At5g18780) as a control. PCR products were separated on 1% agarose gel and visualized with ethidium bromide staining.
Arabidopsis Genome Initiative

Arabidopsis Initiative Numbers for the genes discussed here are as follows: ACGP18 (At1g07870), ER (At2g20330), ERL1 (At5g62230), ERL2 (At5g07180), TMM (At1g08080), YODA (At1g63700), and SKL5 (At4g12420).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Semi-quantitative RT-PCR analysis of ACGP18 and ACTIN transcripts in 12-day-old seedlings.

Supplemental Figure S2. At 30 days growth and development of atgpi8-1/ atgpi8-2 plants is drastically impeded compared to wild type.

Supplemental Figure S3. The expression of ACGP18-EGFP under the endogenous promoter rescued the majority of phenotypes observed in atgpi8-1 mutants.

Supplemental Table S1. Primers used to amplify SSLPs during positional cloning of atgpi8-1.

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LITERATURE CITED


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