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Evaluation of receptor protein TOO MANY MOUTHS (TMM) as a Glycosylphosphatidylinositol-Anchored Protein

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ABSTRACT

Plants rely on valves called stomata for efficient gas exchange. Stomata develop through a lineage controlled by signals both internal and external to the stomatal precursor cell. TOO MANY MOUTHS (TMM) is a receptor like protein that forms heterodimers with members of the ERECTA family (ERf) receptor-like kinases to negatively regulate the stomatal development pathway in *Arabidopsis thaliana*. Mutations in TMM or in the ERECTA family cause stomatal clustering. Various proteins in plants and animals are attached to the plasma membrane by a Glycosylphosphatidylinositol (GPI)-anchor instead of a transmembrane domain composed of amino acids. The phenotype of the *atgpi8-1* mutation in the catalytic subunit responsible for attaching GPI anchors to membrane proteins suggests the involvement of a GPI-anchored protein in stomatal formation. TMM or another protein dependent on TMM is suspected to be GPI-anchored due to the epistasis of *tmm* to *atgpi8-1* in stomatal formation. Expression of the TMM functional domain with various anchors in *tmm* and *tmm agtpi8*-1 suggests TMM function is not dependent on GPI-anchoring. The reinsertion of a TMM with different membrane anchors into both a *tmm* and *tmm atgpi8-1* background suggests that while TMM is GPI-anchored, the protein functions independently of its method of membrane attachment.

BACKGROUND

The addition of a glycophosphatidylinositol-anchor, or GPI-anchor, is a common post-translational modification for membrane proteins. On average, 0.5% of eukaryotic proteins receive a GPI-anchor (Mayor et al., 2004). The GPI-anchored proteins are quite diverse, with roles ranging from immunity to cell adhesion (Paulick & Bertozzi, 2008). When polypeptides directed to the endoplasmic reticulum possess a GPI-anchor signal sequence on their C-terminus, the signal sequence will be cleaved and a GPI-anchor

covalently joined to the terminus of the protein before delivery to the outer leaflet of the cell membrane (Fig 1). This substitute anchor will serve as the membrane attachment in place of the traditional hydrophobic transmembrane domain. The anchor is composed of an oligosaccharide linked glycosidically to phosphatidylinositol (Low et al.,1988). The structure of the GPI-anchor is mostly conserved but can be modified (Paulick & Bertozzi, 2008). The GPI moiety is synthesized on the endoplasmic reticulum by a host of over 20 proteins and attached to the C-terminus of the specified polypeptide at the ω-site (Bundy et al., 2016). Attachment of the GPI moiety to the ω-site is executed by a transamidase enzyme complex with five subunits (Fig 1). GPI-8 is one of the two catalytic subunits of this complex; its analog is known as PIGK in mammals and GPI8 in yeast. Mutation of this catalytic subunit results in lethality in both mammals and yeast.

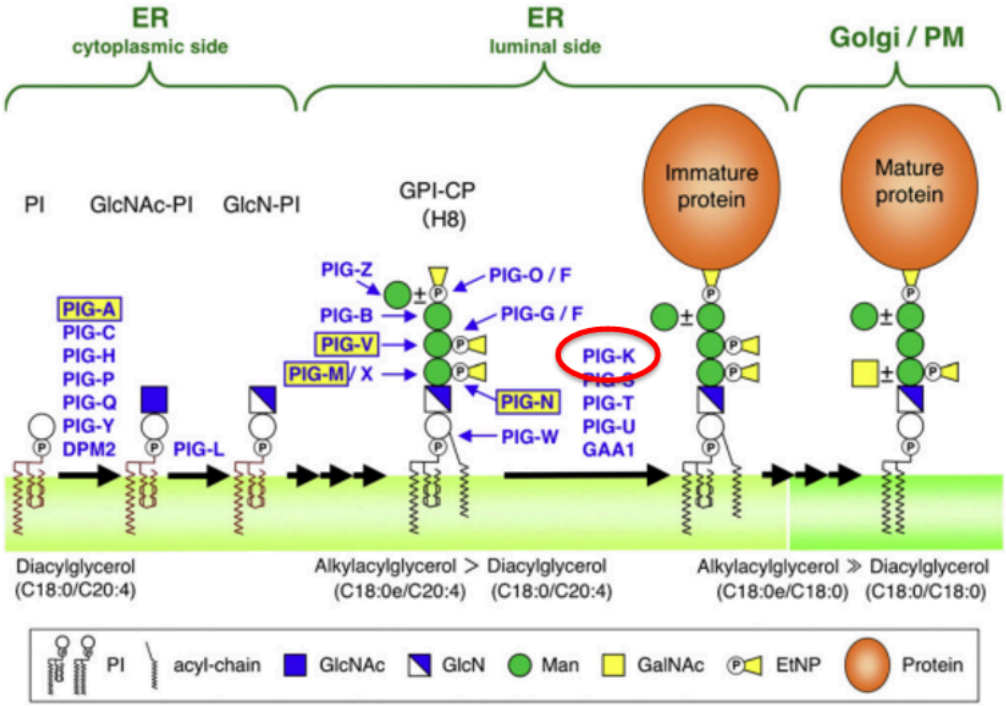


Figure 1: Biosynthesis of a GPI-Anchored Protein. PIG-K is analogous to GPI-8, in *Arabidopsis*, AtGPI-8. The GPI moiety is synthesized at the endoplasmic reticulum and then attached to a protein with a GPI-anchor signal sequence. Modified from Fujita & Kinoshita, 2012.

In *Arabidopsis thaliana*, the GPI-8 protein is named AtGPI-8 (Bundy et al., 2016). Two versions of an AtGPI-8 mutation were used in our lab: the knockdown *atgpi8-1* and the knockout *atgpi 8-2*. The former is a missense mutation that reduces enzyme efficiency, while the latter is a T-DNA insertion that leads to lethality (Bundy et al., 2016). The phenotype of *atgpi8-1* includes slowed growth of root and shoot, delayed flowering, reduced fertility, and changes in epidermal cell patterning as seen through stomata formation (Fig 2).

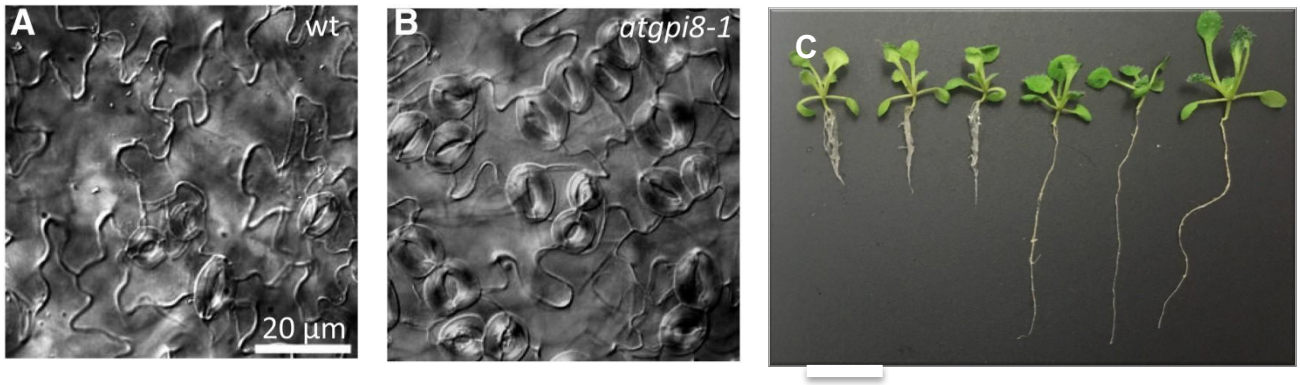


Figure 2: A) Wild type (COL) adaxial cotyledon as compared to cotyledon of B) ***atgpi8-1***. Modified from Bundy et al., 2016. Clustering is noted in the ***atgpi8-1*** sample. C) Seedlings of ***atgpi8-1***, left, and seedlings of wild type (COL) at 12 days post germination. Size bar, 1 cm.

