Invited Expert Review

Plant glycosylphosphatidylinositol (GPI) anchored proteins at the plasma membrane-cell wall nexus

Running title: Plant GPI-anchored proteins

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Abstract

Approximately 1% of plant proteins are predicted to be post-translationally modified with a glycosylphosphatidylinositol (GPI) anchor that tethers the polypeptide to the outer leaflet of the plasma membrane. While the synthesis and structure of GPI anchors is largely conserved across eukaryotes, the repertoire of functional domains present in the GPI-anchored proteome has diverged substantially. In plants, this includes a large fraction of the GPI-anchored proteome being further modified with plant-specific arabinogalactan (AG) \( O \)-glycans. The importance of the GPI-anchored proteome to plant development is underscored by the fact that GPI biosynthetic null mutants exhibit embryo lethality. Mutations in genes encoding specific GPI-anchored proteins (GAPs) further supports their contribution to diverse biological processes occurring at the interface of the plasma membrane and cell wall, including signaling, cell wall metabolism, cell wall polymer cross-linking, and plasmodesmatal transport. Here, we review the literature concerning plant GPI-anchored proteins in the context of their potential to act as molecular hubs that mediate interactions between the plasma membrane and the cell wall and their potential to transduce the signal into the protoplast and thereby activate signal transduction pathways.

Abbreviations: GPI-anchored proteins (GAPs), post-translational modifications (PTMs), phospholipase C/D (PI-PLC/D), arabinogalactan-protein (AGP), plasma membrane (PM), Golgi Apparatus (GA), endoplasmic reticulum (ER), receptor-like kinases (RLKs)
INTRODUCTION

Proteins associated with the plasma membrane are important structural components that also perform a myriad of essential functions in eukaryotes such as facilitating signaling, transport, and metabolism at the cell surface. Whilst many proteins are anchored and/or embedded within the plasma membrane (PM) by domains consisting of hydrophobic amino acid sequences, posttranslational attachment to lipids can also anchor proteins to membranes. On the intracellular face of the plasma membrane, myristoylation, prenylation and S-acylation are classes of post-translational modifications (PTMs) that can confer such associations (Hemsley 2015). On the other hand, the attachment of a GPI anchor during secretion of a protein targets the protein to the outer surface of the plasma membrane. The significance of the GPI anchor as a mode of surface display is thought to stem from two key aspects of its structure. First, a highly saturated lipid moiety can direct proteins to liquid-ordered microdomains, which act as foci for PM functions such as signal transduction. Secondly, cleavage of the GPI anchor can modulate protein function by allowing GPI-anchored proteins (GAPs) to diffuse into the extracellular milieu and therefore participate in additional functions, such as cross-linking of walls polysaccharides/(glyco)proteins.

GAPs were first discovered based on their susceptibility to bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) treatment. Treatment of mammalian cells with PI-PLC resulted in solubilization of specific isoforms of alkaline phosphatase, 5’nucleotidase, and acetylcholinesterase (Low 1989). Concurrently, GAPs were found in Saccharomyces cerevisiae (Conzelmann et al. 1988) and the parasitic protozoans Trypanosoma brucei and Leishmania spp. (Bordier et al. 1986). Several years later, the occurrence of GAPs in plants was demonstrated by similar means in a range of species (Morita et al. 1996; Kunze et al. 1997; Takos et al. 1997; Youl et al. 1998). With the availability of full genome sequences, computational prediction methods, and mass spectrometry-based proteomics in the first decade of the 21st century, the frequency and ubiquity of the GPI anchor became evident: approximately 1% of protein coding genes in eukaryotic genomes are likely to encode GAPs.

Dozens of studies on the structure of specific GPI anchors from diverse eukaryotic lineages has revealed a conserved glycan core with heterogeneity in the lipids and the substitutions (glycosyl and non-glycosyl) on the glycan core structure. The structure of the GPI anchor consists of a core glycan of three α-linked mannose (Man) residues and glucosamine (GlcN) linked to the C-terminal end of the polypeptide by phosphoethanolamine (Figure 1A). The lipid moiety is invariably attached to the glycan by myo-inositol, although the lipid itself can be either a glycerol- or sphingo-lipid. Elaborations of the core GPI structure can include either short mono-/oligo-saccharides or phosphoethanolamine residues attached to several positions on the core glycan (Figure 1A). Finally, palmitate attached to the C2 position of myo-
inositol occurs in some mature GAPs. As discussed below, all GPI anchors transiently contain this additional lipid anchor, but it is usually removed during processing and secretion.

Despite the diversity of structures that have been observed in other kingdoms, to date, the sequence of only a single plant GPI anchor has been resolved. The arabinogalactan-protein (AGP) PcAGP1, isolated from Pyrus communis (pear) cell suspension culture, was shown to possess a GPI anchor with relatively simple structure devoid of phosphoethanolamine sidechains (Oxley and Bacic 1999). The only glycan modification to the conserved core was a β-linked galactose (Gal) sidechain on O4 of Man 1, which was detected with partial occupancy. The lipid moiety of PcAGP1 consists of a ceramide, as has been detected in several fungal GPI anchors (Figure 1B). A ceramide was also detected as the lipid component of the GPI anchor of an AGP isolated from Rosa sp. cell suspension culture (Svetek et al. 1999). There are no subsequent studies of GPI anchor sequences on any plant (glyco) protein.

Over the last two decades, most of the pathway of GPI anchor biosynthesis has been elucidated, primarily using cultured mammalian cells, protozoan parasites, and S. cerevisiae as models. In sum, the GPI biosynthetic pathway is largely conserved in these well-studied systems: Initially, the anchor is assembled on phosphatidylinositol in the ER and transferred en bloc to the processed C terminus of a protein (Figure 2). Subsequent remodeling of the anchor, particularly with regard to its lipid moiety, occurs during secretion. Approximately 30 genes have been identified to be involved in particular steps of GPI anchor assembly and their remodeling, and in most cases, homology between yeast and mammalian genes is evident. A survey of the Arabidopsis genome indicates most of these genes have single-copy homologs in plants (Luschnig and Seifert 2011), and mutation of five of these genes has experimentally supported their functional roles in GPI biosynthesis (Figure 2) (Lalanne et al. 2004; Gillmor et al. 2005; Dai et al. 2014; Bundy et al. 2016). Thus, the synthesis and core structure of GPI anchors appears to be conserved in plant and non-plant eukaryotes. This is not surprising as the evolution of GPI anchors likely predates the origin of eukaryotes and GAPs have been detected in several archaea species, and archaeal genome sequences also indicate a largely conserved biosynthetic pathway (Eichler and Adams 2005). What is still lacking in plants is complete characterization of the biosynthetic pathway to determine if all yeast and human orthologues are functional and if function is conserved. Structural determination of more plant GPI anchors is sorely needed to determine if variations in structure exist.
DELIVERY OF GAPS TO THE CELL SURFACE – ASSOCIATION WITH LIPID MICRODOMAINS

