Molecular Interactions of Arabinogalactan Proteins with Cortical Microtubules and F-Actin in Bright Yellow-2 Tobacco Cultured Cells

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Arabinogalactan proteins (AGPs), a superfamily of plant hydroxyproline-rich glycoproteins, are present at cell surfaces. Although precise functions of AGPs remain elusive, they are widely implicated in plant growth and development. A well-characterized classical tomato (Lycopersicon esculentum) AGP containing a glycosylphosphatidylinositol plasma membrane anchor sequence was used here to elucidate functional roles of AGPs. Transgenic tobacco (Nicotiana tabacum) Bright Yellow-2 (BY-2) cells stably expressing green fluorescent protein (GFP)-LeAGP-1 were plasmolysed and used to localize LeAGP-1 on the plasma membrane and in Hechtian strands. Cytoskeleton disruptors and β-Yariv reagent (which binds and perturbs AGPs) were used to examine the role of LeAGP-1 as a candidate linker protein between the plasma membrane and cytoskeleton. This study used a two-pronged approach. First, BY-2 cells, either wild type or expressing GFP-microtubule (MT)-binding domain, were treated with β-Yariv reagent, and effects on MTs and F-actin were observed. Second, BY-2 cells expressing GFP-LeAGP-1 were treated with amiprophosmethyl and cytochalasin-D to disrupt MTs and F-actin, and effects on LeAGP-1 localization were observed. β-Yariv treatment resulted in terminal cell bulging, puncta formation, and depolymerization/disorganization of MTs, indicating a likely role for AGPs in cortical MT organization. β-Yariv treatment also resulted in the formation of thicker actin filaments, indicating a role for AGPs in actin polymerization. Similarly, amiprophosmethyl and cytochalasin-D treatments resulted in relocalization of LeAGP-1 on Hechtian strands and indicate roles for MTs and F-actin in AGP organization at the cell surface and in Hechtian strands. Collectively, these studies indicate that glycosylphosphatidylinositol-anchored AGP's function to link the plasma membrane to the cytoskeleton.

Arabinogalactan proteins (AGPs) are a class of cell surface plant proteoglycans that may mediate signal transduction at the cell wall-plasma membrane interface (Kjellbom et al., 1997; Gao and Showalter, 1999; Showalter, 2001; Kohorn, 2001). AGPs belong to a superfamily of Hyp-rich glycoproteins that have a wide taxonomic distribution in the plant kingdom (Fincher et al., 1983; Nothnagel, 1997; Showalter, 2001). AGPs are typically composed of 90% carbohydrate and 10% protein (Showalter and Varner, 1989; Showalter, 1993; Nothnagel, 1997). They consist of a peptide backbone on which Hyp residues are glycosylated with type II arabinogalactans and arabinosides (Pope, 1977; Qi et al., 1991). Moreover, several AGPs are characterized by a C-terminal glycosylphosphatidylinositol (GPI) anchor that allows for their attachment to the outer leaflet of the plasma membrane (Youl et al., 1998; Sherrier et al., 1999; Svetek et al., 1999; Sun et al., 2004). Despite the vast information on plant cell wall biochemistry and structure, little is known regarding the molecular components responsible for the dynamic connections between the cell wall, plasma membrane, and cytoskeleton (Knox, 1992; Carpita and Gibeaut, 1993; Wyatt and Carpita, 1993; Roberts, 1994; Darley et al., 2001; Martin et al., 2001; Gouget et al., 2006). Unlike animals, where extracellular matrix (ECM) proteins such as integrins are involved in the ECM-plasma membrane-cytoskeleton continuum and various signaling processes (Stupack and Cheresh, 2002; Katsumi et al., 2004), a number of proteins are suggested to play a role in mediating these connections in plants (Kohorn, 2000). In this context, AGPs may function as potential candidates at the cell surface to mediate signal transduction via the cell wall-plasma membrane-cytoskeleton continuum.