Increased stomatal index and stomata clustering are seen in *atgpi8-1* mutants as compared to wild type. Due to the disruption of the stomata formation by *atgpi8-1*, it is evident a protein in the stomatal lineage pathway relies on the AtGPI-8 transamidase to attach its anchor. Without functional AtGPI-8, the mystery protein cannot travel to and anchor within to the outer membrane to perform its function.

Stomatal differentiation relies on both internal and external, or positional cues; it is highly regulated by the ERECTA family (ERf) receptors and their ligands. Binding of ligands to ERfs causes location-specific differences in stomata differentiation. When positively activated, ERf inhibits stomatal formation. Receptor-like protein TOO MANY MOUTHS (TMM) directly interacts with ERf receptors and promotes

binding of certain ligands (Torii et al., 2012). ERf and TMM act via a MAP kinase (MAPK) pathway that inhibits transcription factor Speechless (SPCH), that allows a protodermal cell to enter the Stomatal Lineage Pathway (Fig 3). Cells that do not enter this pathway or exit the pathway become pavement cells. Once the pathway is entered, the cell is termed a meristemoid mother cell (MMC) and will divide asymmetrically. The MMC gives rise to the smaller cell Meristemoid which typically divides one to three times in a fashion ensuring stomata are not adjacent to each other. After several divisions, the meristemoid will become a guard mother cell (GMC). The GMC divides into two guard cells, together composing a stoma. Due to regulation of cell divisions by ERfs, in the wild type stomata are separated by at least one pavement cell.

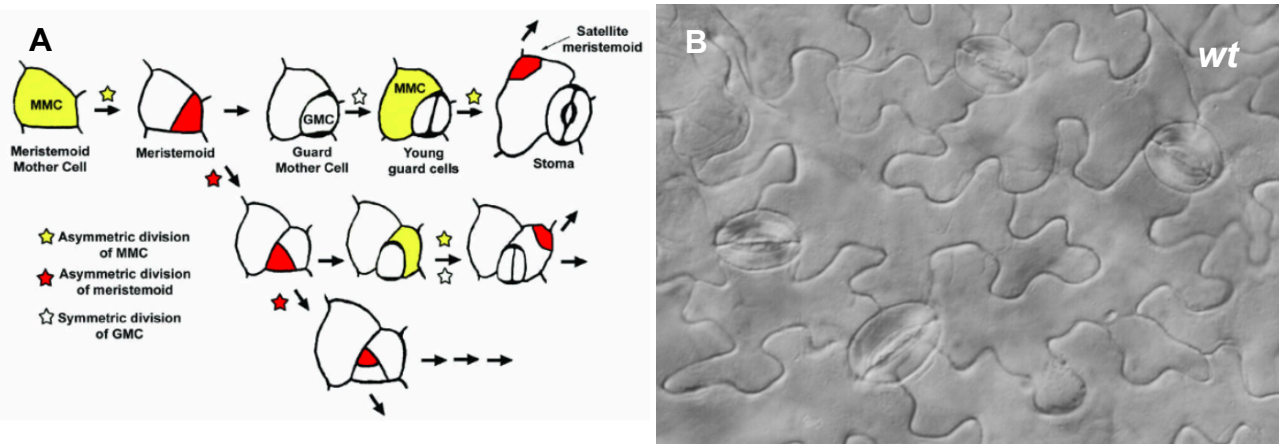


Figure 3: A) Stomatal differentiation pathway in ***Arabidopsis thaliana***. Modified from Sack et al, 2000. B) Wild type epidermal patterning on cotyledon. This illustrates the "one-cell-rule," in which at least one pavement cell lies between stomata. Wild type stomata rarely form adjacently. The image is a result of the process in A).

Many proteins are involved in stomatal differentiation; this project seeks to identify and characterize the protein with a GPI-anchor. The *erecta erl1 erl2* mutant produces similar clustering as in *atgpi8-1*, but ERfs are known to have a transmembrane domain. Internal proteins affecting the stomatal lineage are cytoplasmic (Shimada et al., 2017). Constitutive activation of YODA and therefore the MAPK cascade following the ERfs and TMM rescues the *atgpi8-1* phenotype, indicating the AtGPI-8 dependent protein is upstream in the pathway (Fig 4). The most likely candidate for a GPI-anchored protein in stomatal formation is TMM. TMM forms heterodimers with ERECTA and ERL1 but does not form homodimers (Lee et al., 2012).

Mutations in either the ERfs or TMM increase stomata density and clustering. TMM negatively regulates ERfs at two points, 1) entrance of a protodermal cell into the stomatal lineage pathway, and 2) when a meristemoid differentiates into a guard mother cell (Shpak et al., 2005). TMM functions in an organ dependent manner (Fig 4). In stems, *tmm* shows no stomata, while in cotyledons and leaves, *tmm* shows increased stomatal index and clustering (Shpak et al., 2005, Yang & Sack, 1995). *tmm* does not decrease fertility and therefore is easily passed to later generations. *tmm* is epistatic to *atgpi8-1*, suggesting that the *atgpi8-1* phenotype is being manifested through TMM. In an absence of functional TMM, the *atgpi8-1* phenotype is concealed (Fig. 5).