After GPI anchor attachment by a GPI transamidase to proteins synthesized in the ER lumen (Ellis et al. 2010; Kinoshita 2014) the secretion and further modification (glycosylation) of GAPS begins. GAPS are secreted through the ER and GA to the cell surface and there is evidence to suggest that this is selective rather than ‘bulk-flow’ (Muniz and Riezman 2000; Bonnon et al. 2010; Zurzolo and Simons 2016). Studies in plants, animals and fungi show GAPS associate with membrane domains rich in sphingolipids and sterols, termed lipid rafts or membrane microdomains. Depending on the organism, these start forming in the ER or Golgi apparatus (GA) and facilitate early sorting of GAPS as well as other membrane-associated proteins (Pielsticker et al. 2005; Bonnon et al. 2010). Remodeling of the GPI anchor after transfer onto the protein has been shown to important in yeast and mammalian cells for association with lipids and sorting of GPI-anchored proteins (Fujita and Kinoshita 2012). In yeast, remodeling of the GPI-lipid occurs entirely in the ER and usually results in replacement with a very long highly saturated fatty acid such as ceramide (Muniz and Zurzolo 2014). In mammalian cells, remodeling of the GPI-lipid begins in the ER with GPI-inositol deacylation. Unlike in yeast, subsequent fatty acid remodeling occurs at the GA (Muniz and Zurzolo 2014; Kinoshita and Fujita 2016). Transport to the GA involves p24 proteins, regulators of protein trafficking along the secretory pathway that associate with coat protein complex I (COPI) and COPII vesicles. In yeast, p24 proteins have been proposed to selectively export GAPS in vesicles distinct from other secretory proteins (Muniz and Riezman 2000; Castillon et al. 2011). In mammalian cells, p23 and p24 proteins, along with association with lipids, was found to concentrate GAPS at ER exit sites for packaging into distinct COPII vesicles and efficient ER-GA transport (Fujita et al. 2011). In Arabidopsis p24 proteins traffic between ER and GA and associate with ARF1 and coatamer (Montesinos et al. 2012; Montesinos et al. 2014), suggesting they also have roles in secretion. It is yet to be shown if p24 proteins are also involved in selected secretion of GAPS in plants.

Further sorting of GAPS in the GA is associated with differences in lipid composition of the cisternae. Sorting of GAPS in the GA has been characterized in polarized mammalian epithelial cells where they associate with sphingolipids and sterol rich microdomains that form apical sorting platforms (Lisanti et al. 1988). In the trans-golgi network (TGN), GAPS are segregated into distinct vesicles for delivery to the apical surface (reviewed in Zurzolo and Simons 2016). Lipid asymmetry in the plasma membrane (PM) which is rich in sphingolipids in the outer leaflet and unsaturated phospholipids in the cytosolic leaflet directs apical or basolateral targeting. Association with lipids may not be the only mechanism for apical sorting in epithelial cells. Oligomerization of GAPS is also proposed to assist
segregation from other proteins in the GA, enhance lipid ordering and membrane curvature to promote vesicle budding (Zurzolo and Simons 2016). The GAPs themselves could therefore also act as sorting signals for secretory and endocytic cargo at the TGN (Paladino et al. 2006). In animals, Golgi complexes are commonly located in the perinuclear region and are generally stationary, whereas in yeast, multiple Golgi compartments are found in either ordered stacks or as separated cisternae. Plant GAs are distinct again, being dispersed throughout the cytoplasm, mobile and consisting of a series of cisternae that are polarized into cis- (closest to ER), medial- and trans-cisternae (closest to TGN) (van de Meene et al. 2017). Sequential glycosylation, both N- and O-linked, occurs in the GA. As many plant GAPs are proposed to carry one, or both, of these PTMs they are presumed to be transported through the GA, however if this is via lipid microdomains is unclear.

Trafficking of GAPs in plants has only been characterized for a few proteins. Fusion of a secreted GFP variant to a GPI-anchored pectin methylesterase inhibitor protein (PMEI1) in tobacco showed it trafficked via the ER and GA before reaching the PM and cell wall (De Caroli et al. 2011). Interestingly, secretion of PMEI1 was found to be independent of the conventional secretory pathway. Disruption of the GPI anchor signal sequence or blocking GPI anchor synthesis resulted in accumulation of PMEI1 in the Golgi stacks. Based on this study and others in plants where disruption of GPI anchor synthesis or attachment results in mis-localization (Gillmor et al. 2005; Dai et al. 2014; Xue et al. 2017), the GPI anchor may act as a sorting signal during secretion, similar to yeast and animals. The TGN in plants is suggested to have multiple sub-domains and mediate a diverse array of secretion pathways (van de Meene et al. 2017). Further studies of the secretion of a diverse range of plant GAPs is essential to determine if secretion is based on lipid association and/or other components. For example, many GAPs in plants contain N- and/or O-linked glycans that could reinforce transport to the outer leaflet of the PM due to protein-carbohydrate and/or carbohydrate-carbohydrate interactions within microdomains. Glycosyltransferases (GTs) involved in O-glycosylation have also been found in exocyst-positive organelles (EXPOs) that are part of the unconventional protein secretion pathway (Poulsen et al. 2014; Poulsen et al. 2015). This has led to the experimentally unverified suggestion that GPI-AGPs could be secreted via the EXPO pathway to direct targeted or polarized secretion (van de Meene et al. 2017).

Although it is assumed lipid asymmetry of the PM bilayer also exists in plants, only a few studies have attempted to demonstrate this. A study in oats suggested that, similar to animals, phospholipids and a minor galactolipid component typically predominate on the cytosolic leaflets of the PM. The apoplastic leaflet is enriched in sphingolipids and sterols (Tjellstrom et al. 2010). In plants GlycosylInositol PhosphorylCeramides (GIPCs) are the major sphingolipids and are enriched in the apoplastic face of the PM (Markham et al. 2013; Cacas et al. 2016). Studies in tobacco suggest poly-glycosylated GIPCs promote the formation of microdomains (Cacas et al. 2016) however, it is currently unknown if GAPs are
present in GIPC-enriched microdomains. Targeted delivery to the PM and segregation of membrane regions into microdomains is proposed to facilitate co-existence of signaling partners, adhesion, matrix remodeling/interaction with cell wall components and pathogen responses (Konrad and Ott 2015).

Membrane microdomains have a more liquid-ordered state from the surrounding membrane, have reduced mobility and are resistant to solubilization with non-ionic detergents. This latter feature has enabled their isolation in ‘detergent-resistant membranes’ (DRMs) formed due to the aggregation of the lipid structures. Due to this aggregation, DRMs do not perfectly represent microdomains in vivo and enrichment of two proteins in these fractions is not sufficient evidence of bona fide co-location in the cell. Additionally, lack of a protein in DRMs does not exclude its association with microdomains in vivo as these could be lost during DRM preparation. Despite these caveats, this method, combined with proteomic techniques, is most widely used to study membrane microdomains and the GAPs that have come to characterize them in animals, yeast and plants. In plants, DRM proteomes have been determined for numerous plant species including *Arabidopsis*, tobacco and rice (reviewed in (Takahashi et al. 2013; Tapken and Murphy 2015).