Several approaches are being used to elucidate AGP functions. One approach uses Yariv phenylglycosides (Yariv et al., 1962; Yariv, 1967), which selectively bind and perturb AGPs to probe their functions (Serpe and Nothnagel, 1994; Willats and Knox, 1996; Showalter, 2001; Guan and Nothnagel, 2004). Another approach uses antibodies to track and/or perturb AGPs to provide functional insights. More recently, forward and reverse genetics approaches are being used to analyze specific AGP gene functions (van Hengel and Roberts, 2003; Gaspar et al., 2004). Although the specific functions of AGPs remain elusive, these studies have implicated AGPs in plant growth and developmental processes such as female gametogenesis (Acosta-Garcia and...
Vielle-Calzada, 2004), cell proliferation (Serpe and Nothnagel, 1994; Langan and Nothnagel, 1997), cell differentiation (Schindler et al., 1995), somatic embryogenesis (Thompson and Knox, 1998; van Hengel et al., 2001), cell expansion (Ding and Zhu, 1997), pollen germination and growth (Cheung et al., 1995), root regeneration and seed germination (van Hengel and Roberts, 2003), hormone responses (Park et al., 2003), and programmed cell death (PCD; Chaves et al., 2002).

Little is known regarding the role of AGPs in aspects related to the plant cytoskeleton. A recent study on the Arabidopsis (Arabidopsis thaliana) reb-1 mutant has suggested the connection between AGPs and microtubules (MTs; Andéme-Onzighi et al., 2002). These mutants have a root epidermal bulger phenotype and show decreased amount of AGPs and disorganized cortical MT arrays (Andéme-Onzighi et al., 2002). Ding and Zhu (1997) demonstrated that β-Yariv reagent treatment of the Arabidopsis seedling leads to epidermal cell bulging and phenocopies the reb-1 mutant. Studies are required to demonstrate that cell bulging is related to apparent changes in AGP-cortical MT connections. Another major protein component of the plant cytoskeleton, actin filaments help to maintain cell architecture and control tip growth polarity in most plants (Pierson and Cresti, 1992; Geitmann and Emons, 2000; Staiger, 2000). With studies showing the presence of AGPs on the growing tips of root hairs and pollen tubes (Samaj et al., 1999; Mollet et al., 2002) and a role for actin in signaling pathways initiated at the plasma membrane interface (Volkmann and Baluska, 1999; Staiger, 2000; Samaj et al., 2002), it remains to be demonstrated whether there are any connections between AGPs and actin.

A modular tomato (Lycopersicon esculentum) AGP, LeAGP-1 is a well-characterized Lys-rich AGP and is at our disposal for elucidating molecular interactions of AGPs (Gao et al., 1999; Gao and Showalter, 2000; Sun et al., 2004). A transgenic tobacco (Nicotiana tabacum) Bright Yellow-2 (BY-2) cell line expressing fusion protein green fluorescent protein (GFP)-LeAGP-1 was used to demonstrate that LeAGP-1 is a GPI-anchored plasma membrane AGP with potential roles in cell signaling pathways and matrix remodeling (Sun et al., 2004). Here, we employ this cell line as well as a cell line expressing GFP-MT-binding domain (MBD; of the MT-associated protein 4; Marc et al., 1998; Granger and Cyr, 2000) to study the selective perturbation of the cytoskeleton (MT and actin) and AGPs to demonstrate a cell surface network involving interactions among AGPs, MTs, and F-actin.

RESULTS

Alterations in Localization of AGPs Induced by Cytoskeletal Disruptors and β-Yariv Reagent in BY-2 Cells Expressing GFP-LeAGP-1 Before and After Plasmolysis

GFP-LeAGP-1 was expressed uniformly at the cell surface in transgenic BY-2 cells stably expressing