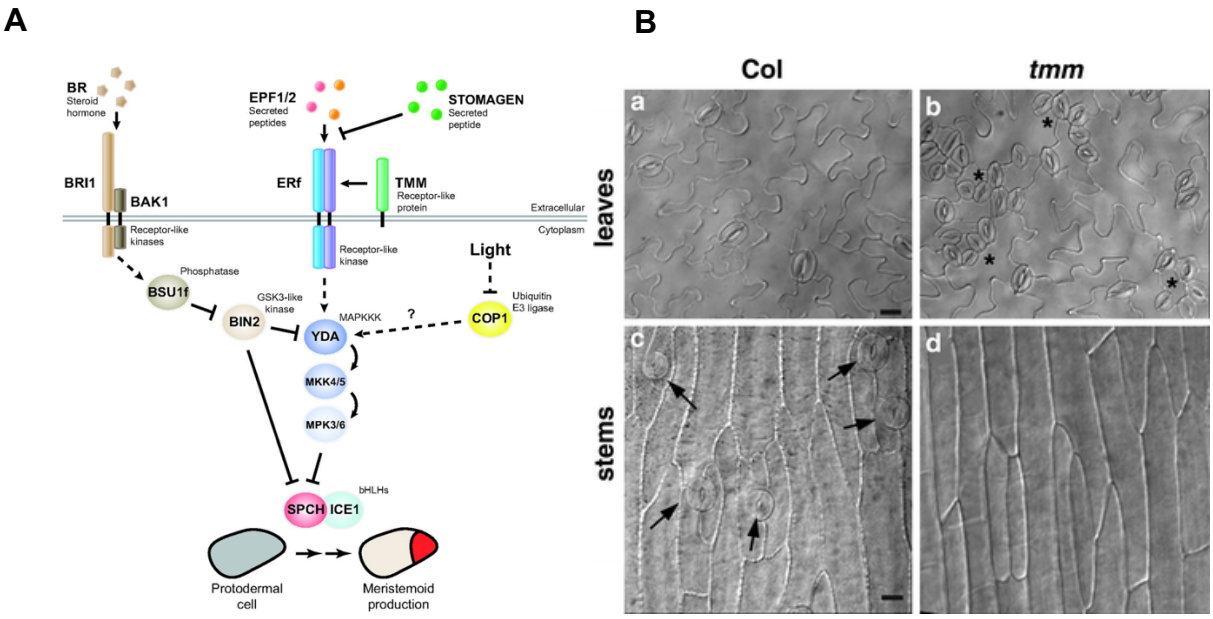


Figure 4: A) MAPK cascade in ***Arabidopsis thaliana.*** B) Phenotype of ***tmm*** as compared to wt. Mutant ***tmm*** shows increased numbers of stomata and clustering in leaves while it causes stomata to be absent altogether on stems. Modified from Sack et al., 2008.

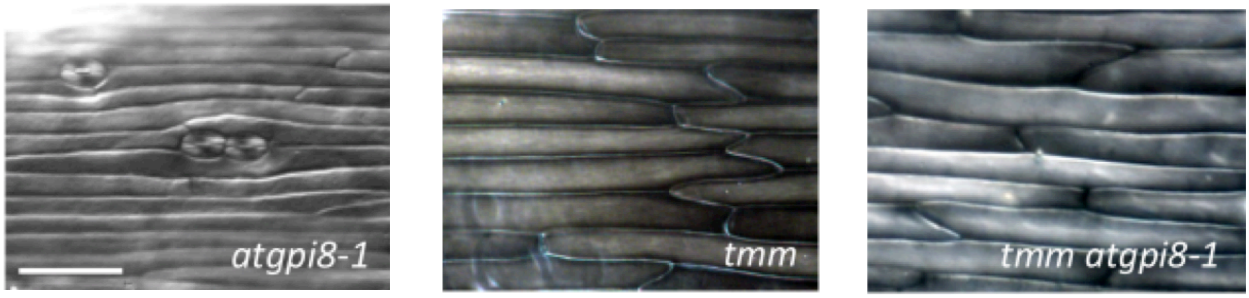


Figure 5: *tmm* is epistatic to *atgpi8-1* in stems. *atgpi8-1* causes clustering of stomata on stems, which are absent on *tmm* mutant stems. When the double mutant is created, the *tmm* phenotype is seen, indicating epistasis. Modified from Bundy, et al., 2016.

OBJECTIVES

The aims of this work were:

1) to determine if TMM functionality depending on its means of attachment to the plasma membrane

2) to identify whether TMM is a GPI-anchored protein

To analyze the first point, four variations of a *TMM* plasmid were created for insertion into a *tmm* background.

A) Endogenous TMM: The normal sequence for TMM was included in its entirety: TMM promoter, TMM coding region, and terminator. This construct is designed to quantify functionality of the normal TMM protein when reinserted.

B) GPI-Anchored TMM: TMM promoter, TMM coding region, the last ~30 bp of TMM replaced with COBRA (COB) GPI-anchor signal sequence, and a terminator. COBRA is a known GPI-anchored protein (Roudier et al., 2005). By taking the 96 bp GPI-anchor signal from COBRA, we aimed to create a GPI-anchored version of TMM.

C) TMM with transmembrane domain: TMM promoter, TMM coding region, the last ~30 bp of TMM

replaced with ERECTA transmembrane domain, and a terminator. ERECTA is a known to be inserted into the plasma membrane via a transmembrane domain composed of amino acids. This known 102 bp domain was attached to the TMM coding region with the aim of creating TMM with an amino acid transmembrane anchor.

D) Extracellular TMM: TMM promoter, TMM truncated by 100 base pairs, and a terminator. The final 100 base pairs were removed from the TMM coding region aiming to make a protein without a membrane anchor.

The second question was investigated by inserting the same four constructs of TMM into a *tmm atgpi8-1* background; this background would cause proteins requiring a GPI-anchor to be produced at a less efficiently. If TMM is GPI-anchored, this reduction in protein would be evident in the plant phenotype for the endogenous TMM and TMM+GPI-anchor constructs.

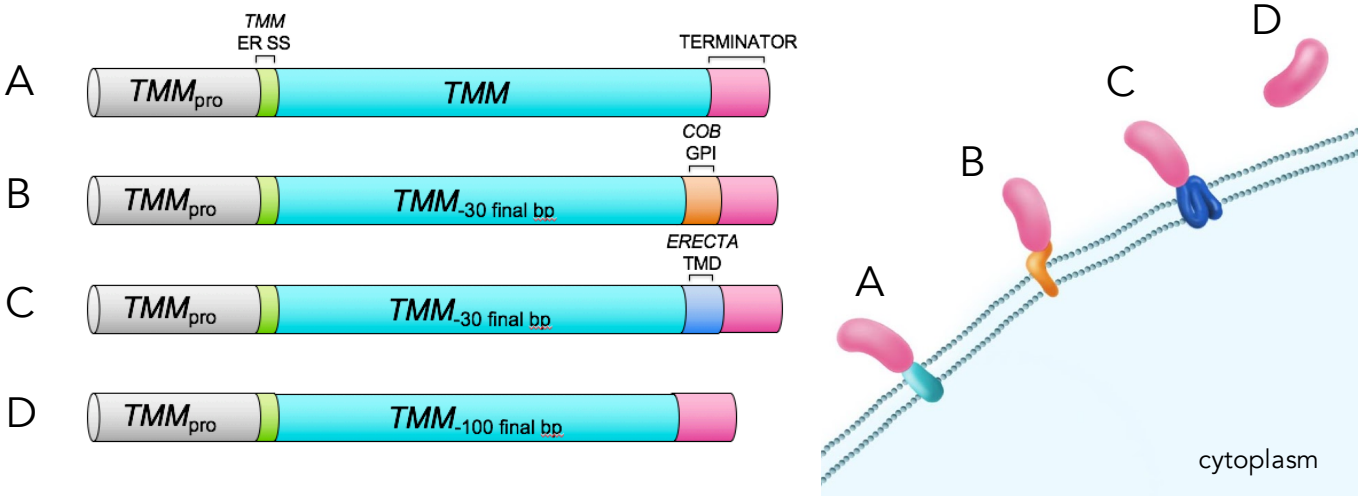


Figure 6: Map of the TMM DNA reinsertions created and theoretical image of the TMM reinsertions if localized to the plasma membrane. A) Endogenous TMM, B) TMM+GPI-Anchor, C) TMM+TMD, D) Extracellular TMM.