GAPs consistently enriched in DRM proteomes include the fasciclin-like arabinogalactan-proteins (FLAs), Glycosyl Hydrolases family 3 (GH3) and 17 (GH17), Plastocyanin-like domain-containing proteins, skewed 5 (SKU5) and SKU5-similar (SKS) proteins, lipid transfer proteins (LTPs) and glycerophosphodiesterase-like proteins (GPDL/SHAVEN3-like proteins [SVL]) (Borner et al. 2005; Morel et al. 2006; Kierszniowska et al. 2009; Takahashi et al. 2012). These proteins largely overlapped with other proteomics approaches that involved enriching GAPs in membrane fractions and releasing them with phospholipase C or D (Borner et al. 2003; Elortza et al. 2006).

Characterizing the spatial resolution and functional partners of GAPs remains challenging. Advanced live-cell imaging techniques, such as super-resolution microscopy, have started to address the relationship between DRMs and microdomains. For example, investigation of the location of remorins, that associate with DRMs and are present on the inner leaflet of the PM, show that distinct sets of microdomains can occur (Jarsch et al. 2014; Konrad and Ott 2015). A number of studies also support the co-existence of proteins with similar functions in distinct microdomains. For example, PIN-formed (PIN) and P-glycoprotein (ABCB/PGP) regulators of auxin transport are proposed to stably associate in discrete microdomains in the PM to ensure coordinated auxin efflux (Titapiwatanakun et al. 2009). Few examples of plant GPI-anchored protein partners and co-location exist. In contrast, interaction of GAPs with components of the cell wall are becoming increasingly apparent. An excellent example of GAPs regulating the cell wall composition/organization in a targeted manner is shown by their role in plasmodesmata (PD).
PD are membrane-rich channels that traverse the cell wall and connect neighbouring cells to enable symplastic transport of small soluble proteins, RNA and solutes. PD therefore contain a distinct membrane for targeting of specific proteins and to generate a curved membrane organization (Mongrand et al. 2010; Bayer et al. 2014). Microdomains are suggested to occur in PD to restrict lateral movement and segregate PD proteins from the rest of the PM (Raffaele et al. 2009; Simpson et al. 2009; Fernandez-Calvino et al. 2011). The lipid composition of PDs is enriched in sterols and sphingolipids with very long chain saturated fatty acids (Grison et al. 2015). This is reminiscent of DRMs and consistent with this finding, members of the Remorin family and GAPs that are enriched in DRMs locate to PD (Raffaele et al. 2009; Fernandez-Calvino et al. 2011; Iswanto and Kim 2017). The sterol composition of PD was found to be important for localization of two GPI anchored PD proteins, Callose Binding 1 (PDCB1) and $\beta$-1,3-glucanase (PdBG2) (Grison et al. 2015). In addition, delivery of PDCBP1 and PdBGs to PD was dependent on a GPI anchor (Grison et al. 2015; Zavaliev et al. 2016). These studies suggest that the specific membrane composition of PD is required for targeted secretion of GAPs to these sites. Interestingly, fusion of only the GPI anchors from PDCBP1 and PdBG2, as well as the GPI anchors from two non-PD localized proteins, AGP4 and LTGP1 to reporter proteins resulted in their enrichment in PDs. In non-PD located GAPs, the other functional domains within the protein are proposed to override the PD targeting (Zavaliev et al. 2016). This is reminiscent of the apical/basolateral sorting in epithelial cells and supports the notion that GPI anchors act as a preliminary sorting signal during secretion and in the GA/TGN other features in the protein either reinforce or redirect microdomain location.

Not only is the membrane surrounding PD specialized, but so is the cell wall that largely lacks cellulose and is rich in pectins and callose (Knox and Benitez-Alfonso 2014). Delivery of PDCBPs and PdBGs is required for their roles regulating callose deposition and cell-to-cell communication. Callose deposited in the neck regions of PD is believed to structurally constrict the PD aperture and therefore inhibit molecular trafficking. Some PDCBP members are predicted to have $\beta$-1,3–glucanase activity in addition to a callose binding X8 domain, providing a link between the PD and cell wall, important not only for PD opening but also for restricting lateral diffusion within the PD-PM.

GAPs within PD are also increasingly being recognized as having a crucial role in defense responses against fungal pathogens. The Lysin motif domain-containing GPI-anchored protein 2 (LYM2) is a chitin receptor-like protein responsible for changes in molecular flux through PD upon chitin perception (Faulkner et al. 2013). LYM2 is able to bind chitin oligosaccharides and acts independently of the receptor kinase for chitin signaling, CERK1 (Shinya et al. 2012; Faulkner et al. 2013). Enriched at PD, LYM2 was identified as a novel and critical component of pathogen-triggered responses and highlights
the key role of GAPs mediating cell-to-cell communication through PD during pathogen perception. These examples emphasize the role of GPI-anchored attachment of proteins in the PM as an important step to enable specialized interactions with cell wall polymers.

**MAKING THE PM – CELL WALL CONNECTION**

In yeast, cross-linking of glycoproteins to cell wall glucans via transglycosylation is mediated by GAPs and is essential for normal development. In *S. cerevisiae* the wall is organized into multiple layers with cell wall proteins located in the outer layer and polysaccharides, largely β−1,6-glucan, β−1,3−glucan and chitin on the inner layers (Kapteyn et al. 1999; Free 2013). Cell wall glycoproteins, which carry N- and/or O-linked glycans are often GPI-anchored and are essential for normal yeast cell wall integrity and morphology (Bowman et al. 2006; Pittet and Conzelmann 2007). GPI-anchored cell wall proteins are released from the wall after digestion by β−1,3−glucanase and β−1,6-glucanase digestion suggesting they were covalently linked to cell wall polysaccharides (Fleet and Manners 1977). GPI-anchored cell wall proteins were further shown to be covalently linked to β−1,6-glucan through phosphoethanolamine and several mannose residues (Kapteyn et al. 1997; Kollar et al. 1997; Fujii et al. 1999). Two GPI-anchored proteins with predicted GT activity, Defective Cell Wall 1 (DCW1) and Defective for Filamentous Growth 5 (DFG5) identified in *S. cerevisiae* were implicated in cleavage of the GPI anchor and transfer as mutants in these genes show weakened cell walls (Kitagaki et al. 2002). The enzymatic activity of DCW1 and DFG5 remains to be verified, however, their effects on cell wall integrity and importance for yeast viability is clear.