Figure 1. CLSM images showing the distribution of GFP fluorescence in tobacco BY-2 cells expressing GFP-LeAGP-1 following treatment with cytochalasin-D and β-Yariv reagent. BY-2 cells (6 d) were washed with fresh SH media and treated with cytochalasin-D or β-Yariv reagent. A, Control cells showed expression of the GFP-LeAGP-1 fusion protein on the cell surface (adjacent cell walls are marked with an arrow). B and C, Cytochalasin-D (20 μM for 2 h) treatment resulted in nonuniform distribution of GFP-LeAGP-1 fluorescence at the cell surface and enhanced accumulation at the cell-cell adhesion zone (arrows). D, Treatment with 80 μM β-Yariv reagent for 7 h showed an uneven distribution of LeAGP-1 on the cell surface. E and F, Prolonged β-Yariv treatment (80 μM for 24 h) resulted in a loss of GFP fluorescence at the cell surfaces and localization of GFP-LeAGP-1 at end wall regions between adjacent cells (arrows). Images are either a single optical section (F) or combined projections of multiple optical sections from a confocal Z-series (A–E). CW, Cell wall. Bars = 20 μm (A, E, and F) and 10 μm (B–D).
GFP-LeAGP-1 (Fig. 1A). These transgenic cells were treated with cytoskeletal disruptors and β-Yariv reagent, and the resulting distribution of GFP fluorescence was analyzed using confocal laser scanning microscopy (CLSM). In the presence of 20 μM cytochalasin-D, an F-actin inhibitor, BY-2 cells showed a nonuniform distribution of GFP-LeAGP-1 at the cell surface and a pronounced accumulation of fluorescence at cell-cell adhesion zones (Fig. 1, B and C). BY-2 cells treated with 80 μM β-Yariv reagent for 7 h bound to GFP-LeAGP-1 on the cell surface and resulted in its nonuniform distribution (Fig. 1D). Prolonged treatment of 6-d-old cells with β-Yariv reagent (24 h) decreased GFP fluorescence on the cell surface (Fig. 1E), but in older cells (13 d) this treatment resulted in GFP-LeAGP-1 localization at end wall regions of adjoining cells (Fig. 1F). This pattern can be attributed to inaccessibility of β-Yariv reagent to the cell surfaces of adjoining cells.

Previous studies employing immunolocalization and western analysis demonstrated that LeAGP-1 was localized to the plasma membrane and Hechtian strands (Sun et al., 2004). These previous results were confirmed using plasmolysis with 4% NaCl to reveal plasma membrane and Hechtian strand localization of GFP-LeAGP-1 (Fig. 2, A and B). This plasmolysis treatment was used as a control treatment in experimental analysis following treatments with cytoskeletal disruptors and β-Yariv reagent to reveal the locations of plasma membranes and Hechtian strands.

When transgenic BY-2 cells stably expressing GFP-LeAGP-1 were treated with β-Yariv reagent and subjected to plasmolysis, GFP-LeAGP-1 was no longer localized to Hechtian strands and was instead dispersed in the periplasmic space (Fig. 2, C and F). BY-2 cells treated with a low concentration of cytochalasin-D (20 μM for 1 h) exhibited a relocalization of LeAGP-1 on Hechtian strands (Fig. 2D), whereas treatment of older BY-2 cells (13 d) with higher concentrations of cytochalasin-D (e.g. 50 μM for 1 h) resulted in a dramatic relocalization of GFP-LeAGP-1 from Hechtian strands to the cytoplasm (Fig. 2E). In contrast, when the transgenic BY-2 cells were treated with amiprophosmethyl (APM; 30 μM for 45 min) followed by plasmolysis, this

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Treatment of tobacco BY-2 cells (6 d) expressing GFP-LeAGP-1 with cytochalasin-D and β-Yariv reagent followed by plasmolysis. A, GFP-LeAGP-1 was localized on the surface of nonplasmolysed BY-2 cells. B, Plasmolysed (4% NaCl for 1 min.) BY-2 cells showed GFP-LeAGP-1 was distributed on Hechtian strands (arrow) and plasma membranes. In the following sections, BY-2 cells were treated with cytochalasin-D or β-Yariv reagent followed by plasmolysis with 4% NaCl during the last 15 min. C and F, β-Yariv reagent treatment (100 μM for 1.5 h) caused relocalization of GFP-LeAGP-1 from Hechtian strands to the periplasmic space (arrow indicates plasmolysed cell). D, BY-2 cells (6 d) treated with a low concentration of cytochalasin-D (20 μM for 1 h) relocalized GFP-LeAGP-1 (arrow) from Hechtian strands to the periplasm. E, BY-2 cells (13 d) treated with a high concentration of cytochalasin-D (50 μM for 1 h) triggered disruption of a significant number of Hechtian strands and relocalized GFP-LeAGP-1 (arrow). Images were single optical sections (B and D) or combined projections of multiple optical sections from a confocal Z-series (A, C, E, and F). CW, Cell wall; PM, plasma membrane. Bars = 20 μm (A, B, D, and E), 50 μm (C), and 10 μm (F).
treatment resulted in a relocalization of GFP-LeAGP-1 from Hechtian strands to a punctate distribution pattern in the periplasmic space (Fig. 3).