RESULTS

TMM Functions Independently of Anchor in *tmm* Background

To evaluate the degree to which TMM reinsertions were rescuing phenotype, we measured stomatal index and percent of stomata clustered in both the cotyledons and stems. These measurements can quantify changes in stomatal differentiation.

$$Percent\ Stomata\ Clustered = \frac{\#\ Clustered\ Stomata}{(\#\ Clustered\ Stomata + \#\ Single\ Stomata)}$$

$$Stomatal\ Index = \frac{\#\ Stomata}{(\#\ Stomata + \#\ Pavement\ Cells)}$$

The TMM reinsertions were compared to the controls wt (COL) and *tmm*. Mutating *TMM* is known to cause stomata clustering and an increased stomatal index in cotyledons as well as an absence of stomata in the stems. A functioning reinsertion would rescue the *tmm* phenotype and have epidermal patterning resembling that of wt, in which clustering is absent and stomata are present in stems. If a GPI-anchor is essential for TMM function then only endogenous TMM and TMM+GPI should rescue the *tmm* phenotype.

However, all four TMM constructs produced multiple independent transgenic lines with stomatal index and stomata clustering resembling wt as observed in cotyledon, indicating rescue of the *tmm* mutant (Table 1 and 2). At the same time, each construct including one encoding endogenous TMM produced lines with only partial rescue with stomatal index and stomata clustering being intermediate between wt and *tmm*. This failure to fully rescue should be expected in some lines; variation exists due to uneven expression levels of transgene in different transgenic lines. This could be caused by the random insertion of constructs into DNA, either in epigenetically blocked or accessible areas, thereby affecting the amount of protein production. As long as even one line rescues, it indicates that the protein functions with its anchor or lack thereof. Similar to the stomatal index data, all versions of TMM show significant rescue of the clustering

phenotype in at least two lines (Table 2). This again suggests that TMM is functional and is able to inhibit stomata production.

In stems, a stomatal index above zero indicates the presence of stomata (Table 3). Because *tmm* lacks stomata on stems, any stomatal index above zero indicates a degree of rescue. Every construct was able to promote stomata formation in the stems, which resembles wt and not *tmm*. The degree of rescue is similar between all constructs. The data do not show that endogenous TMM and GPI-anchored TMM rescue wt more efficiently than TMM with a transmembrane domain or even extracellular TMM. Thus, rescue is achieved even when the protein is secreted into the extracellular space, left to interact with ERfs solely through its extracellular domain. At the same time, for extracellular TMM more variation is seen in percent of stomata clusters suggesting that the function of extracellular TMM might depend more strongly on its efficient expression (Table 4). All constructs possess both transgenic lines with no clustering and transgenic lines with percentage clustered significantly departed from the wt phenotype. In this, the constructs depart from both the wt and *tmm* controls, neither of which create stomata clustering. Overall, stem data shows a partial rescue of epidermal patterning. Stomata are present, as in wt, but are not spatially arranged as according to the "one-cell-rule."

This experiment was designed under the premise that TMM functionality would decrease if not anchored as endogenously coded. This data supports the null hypothesis and suggests TMM is more versatile than expected; the protein does not require a particular membrane attachment for phenotype rescue. The data suggests that only the extracellular domain of TMM is critical for its function and attachment of TMM to the membrane plays a minor role in its function. Because TMM can function independently of its anchor, this data cannot indicate how TMM is endogenously anchored. If TMM only functioned with one type of anchor, the endogenous reinsertion would resemble either TMM+GPI or TMM+TMD, not both. Because this data did not indicate TMM's endogenous anchor, another experiment was designed.

Data in *tmm atgpi 8-1* Background suggest TMM is GPI-anchored

Data for the same TMM constructs within a *tmm atgpi8-1* background were collected to identify the endogenous anchor. When a GPI-anchor is coded for, the *tmm atgpi8-1* plant will fail to complete post-translational modification and delivery of TMM to the cell surface. This would manifest through an *atgpi8-1* phenotype and is expected of TMM+GPI. In *tmm atgpi8-1* plants when no GPI-anchor is required, i.e. TMM+TMD, the effect of *atgpi8-1* on stomata is circumvented. If no GPI-anchor is needed, broken GPI machinery does not matter; functional TMM+TMD would manifest as the wt (COL) phenotype. Endogenous TMM should either appear like TMM+GPI/*atgpi8-1* or TMM+TMD/COL, revealing the endogenous anchor. Extracellular TMM should partially rescue *tmm atgpi8-1*.

The data for cotyledons shows a similar stomatal index and percent clustered between endogenous TMM resembling *atgpi8-1*, as expected of a protein with a GPI-anchor (Tables 5 &6). TMM+GPI does not show similarity to endogenous TMM or *atgpi8-1* as expected; instead it most closely resembles wt (COL) with a stomatal index ~30%. Importantly, TMM+GPI lines do not show clustering consistent with *atgpi8-1* as expected. In fact, the clustering of TMM+GPI is indistinguishable from that of TMM+TMD. While the stomatal index of TMM+TMD lies between that of wt (COL) and *atgpi8-1*, the lack of clustering from the reinsertion as a whole suggests a much stronger resemblance to COL as expected. Finally, extracellular TMM, expected to resemble *tmm atgpi8-1*, continues to rescue more than expected. Extracellular TMM is able to partially rescue the clustering phenotype of *atgpi8-1*. A large amount of variability exists between lines of extracellular TMM.

Again, in the stem data, endogenous TMM continues to most closely resemble *atgpi8-1* in both stomatal index and clustering. Because endogenous stems exhibit significantly more stomata than *tmm atgpi8-1*, it's clear that there has been rescue of the *tmm* phenotype as seen previously in the *tmm* background. Data for TMM+GPI and TMM+TMD resemble wt (COL) as was seen in cotyledons. Both rescue clustering,

indicating that mutating the catalytic subunit AtGPI-8 has not affected them. This concurs with expectations for TMM+TMD but continues to defy those for TMM+GPI, which should be affected by a mutation in AtGPI-8. Extracellular TMM again shows variability.

In summary, endogenous TMM does appear as *atgpi8-1*, suggesting a GPI anchor. However, TMM+GPI-Anchor resembles the COL control as does TMM+TMD, only expected of TMM+TMD. The data indicates that our putative GPI-anchored construct show the phenotype expected of a construct with a transmembrane domain and prevents resemblance between endogenous TMM and TMM+GPI.

DISCUSSION

This project sought to elucidate whether TMM, a membrane protein involved in stomatal formation, is GPI-anchored. Furthermore, is TMM's function dependent on its anchor?

Our data suggest that TMM functionality is not dependent on a GPI-anchor, strongly supported by the fact that even extracellular TMM partially rescues the *tmm* phenotype. TMM does not seem to have a preference for its means of attachment to the outer membrane. Analogous results were found for the putative GPI-anchored protein LRE, which also retains function when it is attached via a transmembrane domain (Palanivelu et al., 2016).

TMM's independent extracellular domain

The data showing extracellular TMM functionality in both *tmm* and *tmm atgpi8-1* backgrounds was unexpected. The data shows variation, likely due to differences protein concentration between lines. Variation in protein production can be explained through epigenetics; different insertion sites lead to different transcription levels and different concentrations of TMM. Because extracellular TMM would be secreted, we predict a certain minimum concentration of TMM permits molecular interaction with ERfs and

therefore phenotypic rescue. Regardless of anchor, TMM functionality may be ensured through molecular interactions with ERfs, which TMM was previously shown to interact with (Lee et al., 2012). A lab we partner with is currently investigating the interactions between the extracellular domains of ERfs and TMM; the resulting data could validate our hypothesis that, at sufficient concentrations, TMM can be tethered in the appropriate area solely by interactions with ERfs.