Transglycosylation of GAPs via their GPI anchors is not known to occur in plants, however, cross-linking of cell wall polysaccharides via transglycosylation is important for wall remodeling during growth (Frankova and Fry 2013). Interestingly, over 40% of the GAPs predicted by bioinformatics studies contain proteins with putative arabinogalactan (AG) or extensin-like glycosylation (Borner et al. 2003). These include classical AGPs, FLAs, plastocyanin-like AGPs (PAGs)/early nodulin-like and lipid transfer protein-like (LTPL) and receptor-like proteins involved in cell wall maintenance. These proteins are characterized by a domain rich in Proline (P) residues which can be hydroxylated to Hydroxyproline (Hyp, O) depending on the surrounding amino acids, and this acts as a signal for O-glycosylation (Kieliszewski and Shpak 2001; Zhao et al. 2002; Tan et al. 2003). In most GAPs, P residues occur in a non-contiguous manner, for example SPTP, such as occurs in AGPs, and this acts as a signal for glycosylation of large branched type II arabinogalactan (AG) polysaccharides (Tan et al. 2003). Covalent linking of AG to wall polysaccharides has been inferred in a number of plant species (Kjellbom et al. 1997) and has been confirmed for ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN1
APAP1 (Tan et al. 2013). APAP1 has been shown to be covalently cross-linked to both pectins and arabinoxylans. How universal AGP-wall polysaccharide interactions are remains unclear and is difficult to address due to the large heterogeneity of family members and the inability to identify them due to both glycosylation masking the protein backbones and the N-termini of many mature proteins being blocked by pyroglutamate (Johnson et al. 2017). AG glycans have also been suggested to act as ligands in signaling pathways due to the heterogeneity of their glycan structures. This intriguing association of GPI anchors with proteins proposed to contain AG glycans suggests plants have adapted unique mechanisms to make PM-cell wall connections. Further non-covalent interactions between GAPs and cell walls may be mediated by protein domains, for example, COBRA and COBRA-like GAPs have been shown to bind cellulose via a carbohydrate-binding module (CBM)-like domain (see below; (Li et al. 2013; Liu et al. 2013). Although it cannot be excluded that the GPI anchor covalently links to wall polysaccharides in plants, no clear DCW1 or DFG5 orthologues have been identified. It seems more likely therefore that GAPs associate with wall polysaccharides via protein/or O-glycan interactions and that release of the GPI from the PM occurs via another mechanism.

**CELL WALL VS. PM LOCALIZATION**

In animals, substrate-specific mechanisms have been proposed to release GAPs from the cell surface. Two classes of phosphotidylinositol-specific phospholipases (PI-PLs), PI-PLCs and PI-PLDs, are present in the ECM and are proposed to cleave the GPI anchor at different sites within the GPI moiety (Udenfriend and Kodukula 1995). For example, the GPI-specific PI-PLD in mammals is involved in cellular processes including adhesion, differentiation, proliferation, survival and oncogenesis (reviewed in (Fujihara and Ikawa 2016). PI-PLCs are known to release GAPs in unicellular organisms. A study of Paramecium PI-PLCs show that in addition to a role in intercellular signaling pathways they can also be translocated to the cell surface to act on cell surface GAPs (Staudt et al. 2016). The role of PI-PLCs in multicellular organisms is less clear. A study of GAPs secreted by mammalian gastric tumor cells showed increased stability at the membrane in the presence of PI-PLC inhibitors (Song et al. 2006). This suggests that the function of PI-PLCs to release GAPs from the PM may have been conserved.

The mammalian protein GDE2 cleaves two different GAPs in order to regulate neurogenesis. GDE2 is anchored to the PM by 6 transmembrane helices with an extracellular catalytic domain. Although the protein was initially annotated based on sequence similarity to be a glycerophosphodiester phosphodiesterase, it was experimentally shown to catalyze the cleavage of the GPI-anchored protein RECK (reversion-inducing cysteine-rich protein with kazal motifs) by a PI-PLC-like mechanism (Park et al. 2013). Cleavage of RECK relieves its inhibition of ADAM (a disintegrin and metalloproteinase), a
protease that releases peptide ligands that are perceived by the Notch receptor in a non-cell autonomous manner in order to promote neuronal differentiation. More recently, a second substrate of GDE2 was identified that is also involved in neurogenesis. The GPI anchor of heparan sulfate proteoglycan glypican-6 (GPC6) was also shown to be cleaved by GDE2. Although specific details of GPC6’s role in neuronal differentiation are unclear, it is thought that this and other glypicans may play a role in this process by either recruiting and redistributing growth factors or acting as signaling ligands (Matas-Rico et al. 2016).

A second enzyme involved in substrate-specific GPI anchor cleavage by a different mechanism was recently identified in mammals. In contrast to GDE2, PGAP6 exhibits phospholipase A2 activity that cleaves an acyl lipid group at the sn-2 position of glycerol, yielding a lyso-phosphatidylinositol-containing GPI anchor that is shed from the PM and further cleaved by extracellular phospholipase D activity (Lee et al. 2016a). PGAP6 was shown to exhibit substrate specificity for CRIPTO, a GPI-anchored co-receptor involved in embryo development, as the GPI anchor of its close homolog CRYPTIC was not processed (Lee et al. 2016a).

To date, no GPI-specific phospholipases have been characterized from plants although there are many candidates with sequence similarity to phospholipases and the requisite localization to the secretory system, PM, and/or extracellular space. The structural characterization of the GPI anchor present on the soluble isoform of Pyrus communis AGP1 indicated the absence of a terminal phosphate, suggesting that this GPI lipid was cleaved either by a phospholipase D or a phospholipase C with subsequent phosphatase action (Oxley and Bacic 1999). The identification of both specific and general GPI cleavage mechanisms in plants and their significance is an interesting area of future study.

A number of GAPs present in DRMs could be released by treatment with PI-PLC supporting the idea that their PM location could be regulated by the action of PI-PLs (Borner et al. 2003; Lalanne et al. 2004). Visualization of GAP release in vivo is needed to confirm such events yet very few examples exist. Using immuno-gold labelling of a COBRA-like protein in rice, BRITTLE CULM 1 (BC1), the authors showed BC1 located to the PM in cells with thin walls, and into the cell wall in cells with thickened, secondary walls (Liu et al. 2013). BC1 was also shown to be PI-PLD sensitive. Similarly, the GAP ZERZAUST (ZET) localized not to PM but to the cell wall (Vaddepalli et al. 2017). A number of studies have also shown that although removing the GPI-signal sequence disrupts trafficking to the surface, limited release of protein into the cell wall can still occur and, interestingly, recover protein function when used in complementation studies (Liu et al. 2016; Vaddepalli et al. 2017; Xue et al. 2017). The activity of PI-PLs is therefore likely to influence the release and function of GAPs. Further work is needed to understand the temporal and spatial dynamics of GAP release in response to PI-PL activity.
THE GPI-ANCHORED PROTEOME - PREDICTION OF GPI ANCHOR SIGNALS

The pro-sequence of GAPs typically contains two signal sequences at their N- and C- termini that are both cleaved in the mature GPI-anchored protein (Figure 1A). The N-terminal peptide is a typical secretory signal peptide that is recognized for SRP-dependent co-translational insertion of the polypeptide into the endoplasmic reticulum (ER). Eukaryotic secretory signal peptides are typically 15-30 amino acids in length, and while the amino acid sequence is not conserved, the general pattern of charged and hydrophobic amino acids in these peptides make their prediction by machine learning-based computer programs such as SignalP straightforward (Petersen et al. 2011). While approximately 10% of the plant proteome contain such signal peptides, only 10% of these are potentially further modified by attachment of a GPI anchor as specified by their C-terminal signal peptides.