Alterations in Cortical MT Arrays and Cell Morphology Induced by β-Yariv Reagent in BY-2 Cells Expressing GFP-MBD

To visualize cortical MTs, BY-2 cells expressing GFP-MBD were used either directly (unwashed) or washed with fresh *N. tabacum* (NT-1) media prior to treatments with Yariv reagent and APM. Compared to unwashed control cells (Fig. 4A), unwashed cells treated with 100 μM Yariv for 24 h exhibited characteristic terminal cell bulger phenotypes (Fig. 4, B–D). Optical sectioning of these bulged cells revealed the formation of depolymerization products, or puncta (Fig. 4E). Treatment of unwashed control cells with 100 μM β-Yariv for 5 h resulted in disorganization and depolymerization of cortical MTs (Fig. 4F). Depolymerization of cortical MTs was evident by their decreased number and observation of oligomers of tubulin subunits, as reported by Sivaguru et al. (2003).

Control BY-2 cells expressing GFP-MBD were washed with fresh NT-1 media and revealed transverse arrays of cortical MTs (Fig. 5, A–C) similar to those seen in unwashed control cells. However, in contrast to unwashed cells, washed BY-2 cells treated with 5 μM Yariv reagent for 5 h displayed disorganization of cortical MTs and also displayed fluorescence decoration at the walls of adjoining cells (Fig. 5D). The washing treatment effectively lowers the concentration of Yariv reagent required to elicit cortical MT disorganization by washing away AGPs secreted into the media. Washed cells treated with higher concentrations of β-Yariv reagent (100 μM for 5 h) caused disorganization of cortical MTs resulting in terminal cell bulging (Fig. 5F) as well as enhanced fluorescent labeling of cortical MTs underlying the end walls of adjacent cells (Fig. 5E). A positive control treatment with APM demonstrated extreme depolymerization of cortical MTs with characteristic depolymerization products appearing within 45 min (Fig. 5G).

Alterations in F-Actin Arrays Induced by β-Yariv Reagent in BY-2 Cells

Wild-type BY-2 cells were fluorescently labeled with rhodamine-phalloidin to localize F-actin (Fig. 6A). BY-2 cells were washed with NT-1 media prior to treatment. BY-2 cells were treated with β-Yariv reagent or cytochalasin-D, fixed, and immunolabeled to observe the distribution of F-actin. Treatment with β-Yariv reagent (80 μM for 1 h) showed formation of thicker cortical actin filaments (Fig. 6B). A positive control treatment with cytochalasin-D, an F-actin inhibitor, depolymerized F-actin arrays (Fig. 6, C–E).

DISCUSSION

Although AGP mutants in Arabidopsis have been reported with specific phenotypes, a clear understanding of AGP function, mode of action, and
molecular interactions remains elusive (Park et al., 2003; Shi et al., 2003; van Hengel and Roberts, 2003; Acosta-Garcia and Vielle-Calzada, 2004; Gaspar et al., 2004; Motose et al., 2004). Consistent with the approach of identifying molecular interactions of AGPs, we employed β-Yariv reagent and cytoskeleton inhibitors to conduct in vivo studies at the cellular level to examine molecular interactions between AGPs and the cytoskeleton (MTs and F-actin). Specifically, we demonstrate that β-Yariv treatment triggers responses in cortical MT and F-actin networks; conversely, cytoskeleton inhibitors relocalize LeAGP-1 in Hecthan strands and the periplasmic space.