Reevaluation of the GPI-anchor signal sequence

Endogenous TMM in the *tmm atgpi8-1* background resembles *atgpi8-1*, suggesting TMM is GPI-anchored. However, the data for TMM+GPI must also show similarity to Endogenous TMM and to *atgpi8-1* to support our hypothesis that TMM is a GPI-anchored protein. The original construct was created with the GPI anchor signal sequence beginning at ω-11 site, 11 amino acids upstream of the point of signal cleavage (Fig. 7). As this was a novel method, this may not have provided enough area for the GPI transamidase complex to successfully recognize the signal or dock on the polypeptide. Essentially, we suspect the signal sequence used is not signaling for a GPI-anchor. If the construct fails to obtain a GPI-anchor, we suspect the hydrophobic signal region will function as a transmembrane domain and insert into the membrane, rendering our TMM+GPI-Anchor and TMM-Transmembrane Domain constructs - the same. To evaluate this hypothesis, we will recreate the TMM+GPI construct, beginning the GPI anchor signal sequence around ω-20. This will allow the presence of 9 additional amino acids around the cleavage site which we suspect will aid in recognition and docking of the AtGPI8-1 complex and therefore clearly distinguish the protein We will transform *tmm atgpi8-1* plants with the new construct and grow into T3/T4 generation. The exact span of residues required to create a GPI-anchor signal sequence in *Arabidopsis* have not been determined in external research. If recreation of the plasmid with more residues shows the expected data, it will provide a map of the required GPI-anchor signal sequence that could prove useful to many researchers undertaking similar transformations and inform the plant biology community as a whole.

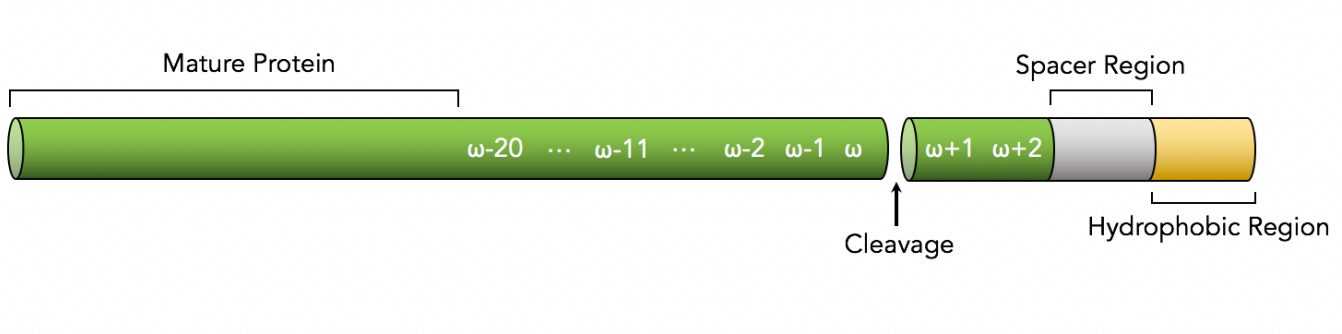


Figure 7: GPI Signal Sequence. Instead of beginning the GPI-anchor signal sequence at the ω–11 site, we will recreate the TMM+GPI construct beginning at the ω–20 site. This may provide the extra signal sequence necessary to attach a true GPI-anchor to TMM and will be evaluated through upcoming data.

Implications of findings

Why are these proteins GPI-anchored when it seems a transmembrane domain would suffice? Attaching a GPI-anchor requires a cell to use more energy and create more enzymatic machinery. Maybe under certain environmental stresses *Arabidopsis thaliana* requires GPI-anchoring for proper function of proteins like TMM and LRE. If TMM is not GPI-anchored, there exists another protein involved in stomatal determination whose function is dependent on TMM.

DATA FIGURES

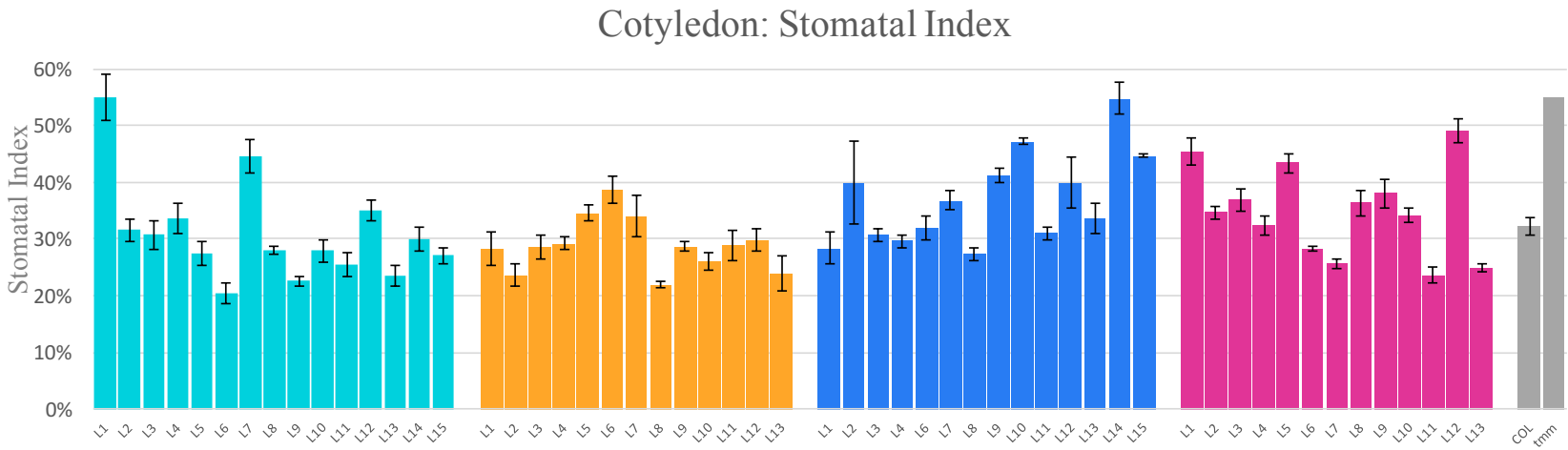


Table 1: Cotyledon Stomatal Index by TMM reinsertion in ***tmm*** background, T1 generation plants, 1 plant per bar.