The C-terminal signal peptide of GAPs is recognized by the GPI-transamidase complex, which cleaves the peptide at a site designated as the ω site and attaches the GPI anchor to the protein via an amide linkage. Although the specific sequence around this site is not conserved, significant features have been characterized. First, the amino acid sequence from ω-11 to ω-1 is typically unstructured. The region surrounding the ω site, from ω-1 to ω+2 tends to be composed of small residues. This is followed by a spacer region (ω+3 to ω+9) of moderate polarity and finally a hydrophobic region that spans the remaining peptide sequence. Based on these features and training sets of bona fide GAPs, a number of computer programs have been developed for genome-wide prediction of GPI anchor C-terminal signal sequences. These include BIG-PI (Eisenhaber et al. 1999), PredGPI (Pierleoni et al. 2008), GPI-SOM (Fankhauser and Maser 2005), and FragAnchor (Poisson et al. 2007). However, at present, these methods are universally limited by false positive rates that are of the same magnitude as the genome-wide frequency of genes that encode GAPs. Thus, published genome-wide censuses of predicted GAPs, such as Borner et al. (2003), are likely incomplete in addition to containing a number of false positives. For example, Borner et al. (2003) list At4g28560 (also known as Rac-interactive binding motif-containing protein 7 [RIC7]) as a predicted GAP, although further characterization of this protein indicated dynamic relocation between the nucleus and plasma membrane that is not characteristic of GAPs (Jeon et al. 2008). A further complication is suggested by the possibility of GAPs without signal peptides that are “double anchored” by internal transmembrane sequences. For example, the Arabidopsis gene NON RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) encodes a protein with two predicted transmembrane domains, although the mature protein’s C terminus is cleaved and contains inositol, a likely marker of GPI anchor attachment (Coppinger et al. 2004). However, as this protein is not released from membrane fractions by treatment with PI-PLC and it does not contain a signal peptide, it would not be detected by most experimental or computational approaches for identifying GAPs. Thus, while the
majority of the GPI-anchored proteome can be predicted, some of the most intriguing cases may escape high throughput experimental or computational detection.

THE PLANT GPI-ANCHORED PROTEOME

Despite the conservation of GPI biosynthetic processes across eukaryotes, there are no obvious GPI-anchored protein homologs shared between the different kingdoms of eukaryotic life. However, within the green plant lineage, the repertoire of GAPs is generally well conserved, with many GPI-anchored protein-encoding genes forming gene families within each given species. While the majority of experimental evidence with regard to GPI-anchored protein function is from the *Arabidopsis* system, there are several examples of homologous genes having similar functions in other species. A study of the hydroxyproline-rich glycoproteins (HRGP) superfamily in the 1000 plant transcriptomics data (1KP) showed that GPI anchored AGPs (GPI-AGPs) can be identified early in land plant lineages, being present in Glaucophyta, Chromista and green algae (Johnson et al. 2017) and were found throughout land plant evolution in all 1KP groups. Evolutionary studies of chimeric AGPs, many of which are predicted to be GPI anchored, could also be identified in the genomes of 47 plant species (Ma et al. 2017). In addition, studies of GPI-LTPs, proposed to function in the synthesis and/or deposition of cutin and cuticular waxes, in the moss *Physcomitrella* suggest they share similar features and function to GPI-LTPs found in flowering plants (Edstam and Edqvist 2014; Edstam et al. 2014).

Phylogenetic studies suggest that individual GPI-AGP members can be traced throughout evolution suggesting conservation of function. For example, orthologues of the *Arabidopsis* pollen-specific AGP6 and AGP11 (Coimbra et al. 2009) could be robustly identified in samples that contained floral tissue in angiosperms (Johnson et al. 2017). Similarly, the GPI-anchored AG peptide, AGP23 has been shown to be predominantly expressed in pollen in *Quercus suber* (cork oak), *Prunus persica* (peach) and *Populus trichocarpa* (Western balsam poplar) (Costa et al. 2015). These studies suggest that, at least in angiosperms, the function of GPI-AGPs and other GAPs may be conserved in reproductive development.

FUNCTION OF PLANT GAPs

Loss of GPI-anchoring in plants results in lethality, either gametophytic or embryogenic. For example, mutations in the *Arabidopsis* homolog of mammalian PIG-M, an ER-localized mannosyltransferase required for synthesis of GPI anchors show defects in cell wall synthesis in the embryo and is lethal (Gillmor et al. 2005). Phenotypes associated with specific GAPs in plants are
summarized in Table 1. These studies highlight the role of GAPs in plant development, and we provide specific examples of two key areas that are emerging, roles in signaling and cell wall maintenance.

**CELL WALL INTEGRITY MAINTENANCE AND SIGNALING?**

Although proposed for many years based on the signaling function of GAPs in other organisms, direct interaction of GAPs in plants with signaling proteins has only recently been experimentally shown. An elegant example is in the female gametophyte, where LORELEI (LRE), a GAP expressed exclusively in the ovule functions in female fertility (Liu et al. 2016). A related protein LRE-like GAP (LLG1) is more universally expressed and mutants show defects in seedling development. The similarity of *llg1* mutants phenotypes to that of mutants in FERONIA (FER), a receptor-like kinase implicated in cell wall integrity sensing, suggested they may act in the same pathway (Huck et al. 2003; Wolf and Hofte 2014; Li et al. 2015). Investigation of double mutants of *llg1* and *fer* confirmed a genetic interaction. Both LLG1 and LRE are essential for membrane localization of FER, with FER-GFP located to ER in *llg1* and *lre* mutants. LLG1 and LER physically interact with FER and are proposed to act as molecular chaperones to regulate trafficking and modulate its activity in multiple signaling pathways at the PM (Li et al. 2015). Further research investigating the specific interaction between LRE and FER in the filiform apparatus of synergid cells showed they jointly function in pollen tube reception and the GPI anchor of LRE was required for its localization in the filiform apparatus but not its function (Liu et al. 2016). Recently, an additional role of of LRE-like proteins in defense signaling has been demonstrated. LLG1 forms complexes with FLAGELLIN SENSING 2 (FLS2), the elongation factor-Tu receptor (EFR), and BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) in order to regulate innate immunity in response to pathogen-associated molecular patterns (PAMPs; (Shen et al. 2017)).