Previous studies have shown that β-Yariv disruption of AGPs at the cell wall plasma membrane interface results in inhibition of growth in cell cultures (Serpe and Nothnagel, 1994; Gao and Showalter, 1999; Guan and Nothnagel, 2004) and plants (Ding and Zhu, 1997; Roy et al., 1998). In nonplasmolyzed BY-2 cells, β-Yariv and cytochalasin-D treatment affects localization of GFP-LeAGP-1 and results in pronounced fluorescence at end wall regions (Fig. 1F) and cell-cell adhesion zones (Fig. 1, B and C), respectively. This pronounced fluorescence may result from β-Yariv’s inaccessibility to end wall regions. Although the exact mechanism of AGP-Yariv binding is unknown, Yariv’s structural configuration allows it to associate with AGP molecules to form aggregated AGP-Yariv complexes. Moreover, Yariv enters into the cell wall where it binds AGPs at the cell wall-plasma membrane interface, thereby disrupting interactions of AGPs with other molecular components in the cell wall and plasma membrane (Serpe and Nothnagel, 1994). A recent report demonstrated that sonic disruption of tobacco BY-2 cells results in a large pool of cell surface soluble AGPs that are localized at the zone of plasma membrane-cell wall interface known as periplasm (Lamport et al., 2006).

In plant cells, signal transduction of developmental and environmental cues is believed to be perceived through a route of cell wall, plasma membrane, and cytoskeleton (Wyatt and Carpita, 1993; Baluska et al., 2003). Unlike animal cells where a family of transmembrane receptors termed integrins participate in the ECM-plasma membrane-cytoskeleton continuum (Hynes, 1992), plant cells lack integrin homologs (Baluska et al., 2003). Integrins in animal cells interact with ECM proteins such as vitronectin, fibronectin,

Figure 4. Confocal images showing depolymerized cortical MT arrays and the “terminal bulger” phenotype in unwashed tobacco BY-2 cells (4 d) expressing GFP-MBD in response to β-Yariv treatment. A, Control BY-2 cells expressing GFP-MBD displayed transverse arrays of cortical MTs. B, C, D, and E, Treatment of BY-2 cells expressing GFP-MBD with 100 μM β-Yariv reagent for 24 h resulted in cell bulging (arrows in B indicate the terminal cell bulger) and characteristic depolymerization products, puncta, in C and E (arrows). D and E, MTs within the bulged cell (inset box in D) are shown as a single optical section in image (E). F, Fluorescent image showing disorganized and depolymerized MTs (arrows) after β-Yariv treatment (100 μM for 5 h). Images were single optical sections (A, E, and F) or combined projections of multiple optical sections from a confocal Z-series (B–D). CW, Cell wall; N, nucleus; PM, plasma membrane. Bars = 20 μm (A, B, D, and F), 10 μm (C), and 5 μm (E).
collagen, and laminin via an Arg-Gly-Asp motif (Hynes, 1999). In plant cells, plasmolysis reveals the existence of adhesion zones between the cell wall and protoplast (Lang-Pauluzzi, 2000). Thread-like structures known as Hechtian strands are seen in the adhesion zones that connect the cell wall to the plasma membrane (Hecht, 1912; Lang-Pauluzzi, 2000). Hechtian strands are suggested to play significant roles in signal transduction and cell-cell communication events (Zandomeni and Schopfer, 1994; Reuzeau et al., 1997; Canut et al., 1998; Glass et al., 2000). In plants, application of Arg-Gly-Asp peptides results in disruption of Hechtian strands accompanied by increased pathogen susceptibility (Mellersh and Heath, 2001) and loss in signaling between cell wall and plasma membrane (Kiba et al., 1998). Studies indicate that connections between the plasma membrane and cell wall can be mediated by a number of proteins, such as AGPs, wall-associated kinases, endo-1-4-β-D-glucanases, and cellulose synthases (Kjellbom et al., 1997; Kohorn, 2000, 2001). A Lys-rich tomato AGP, LeAGP-1, is plasma membrane bound via a C-terminal GPI anchor that tethers the protein to the plasma membrane (Sun et al., 2004). Consistent with this study, we show that plasmolysis of transgenic BY-2 cells localizes GFP-LeAGP-1 on the plasma membrane and in Hechtian strands (Figs. 2B and 3A). More importantly, we show that treatment of BY-2 cells with β-Yariv, a reagent that crosslinks AGPs, relocates GFP-LeAGP-1 within the periplasmic space from the Hechtian strands (Fig. 2, C and F).