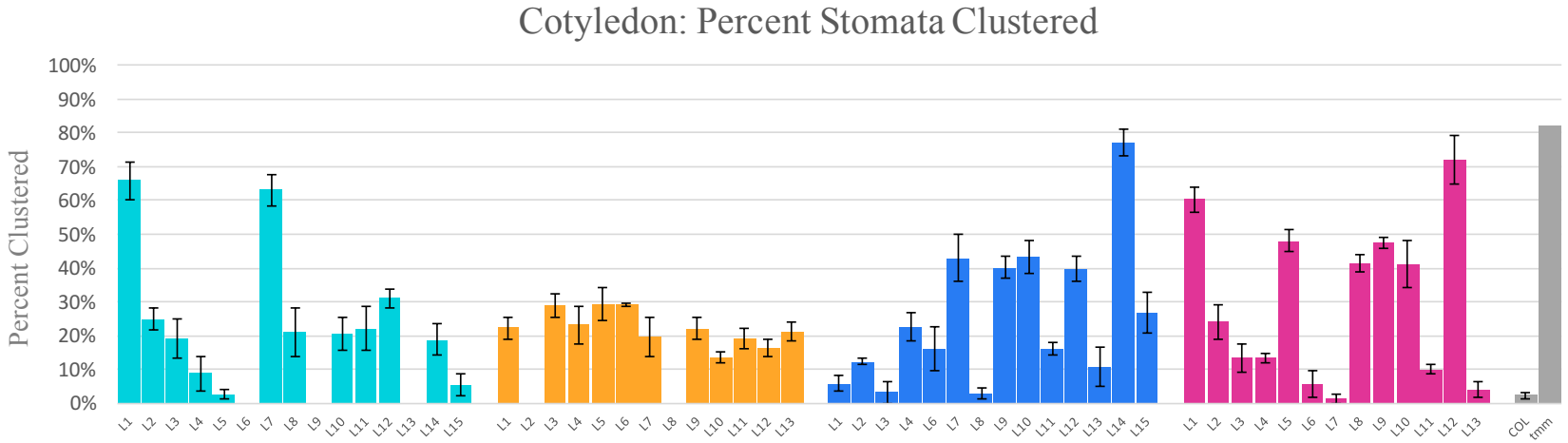


Table 2: Cotyledon Percent Stomata Clustered by TMM reinsertion in ***tmm*** background, T1 generation plants, 1 plant per bar.

Endogenous TMM TMM+GPI-Anchor TMM+Transmembrane Domain Extracellular TMM Control

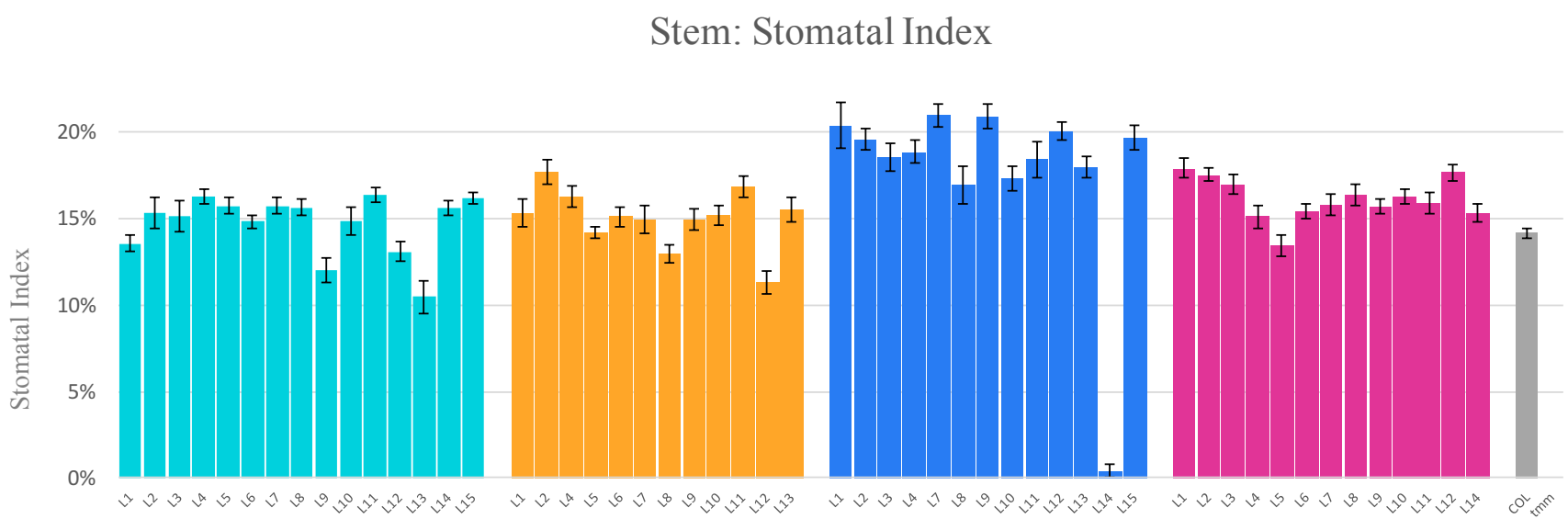


Table 3: Stem Stomatal Index by TMM reinsertion in ***tmm*** background, T1 generation plants, 1 plant per bar.