Also shown to interact with FER is ENOD-like 14 (ENODL/EN14). Containing a plastocyanin-like domain, AG glycomotifs and GPI anchored, ENODLs 11-15 are predominantly expressed in ovules and accumulate at the filiform apparatus (Hou et al. 2016). Reduced levels of *enodl11-15* through RNAi resulted in reduced pollen tube rupture and attraction of multiple pollen tubes. Potentially working in a larger FER complex, further elucidation of ENODLs, LRE and LLG1 in signaling pathways and the importance of GPI anchoring in this process is required.

The role of GAPs in pollen tube growth is also exemplified by disrupting SETH1 and SETH2, involved in the first step of GPI biosynthetic pathway. Loss of SETH1 and SETH2 severely affected pollen germination efficiency and pollen tube growth, likely associated with altered callose deposition (Lalanne et al. 2004). A combined transcriptomics and proteomics approach to identify putative pollen-
specific GAPs that might be disrupted in *seth1* and *seth2* detected β-1,3–glucanases, phytocyanins, FLAs and LTPs, many of which have members implicated in regulating cell walls. Reminiscent of PD, a specialized wall and membrane occurs in pollen tubes for growth that encompasses significant elongation whilst maintaining mechanical strength (Hepler et al. 2013; Ischebeck 2016). GAPs appear to have evolved important roles in maintaining both cell wall integrity and signaling during pollen tube growth and reception.

The link with PD and floral development is further emphasized by investigation of ZERZAUST (ZET), a GAP with similarity to β-1,3–glucanases (Vaddepalli et al. 2017). ZET likely acts together or in parallel with the atypical receptor-like kinases (RLKs) STRUBBELIG and QUIRKY, both localized to PD, to regulate development of ovules, flowers, stems and root hairs. Although localized to the cell wall, ZET is shown not to act on callose but rather acts non cell-autonomously to influence cell wall composition. ZET is potentially a mobile extracellular signaling protein that has a regulatory role controlling cell wall biology and tissue morphology.

In the seed coat, another glycoprotein, FLA4/SOS5 is proposed to act in the same pathway as two leucine-rich repeat receptor like kinases, FEI1 and FEI2, through genetic interaction studies of the mutants (Basu et al. 2016; Showalter and Basu 2016). Basu et al., 2016 suggest they physically interact through the AG glycans on FLA4, however this has not been biochemically confirmed. FLA4 lacking O-glycan structures was still able to complement a *fla4* mutant and FLA4 could function as a soluble extracellular factor suggesting it may only transiently act as a ligand for RLKs (Xue et al. 2017). The association of proteins with AG glycans and GPI anchors in plants suggests that they may play multiple roles. The potential for AG glycans to cross-link to other cell wall polymers and influence the wall matrix supports this view (see earlier section). In addition, FLA11 and FLA12 in *Arabidopsis* and their orthologues in other species have been shown to influence the microfibril angle of cellulose in secondary walls (MacMillan et al. 2010; MacMillan et al. 2015). In *fla11 fla12* mutants, this is proposed to result in changes to stem biomechanical properties (MacMillan et al. 2010). Further work is needed to investigate GAPs with AG glycans and their proposed multifunctional roles in maintenance and perception of wall integrity.

**GAPs AND CELL WALL BIOSYNTHESIS AND MAINTENANCE**

GAPs that act to modify cell wall polymers are well characterised in fungi. For example, two putative GPI-anchored glycoside hydrolases, BGT-1 and BGT-2, in *Neurospora crassa* are proposed to be important for cell wall re-modelling at the hyphal dome and during conidiation to ensure controlled growth and cell wall assembly (Martinez-Nunez and Riquelme 2015). In plants, callose binding GPI-
anchored glucanases are involved in modifying the cell walls of PD (see above). In addition, GAPs that act on other wall polymers, such as xyloglucan and cellulose are also known to exist and can be involved in modulating both the early synthesis and later re-modelling of polysaccharides.

Cellulose is the main structural component of the plant cell wall, forming strong microfibrils (MFs) most likely composed of 18-24 β-(1,4)-glucan chains. Synthesized by cellulose synthases (CESAs) at the PM, a number of proteins that associate with the CESA complexes (CSCs) can alter cellulose biosynthesis, for example, by regulating the activity of CESAs or the crystallization of MFs. It is proposed that CSCs have a specialized membrane environment due to the high levels of S-acylation on CESAs and their hydrophobicity (Kumar et al. 2016). Similar to GPI-addition, S-acylation is known to facilitate microdomain formation and/or protein partitioning (Konrad and Ott 2015). A known CESA interacting protein, KORRIGAN, has also been shown to be S-acylated and it is possible that GAPs, such as COBRAs, partition into CSCs microdomains to form large regulatory complexes (Hemsley et al. 2013).

Members of the COBRA-like family of GAPs are known to regulate the deposition of cellulose into the wall (Li et al. 2013; Ben-Tov et al. 2015). A COBRA-like protein, BC1 has been shown to directly bind cellulose to affect MF crystallinity (Liu et al. 2013). A subset of COBRAs appear to be specialized for function in secondary cell walls, which typically consist of 40-50% cellulose and are thickened to provide rigidity (Zhong and Ye 2015). COBRAs are therefore implicated in regulating wood and fiber quality (Gritsch et al. 2015; Niu et al. 2015). Loss of COBRA-like protein activity has been shown to cause plant growth defects, such as dwarfing and reduced tiller number in the brittle culm mutants in rice (Dai et al. 2011; Liu et al. 2013), altered leaf architecture in tobacco (Gao et al. 2013), disrupted seed coat mucilage (Ben-Tov et al. 2015) and pollen tube guidance (Li et al. 2013) in Arabidopsis. In pollen tubes, COBRA-like 10 (COBL10) is localized at the most apical region, where newly synthesized cell wall material is deposited, and this localization is dependent on an intact GPI anchor. COBL10 is proposed to regulate organization of pectins in the wall in response to sensing female signals. Recently, two putative GPI-anchored aspartic proteases, A36 and A39, were shown to co-localize with COBL10 (Gao et al. 2017a; Gao et al. 2017b). In the apical cell walls of pollen tubes in double a36 a39 mutants, increased levels of highly methyl esterified homogalacturonan pectins and xyloglucans were detected. GPI-anchored aspartic proteases in yeast have also been shown to function in cell wall integrity and/or remodeling (Krysan et al. 2005; Kaur et al. 2007).

GAPs may also be important for defense against fungal pathogens by modulating the cell wall and limiting penetration. For example, PMR6 encodes a GPI-anchored pectate lyase protein involved in pectin degradation and potentially release of pectic oligosaccharides. Increased pectin content in epidermal cell walls of pmr6 mutants has been shown to increase resistance against powdery mildew and Colletotrichum higginsianum (Vogel et al. 2002; Engelsdorf et al. 2017). It is proposed that this may
result in higher mechanical resistance during infection or alter signaling. Despite being only a minor component of the extracellular matrix, the conservation of roles for GAPs fine tuning the cell wall for growth, development, defense and signaling emphasizes their importance.