There are contrasting reports on the presence of microfilaments and MTs within Hechtian strands. Certain studies indicate existence of microfilaments and MTs within the Hechtian strand (Lang-Pauluzzi, 2000; Lang-Pauluzzi and Gunning, 2000), whereas another study demonstrates their absence (Domozycz et al., 2003). Consistent with our hypothesis that cytoskeleton inhibitors would disrupt the localization of LeAGP-1 on Hechtian strands, treatment of BY-2 cells with APM and cytochalasin-D relocates GFP-LeAGP-1 in the periplasmic space. APM is a specific depolymerization agent for cortical MTs (Granger and Cyr, 2000; Kundelchuk et al., 2002), while cytochalasin-D is an F-actin inhibitor used to probe microfilaments in eukaryotes (Cooper, 1987) and study the role of actin depolymerization in vesicle trafficking and cytoplasmic streaming. In this study, APM treatment relocates GFP-LeAGP-1 on Hechtian strands and results in a punctate distribution (Fig. 3, B and C). Interestingly, this APM treatment does not disrupt

**Figure 5.** β-Yariv treatment of washed BY-2 cells (4 d) expressing GFP-MBD. Control BY-2 cells showing transverse arrays of cortical MTs using a fluorescent filter (A) and the phase contrast image (B). Superimposed image of A and B is shown in image C. D, BY-2 cells treated with 5 μM β-Yariv reagent for 5 h showed disorganization (arrow) of the cortical MTs in most (70%) of the cells. E, Treatment with 80 μM β-Yariv reagent for 5 h resulted in disorganization of cortical MT arrays (arrow points to the deposition of fluorescence in the end wall region). F, Prolonged exposure (80 μM; 5 h) to β-Yariv reagent produced a terminal cell bulger phenotype (arrow). G, Positive control treatment with 30 μM APM for 1 h resulted in extreme depolymerization (arrow) of cortical MTs. Images are single optical sections (A–E and G) or combined projections of multiple optical sections from a confocal Z-series (F). CW, Cell wall; N, nucleus. Bars = 5 μm (A, E, and F), 10 μm (B–D and H), and 20 μm (G).
Hechtian strand formation (data not shown), consistent with previous studies that demonstrated no effects of cytoskeletal destabilizing drugs on the formation of Hechtian strands (Lang-Pauluzzi, 2000; Lang-Pauluzzi and Gunning, 2000). Treatment of BY-2 cells with both low and high concentrations of cytochalasin-D results in relocalization of GFP-LeAGP-1 on Hechtian strands (Fig. 2D) and in the cytoplasm (Fig. 2E). The appearance of GFP-LeAGP-1 in the cytoplasm most likely indicates disruption of vesicle trafficking resulting from actin depolymerization (Nebenführ et al., 1999; Gallagher and Benfey, 2005), although endocytosis of GFP-LeAGP-1 cannot be excluded as another alternative leading to this cytoplasmic localization.

Studies employing Yariv reagent in suspension cultured cells (Serpe and Nothnagel, 1994; Willats and Knox, 1996) and Arabidopsis seedlings (Ding and Zhu, 1997) indicate a connection between AGPs and cell expansion. Another study with the Arabidopsis reb-1 mutant reinforced this connection and reported phenotypic variations, including root epidermal cell bulging in the elongation zone (exclusively within the trichoblasts), immunolocalization of AGPs in the atrichoblast of the roots, and disrupted MTs within the swollen trichoblast cells (Andème-Onzighi et al., 2002). The REB-1 gene encodes a UDP-α-Glc-4-epimerase, which functions in α-Gal synthesis (Seifert et al., 2002). A recent immunocytochemical and biochemical study on reb-1 suggested a role for UDP-α-Glc-4-epimerase in galactosylation of AGPs and xyloglucans (Nguema-Ona et al., 2006).