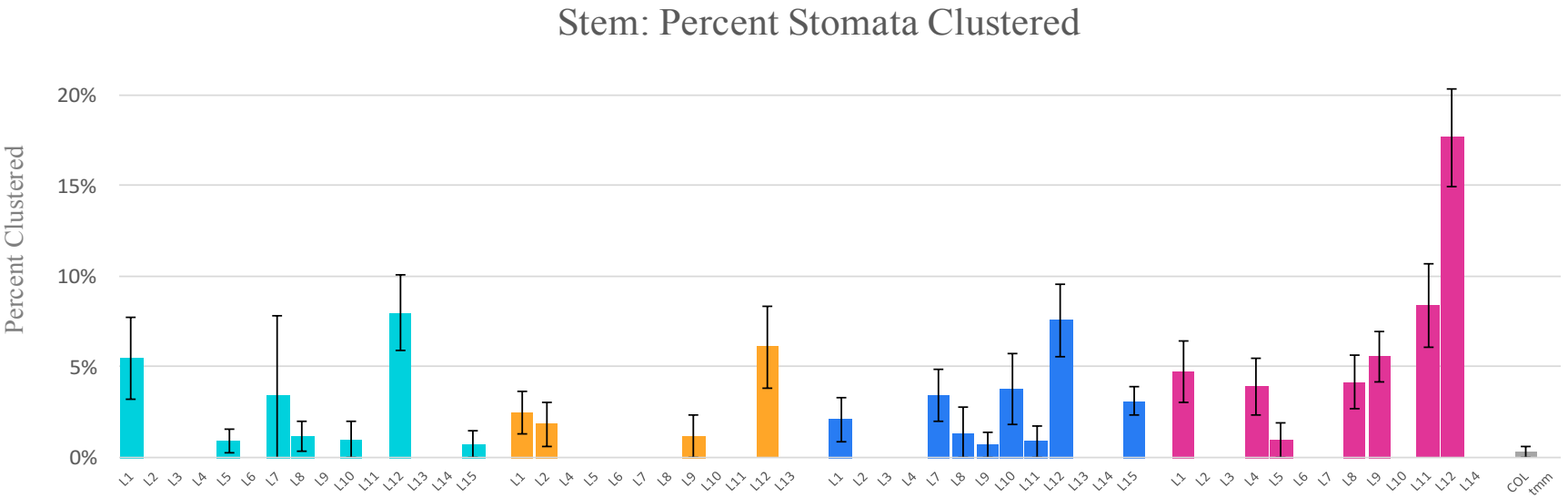
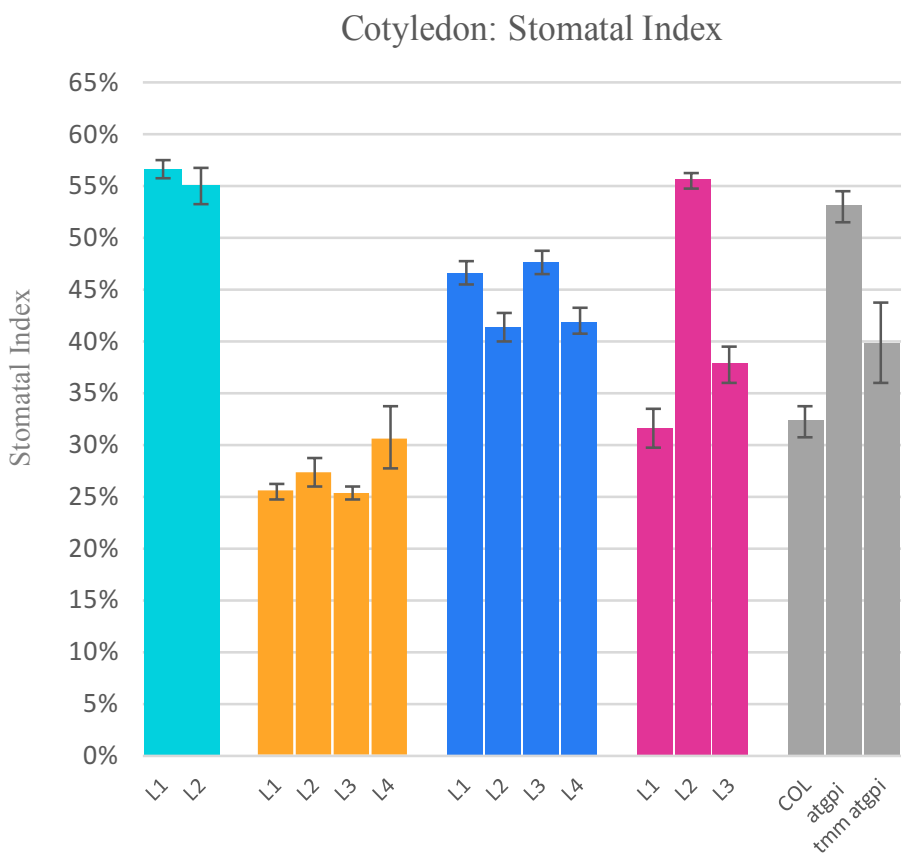
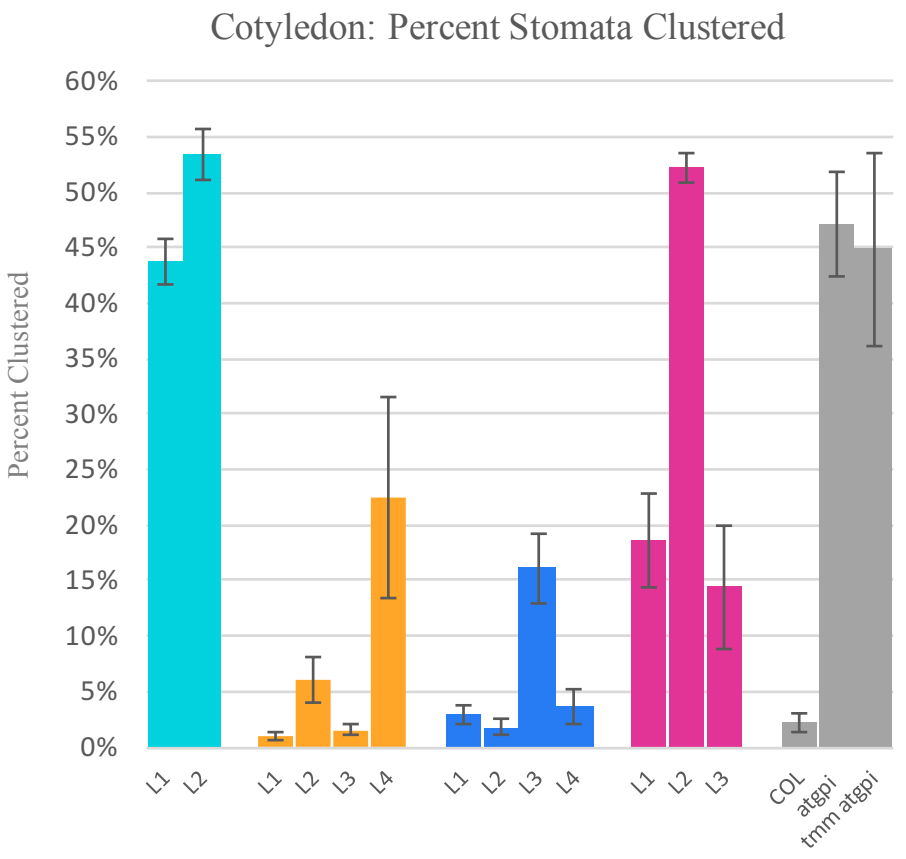


Table 4: Stem Percent Stomata Clustered by TMM reinsertion in ***tmm*** background, T1 generation plants, 1 plant per bar.

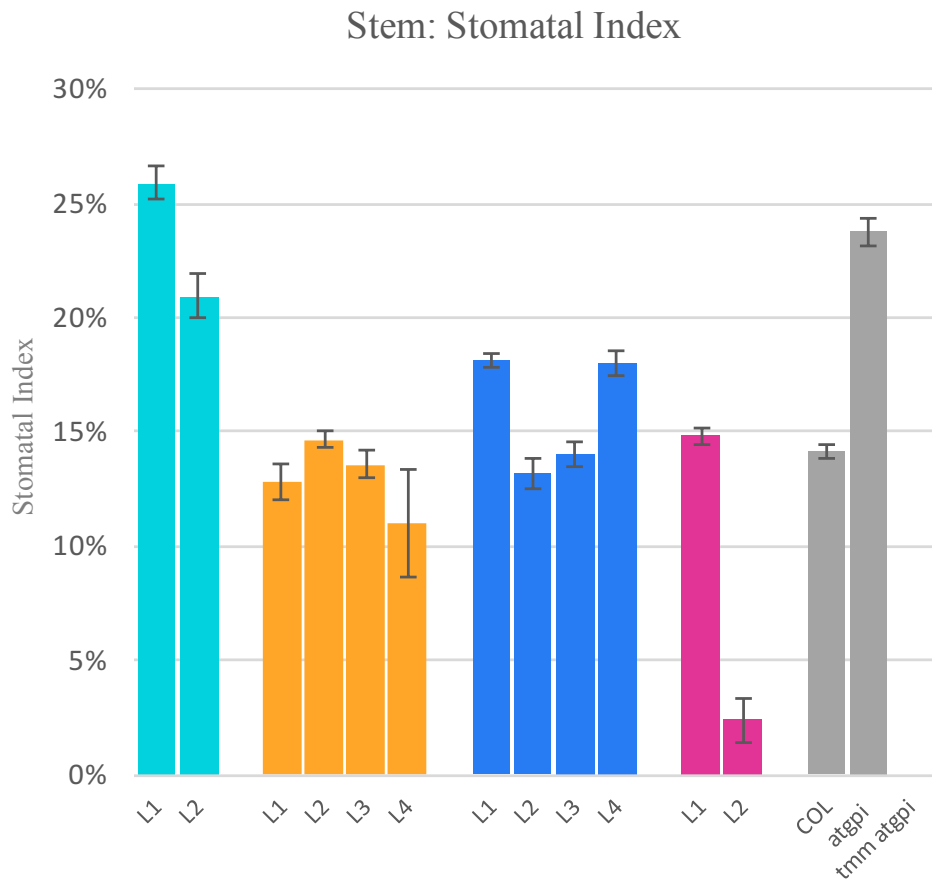
Endogenous TMM TMM+GPI-Anchor TMM+Transmembrane Domain Extracellular TMM Control