CONCLUSIONS AND FUTURE DIRECTIONS

Twenty years after their discovery in plants there are many remaining questions as to the function of GAPs. Recent work has assigned biological significance to dozens of these proteins by way of a range of mutant phenotypes. We have attempted to highlight the broad significance of various GAPs to the biology of plants. The long list of essentially ‘unknown proteins’ that constitute the plant GPI-anchored proteome as described by Borner and others in the early 2000’s now has mutant phenotypes associated with many of these genes. However, the biochemical function of most GAPs remains obscure but with advances in both cell/molecular techniques and modern instrumentation should now be tractable. Notably, many plant GAPs are spuriously annotated based on sequence similarity to biochemically characterized proteins, although closer examination often reveals a loss of conserved amino acids involved in ligand binding (Sedbrook et al. 2002) or catalysis (Hayashi et al. 2008; Yeats et al. 2016). As many plant GAPs are encoded by gene families that are taxonomically conserved, neo-functionalization likely occurred early in the evolutionary history of green plants. An intriguing area for future study is the biology of GAPs in early diverging lineages of green plants, such as the charophycean green algae (Domozych et al. 2016).

Another consequence of plant GAPs being encoded by gene families is that redundancy may obscure phenotypic effects of single gene mutations. For example, disruption of five genes by a combination of RNA interference and T-DNA insertion was required to demonstrate the role of EARLY NODULIN-like proteins in pollen tube reception (Hou et al. 2016). Multiplexed targeted mutagenesis by CRISPR is likely to facilitate the generation of such higher order mutants, both in Arabidopsis and in other plant species (Cermak et al. 2017).

While analysis of mutant phenotypes provides strong evidence for the significance of GAPs to specific biological processes, deeper mechanistic understanding will come from the identification of physical protein interactions. GAPs, like other secreted proteins, are recalcitrant to high-throughput methods such as yeast two hybrid screens. In several cases, co-immunoprecipitation has been used to demonstrate in vivo protein-protein interactions of specific GAPs (Li et al. 2015; Hou et al. 2016; Shen et al. 2017). As discussed earlier, intermolecular interactions involving GAPs are likely to be facilitated in part by lipid interactions that involve the GPI anchor. New technologies for detergent-free solubilization of membrane-protein complexes in their native lipid environment may be a means of directly testing this
and improving the specificity of co-immunoprecipitation experiments. For example, linear copolymers of styrene and maleic acid form amphiphilic loops that can solubilize two dimensional discs of membranes and associated proteins that are termed styrene maleic acid-lipid particles (SMALPs). SMALPs behave essentially as soluble protein-lipid complexes that are amenable to downstream biochemical purification and characterization (Lee et al. 2016b).

To date, the structure of the GPI anchor has only been resolved for a single, highly abundant plant GPI-anchored protein, PcAGP1 (Oxley and Bacic 1999). Results from other systems suggest that a diversity of GPI anchor structure should be expected (Tsai et al. 2012). The increased sensitivity and resolution of mass spectrometry are likely to facilitate further characterization of plant GPI anchor structure. It will be interesting to see whether the relatively simple structure of PcAGP1’s anchor is typical, or if glycan and PEtN sidechains are frequent modifications of other GPI anchors in plants as they are in other systems. Notably, putative orthologs of genes encoding enzymes involved in the attachment of PEtN sidechains are present in plant genomes (Luschnig and Seifert 2011). Another point that can be addressed by further characterization of plant GPI structures is the nature of the lipid groups. In the GPI anchor of PcAGP1, the lipid group was shown to be a ceramide consisting primarily of phytosphingosine and tetracosanoic acid (Oxley and Bacic 1999). The predominant form of ceramide in GPI anchors in suspension cultured cells of rose (Rosa sp.) was composed of tetracosanoic acid and 4-hydroxysphinganine (Svetek et al. 1999) suggesting some variation exists. Whether sphingolipids like this are a universal feature of plant GPI anchors, or if other lipids might confer unique properties to the GPI anchor is an intriguing area for future investigation.

As the GPI-anchored proteomes of plants appear to be distinct from other eukaryotes, it will be interesting to see how plants have adopted this post translational modification to function in the many biological processes occurring at the interface of the plasma membrane and cell wall. Many aspects of growth, development, and signaling ultimately converge here, and genetic evidence indicates the essential contribution of GAPs to all of these processes. We suggest that research efforts should now focus on the definition of what aspects of GPI biology are distinctive or uniquely important to plants. Technical advances in microscopy, mass spectrometry, and genome editing now provide the means to address these questions experimentally. Understanding how GAPs regulate signaling and cell wall metabolism will enable us to better understand their roles in fine tuning plant growth and development.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST
The authors have no conflicts of interest to declare

AUTHOR CONTRIBUTIONS
T.H.Y., A.B. and K.L.J. planned and wrote the article.