In our studies, treatment of BY-2 cells expressing GFP-MBD with Yariv reagent demonstrates a terminal cell bulger phenotype that phenocopies the reb-1 epidermal cell bulger (Figs. 4, C–F). Yariv-induced terminal cell bulging of BY-2 cells indicates a role for AGPs in this event. Anisotropic growth in plant cells is maintained by the turgor pressure (internal and isotropic) exerted on the cell wall. Cellulose microfibrils play an important role in controlling the anisotropic growth (Williamson et al., 2001; Scheible et al., 2003). The direction of growth is controlled by deposition of cellulose microfibrils, which in turn are directed by the organization of cortical MTs (Baskin, 2001; Camilleri et al., 2002; Sugimoto et al., 2003; Lloyd, 2006). Previously, a study on a GPI-anchored protein, COBRA, revealed its role in anisotropic expansion and orientation of cellulose microfibrils (Roudier et al., 2005). In certain studies, it was also shown that loss in anisotropic growth is accompanied by abnormal cell swelling (Lane et al., 2001; Williamson et al., 2001). Our studies show that bulging takes place only in the terminal cells and not in the cells between them. In an elongating cell, growth occurs along the length of the cell, and the cellulose microfibrils in these cells provide structural support and shape by being deposited perpendicularly to the direction of growth and expansion. Compared to nonterminal cells, terminal cells are more exposed to Yariv reagent and thereby readily show a bulger phenotype. Although the exact mechanism underlying this cell bulging is not clear, the depolymerized MTs, cell bulging, and defects in directional growth (Fig. 4) indicate a connection between

**Figure 6.** F-actin (labeled with rhodamine-phalloidin) distribution in wild-type BY-2 cells (3 d) in response to treatment with Yariv reagent and cytochalasin-D. A, Control CSLM image showing F-actin arrays (arrow) in BY-2 cells. B, Yariv reagent treatment (80 μM for 1 h) resulted in formation of thicker actin cables (arrow). C to E, Positive control treatment with cytochalasin-D (20 μM for 1 h) depolymerizes actin strands. Fluorescent image (C) and phase contrast image (D) show depolymerized actin strands and their corresponding superimposed image is shown as image E. CW, Cell wall; PM, plasma membrane. Bars = 20 μm (A, C–E) and 10 μm (B).
AGPs, MTs, terminal cell bulging, and anisotropic growth.

Yariv reagent depolymerizes and disorganizes cortical MTs in washed or unwashed GFP-MBD-expressing cells (Figs. 4 and 5). In comparison to the unwashed cells (Fig. 4), the washed cells (Fig. 5) demonstrate no reduction in the fluorescence of GFP-MBD, and any differences observed in fluorescence intensity are due to BY-2 cells present in different focal planes or low fluorescent filter settings. Similarly, Yariv reagent also affects the organization of the F-actin. Although Yariv does not result in depolymerization of the F-actin, it results in thicker cortical F-actin filaments (Fig. 6). Previously, Gao and Showalter (1999b) reported that β-Yariv treatment of Arabidopsis cells for 72 h results in cytoplasmic shrinkage and absence of nucleus, structural changes that are characteristic of PCD. In another study, treatment of unwashed BY-2 cells with higher concentration (approximately 80 μM) of Yariv reagent results in PCD within 72 h (Chaves et al., 2002). Our studies show that the architectural changes in the cell shape and cytoskeleton take place within 5 h (for washed cells; Fig. 5) and 24 h (for unwashed cells; Fig. 4) of β-Yariv treatment. Thus, defects in the cytoskeleton precede PCD and may be responsible for PCD similar to the way disruption of the ECM-plasma membrane-cytoskeletal continuum can lead to PCD in animals (Ku et al., 1999; Bursch et al., 2000; Suetsugu and Takenawa, 2003).