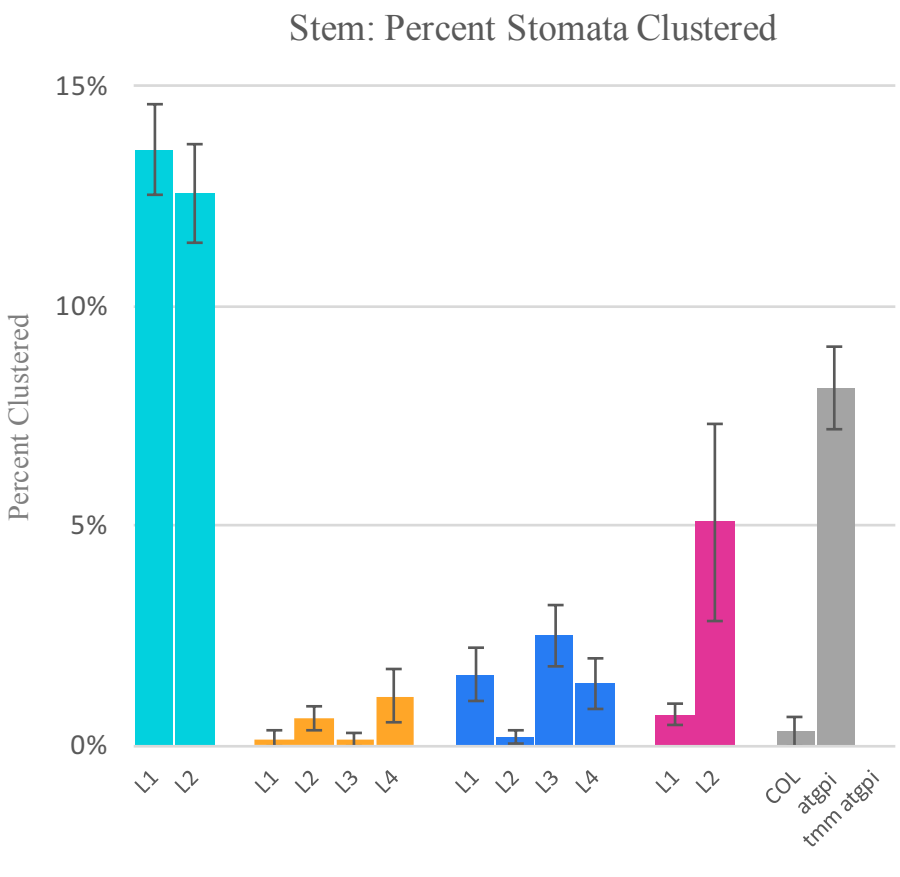




Tables 5 and 6: Cotyledon data by TMM reinsertion in ***tmm atgpi8-1*** background, T4 generation plants, ~10 plants per bar.

Endogenous TMM | TMM+GPI-Anchor | TMM+Transmembrane Domain | Extracellular TMM | Control





Tables 7 and 8: Stem data by TMM reinsertion in ***tmm atgpi8-1*** background, T3 generation plants, ~10 plants per bar.

Endogenous TMM | TMM+GPI-Anchor | TMM+Transmembrane Domain | Extracellular TMM | Control

MATERIALS + METHODS

Plant background and transformation

The Arabidopsis ecotype Columbia (COL) was used as the wild type.

The mutant atgpi8-1 was obtained from an EMS (ethyl methanesulfonic acid)-mutagenized (0.3% for 14h) screen in an erl1-2 erl2-1 population (Shpak et al., 2004). tmm-1 (CS6140) was obtained from the Arabidopsis Biological Resource Center (ABRC). Crosses were performed to obtain *tmm atgpi8-1* +/- plants. The four inserts were constructed by performing PCR on Col genomic DNA with KpnI TMM pro and TMM 1320 ERTM.rc as primers. The inserts generated by PCR were double digested with RsrII and KpnI restriction enzymes and ligated into pMAB 302, pMAB 306, pMAB 308, and pMAB 310. Both *tmm* and *tmm atgpi8-1*+/- plants were transformed using agrobacterium. Transformations resulted in plant lines containing one of the following TMM constructs: endogenous TMM, TMM+GPI-anchor, TMM+transmembrane domain, extracellular TMM.

Plant materials and growth conditions

Following transformation, seeds from the T1-T4 generations were grown to purify gene lines and obtain samples for microscopy. Seeds were sterilized using either bleach sterilization or chlorine gas sterilization and incubated in a cold room for 48 hours before being moved to growth conditions. Growth conditions were 20°C under long-day conditions (18h light / 6h dark). All seed lines containing a plasmid were grown on modified Murashige and Skoog (MS) media plates supplemented with 1xGamborg B5 vitamins and 1% w/v sucrose and gentamicin while COL, *tmm,* and *tmm atgpi8-1* were grown on the same plates sans antibiotics. Plasmids contained antibiotic resistance and therefore allowed for selection of plants with the TMM constructs present. Plants were transferred from plates to soil pots at approximately 10 days post

germination. The soil was a mixture of a 1:1 ratio of Promix PGX and Vermiculite. Plants were supplemented with Miracle-Gro (Scotts) and approximately 3.5mg/cm3 of Osmocoat 15-9-12 (Scotts).

Selection of plants

Because *tmm* does not reduce fertility, all plants used were of the *tmm* -/- genotype. Upon maturity, plants were selected for *atgpi8-1* -/- via phenotypic selection. In contrast to COL wild type*, atgpi8-1* -/- mutants are known to have a phenotype of stunted growth and shortened siliques with low fertility. Despite low fertility, seeds were isolated from T2 *tmm atgpi 8-1* -/- plants and grown in the T3 and T4 generations for sample collection. DNA was isolated from plants used for sample collection and evaluated via PCR and gel electrophoresis as well as Sanger sequencing to ensure that the appropriate plasmid was present.

Microscopy of cotyledons and stems

In order to quantify deviations from stomata in the wt Columbia, two measurements are taken: percent of stomata clustered and stomatal index (SI). Stomatal index represents the density of stomata. Clustered stomata, measured through percent clustered, represent a loss of negative regulation over epidermal cell fate, in this experiment, through the TMM/ERf pathway.

Stomatal clustering and stomatal index of mature plant organs were measured using Differential Interference Contrast (DIC) light microscopy. Cotyledons were collected after 15 days post germination and stems after at least 3 weeks post germination. Samples were prepared for DIC microscopy though incubation in ethanol:acetic acid (9:1) for 24 h, ethanol:water (9:1) for 24 hours, and ethanol:water (7:3) and finally clearing in chloral hydrate solution. Plant tissues were cleared in a chloral hydrate solution (chloral hydrate:water:glycerol 8:1:1) for at least 24 hours prior to microscopy. Structure of the epidermis was observed using a Nikon Eclipse 80i microscope with DIC optics and images were obtained with a 12 megapixel cooled color DXM-1200c (Nikon) camera. Epidermal cells were quantified using NSI-Elements BR 2.30 software. Percent stomata clustering and stomatal index (SI) were measured for each sample. One cotyledon was used from each plant and measured on both sides yielding two sets of measurements per

cotyledon. Six sets of measurements were recorded from the epidermis of each plant stem. In *tmm* measurements, the T1 generation was used, so each data point represents one plant. In *tmm atgpi8-1* measurements, T3 and T4 generations were used with approx. n of 10 plants per data point. See formulas in Results section.

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