<p>| Table 1. Plant GPI-anchored proteins and respective mutant phenotypes |
|--------------------------|-----------------|------------------|------------------|------------------|
| Name                     | Locus ID(s)     | Gene family      | Phenotype/notes   | Reference         |
| <em>Arabidopsis thaliana</em>   |                 |                  |                  |                  |
| ATBG_PPAP                | AT5G42100       | Beta-glucosidase | Control of symplastic connectivity | (Levy et al. 2007) |
| AGP4                     | AT5G10430       | Classical AGP    | Synergid degeneration | (Pereira et al. 2016) |
| AGP6/AGP11               | AT5G14380/AT3G01700 | Classical AGPs   | Pollen development | (Coimbra et al. 2009) |
| AGP17                    | AT2G23130       | Classical AGP    | Biotic responses  | (Gaspar et al. 2004) |
| AGP18                    | AT4G37450       | Classical AGP    | Female gametogenesis and megaspore selection | (Acosta-Garcia and Vielle-Calzada 2004; Demesa-Arevalo and Vielle-Calzada 2013) |
| A36 and A39              | AT5G36260/AT1G65240 | Aspartic protease | Pollen and ovule development | (Gao et al. 2017a) |
| COBRA                    | AT5G60920       | COBRA-like       | Primary wall cellulose | (Schindelman et al. 2001) |
| COBRA-like 10            | AT3G20580       | COBRA-like       | Pollen tube       | (Li et al. 2013) |
| COBRA-like 2             | AT3G29810       | COBRA-like       | Seed coat mucilage | (Ben-Tov et al. 2015) |
| COBRA-like 4/IRX6        | AT5G15630       | COBRA-like       | Secondary wall cellulose | (Brown et al. 2005) |
| COBRA-like 9             | AT5G49270       | COBRA-like       | Root hair development | (Jones et al. 2006) |
| ENODL11-15               | AT2G23990/AT4G30590/AT5G25090/AT2G25060 | Early-nodulin like/plastocyanin | Pollen tube reception | (Hou et al. 2016) |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLA1</td>
<td>AT5G55730</td>
<td>Fasciclin-like AGP</td>
<td>Reduced shoot regeneration in tissue culture (Johnson et al. 2011)</td>
</tr>
<tr>
<td>FLA11 and FLA12</td>
<td>AT5G03170</td>
<td>Fasciclin-like AGP</td>
<td>Secondary cell wall mechanics and cellulose (MacMillan et al. 2010)</td>
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<td>FLA3</td>
<td>AT2G24450</td>
<td>Fasciclin-like AGP</td>
<td>Microspore development, cellulose deposition defect (Li et al. 2010)</td>
</tr>
<tr>
<td>FLA4/SOS5</td>
<td>AT3G46550</td>
<td>Fasciclin-like AGP</td>
<td>Cell expansion; salt sensitivity (Shi et al. 2003)</td>
</tr>
<tr>
<td>FLA9</td>
<td>AT1G03870</td>
<td>Fasciclin-like AGP</td>
<td>Seed abortion under drought stress (Cagnola et al. 2018)</td>
</tr>
<tr>
<td>LORELEI</td>
<td>AT4G26466</td>
<td>Lorelei-like</td>
<td>Pollen tube reception (Capron et al. 2008)</td>
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<td>LLG1</td>
<td>AT5G56170</td>
<td>Lorelei-like</td>
<td>Rapid alkalinization factor (RALF) perception (Li et al. 2015)</td>
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<td>LTPG</td>
<td>AT1G27950</td>
<td>Lipid-transfer protein</td>
<td>Cuticular wax export (Debono et al. 2009)</td>
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<tr>
<td>LTPG2</td>
<td>AT3G43720</td>
<td>Lipid-transfer protein</td>
<td>Cuticular wax export (Kim et al. 2012)</td>
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<td>LYM1 and LYM3</td>
<td>AT1G21880</td>
<td>Lysin-motif (LysM)-domain containing Peptidoglycan sensing (Willmann et al. 2011)</td>
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<td>LYM1 and LYM3</td>
<td>AT1G77630</td>
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<tr>
<td>NDR1</td>
<td>AT3G20600</td>
<td>Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family</td>
<td>Mediates SAR, possibly double-anchored (Coppinger et al. 2004)</td>
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<tr>
<td>PdBG1 and PdBG2</td>
<td>AT3G13560</td>
<td>Beta-glucosidase</td>
<td>Control of symplastic connectivity, lateral root formation (Benitez-Alfonso et al. 2013)</td>
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<td>PdBG1 and PdBG2</td>
<td>AT2G01630</td>
<td></td>
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<td>PMR6</td>
<td>AT3G54920</td>
<td>Pectate-lyase-like</td>
<td>Powdery mildew resistance (Vogel et al. 2002)</td>
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<td>SHAVEN3</td>
<td>AT4G26690</td>
<td>SHAVEN3-like</td>
<td>Root hair defective (Jones et al. 2006)</td>
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<td>SHAVEN3-like 1</td>
<td>AT5G55480</td>
<td>SHAVEN3-like</td>
<td>Cellulose-deficient and (Hayashi et al. 2008;</td>
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<td>SKU5</td>
<td>AT4G12420</td>
<td>skewed S-like/cupredoxin-like</td>
<td>Root skewing</td>
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<td>XYP1 and XYP2</td>
<td>AT5G64080 Lipid-transfer protein (xylogen)</td>
<td>Promotion of xylem differentiation, vascular development defects in mutant</td>
<td>(Motose et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>AT2G13820</td>
<td></td>
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<tr>
<td>ZERZAUST</td>
<td>AT1G64760</td>
<td>Beta-1,3-glucanase</td>
<td>Aberrant cell morphogenesis</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>SRL1/CLD1</td>
<td>Os07g01240 Unknown</td>
<td>Curled leaves, dwarf, reduced cellulose, enhanced water loss</td>
</tr>
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<td></td>
<td>BRITTLE CULM 1</td>
<td>Os03g30250 COBRA-like</td>
<td>Secondary cell wall synthesis</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>BRITTLE STALK 2</td>
<td>COBRA-like</td>
<td>Secondary cell wall synthesis</td>
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**Figure Legends**

**Figure 1. Structural features of GPI-anchored proteins**

(A) The nascent polypeptide of proteins that will be GPI-anchored include a N-terminal secretory signal peptide (SP) and a C-terminal GPI-specifying hydrophobic signal sequence (SS) that are cleaved in the ER during maturation. Approximately 40% of plant GPI-anchored protein encoding genes also contain predicted arabinogalactan (AG) glycomodule domains, and mature GPI-anchored proteins may also contain N-linked glycans. The general structure of the GPI anchor with glycosyl linkages is shown. Variable structural features that
have been observed in a range of eukaryotic species are indicated with parentheses. (B) The structure of *Pyrus communis* AGP1 GPI anchor as reported by Oxley and Bacic (1999). Aside from the core GPI glycan structure, only a \( \beta-1,4 \)-linked Gal sidechain was observed with 0.54 occupancy, as indicated. The lipid was found to be a ceramide consisting primarily of phytosphingosine and tetracosanoic acid, with minor isoforms consisting of 4-hydroxy-8-sphingenine and/or docosanoic acid. Other abbreviations used are Man = mannose, PetN = phosphoethanolamine, Gal = galactose, GalNAc = N-acetyl galactosamine, GlcN = glucosamine, PI-PLC = phosphatidylinositol-specific phospholipase C, PI-PLD phosphatidylinositol-specific phospholipase D, PLA2 = Phospholipase A2.

**Figure 2. Biosynthesis of GPI-anchored proteins in plants**

This scheme is based on the pathway as elucidated in mammalian, protozoan and yeast systems. GPI anchor biosynthesis is initiated by generation of \( N \)-glucosamine-phosphoinositide (steps 1-2) on the cytoplasmic surface before flipping by an unknown mechanism (proposed to be a “flippase”) to the ER lumen (step 3). Following acylation of inositol (step 4) and synthesis of the trimannosyl core (steps 5-7), elaboration of the GPI-anchor in plants is proposed to include galactosylation (step 8) and potentially addition of PEtN sidechains (not shown). A GPI transamidase complex transfers the GPI anchor to the protein (step 10) before export from the ER. Inositol deacylation likely occurs before plant GPI-anchored proteins reach the cell surface, but it is unknown if further fatty acid remodeling occurs as in yeast, protozoa and mammals. Most yeast and mammalian GPI biosynthetic genes have single copy orthologs in the *Arabidopsis thaliana* genome, and mutants deficient in five of these genes have been characterized (*seth1, seth2, pnt1, aptg1, and atgpi8*). The orthologous human (PIG) and yeast
(Gpi) proteins are indicated. *Arabidopsis* genes putatively involved in GPI biosynthesis based on sequence homology are outlined in Luschnig and Seifert (2011) and Ellis et al. (2010).

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**Figure 1**
Figure 2