Based on our studies, we propose a cell surface network model involving interactions between AGPs and the cytoskeleton (i.e. MTs and F-actin). GPI-anchored LeAGP-1 is localized to lipid rafts, which contain lipids such as glycosphingolipids and sterols (such as stigmasterol, campesterol, and β-sitosterol), and interacts with MTs and F-actin in the cytoplasm either by a transmembrane protein (in the phospholipid bilayer) or by molecules (A and B) associated with the lipid rafts. GPI-anchored LeAGP-1 in lipid rafts may mediate interactions in two possible ways: A, binding of LeAGP-1 to a transmembrane receptor in the lipid microdomain mediates the interactions; and B, ligand-LeAGP-1 receptor complex-induced translocation of a signal molecule outside the lipid raft to activate a cytoplasmic kinase. Also shown are the different constituents of LeAGP-1 that include a Pro/Hyp-rich protein backbone decorated with arabinogalactan polysaccharides, short arabinosides, and an unglycosylated Lys-rich peptide region.

Figure 7. A hypothetical cell surface network model involving interactions between LeAGP-1 and the cytoskeleton (i.e. MTs and F-actin). GPI-anchored LeAGP-1 is localized to lipid rafts, which contain lipids such as glycosphingolipids and sterols (such as stigmasterol, campesterol, and β-sitosterol), and interacts with MTs and F-actin in the cytoplasm either by a transmembrane protein (in the phospholipid bilayer) or by molecules (A and B) associated with the lipid rafts. GPI-anchored LeAGP-1 in lipid rafts may mediate interactions in two possible ways: A, binding of LeAGP-1 to a transmembrane receptor in the lipid microdomain mediates the interactions; and B, ligand-LeAGP-1 receptor complex-induced translocation of a signal molecule outside the lipid raft to activate a cytoplasmic kinase. Also shown are the different constituents of LeAGP-1 that include a Pro/Hyp-rich protein backbone decorated with arabinogalactan polysaccharides, short arabinosides, and an unglycosylated Lys-rich peptide region.
were treated with 100 mM (three times) and treated with different concentrations of respective pharmacological treatments.

**NT-1 media and treated with 4% NaCl solution for 10 to 15 min at the end of times** and expressing GFP-LeAGP-1 were washed with fresh SH media (three times). This GPI anchor in the lipid microdomain can potentially interact with receptors for the signaling ligands. Based on studies in animal cells (Zajchowski and Robbins, 2002), GPI-anchored AGPs in lipid rafts may mediate signaling in plants. Two possible scenarios come to mind, as depicted in Figure 7: (A) binding of the GPI-anchored protein to a transmembrane receptor present within the lipid microdomain may initiate signaling; and (B) binding of an extracellular ligand to LeAGP-1 receptor present in rafts may translocate another signal molecule out of the lipid microdomain leading to activation of intracellular/cyttoplasmic kinase. While testing of this model remains to be done, our studies to date clearly indicate that GPI anchored-AGPs play a role in the plasma membrane-cytoskeleton connections.

**MATERIALS AND METHODS**

**Cell Cultures and Growth Conditions**

Two transgenic tobacco (Nicotiana tabacum) BY-2 cell lines were used to conduct the studies, a cell line expressing GFP-LeAGP-1 and another cell line expressing GFP-MBD. The BY-2 suspension cell cultures expressing GFP-MBD were maintained in liquid Murashige and Skoog media (4.3 g/L Murashige and Skoog salts [Sigma], 30 g/L Suc, 1 mg/mL thiamine HCl, 100 mg/L myoinositol, 0.44 mg/L 2,4-dichlorophenoxyacetic acid, pH 5.8) and BY-2 cell line expressing GFP-LeAGP-1 were maintained in liquid Schenk and Hildebrandt (SH) media (3.2 g/L SH basal salt; Sigma), 1 g/L SH vitamin powder, 1 mg/mL kinetin, 1 mg/mL p-chlorophenoxy acetic acid, 1 mg/mL 2,4-dichlorophenoxyacetic acid, 34 g Suc, pH 5.8) on a rotary shaker (120 rpm) at 24°C and subcultured weekly (1:10) into fresh culture media.

**Plasmolysis Treatment**

BY-2 cells expressing GFP-LeAGP-1 were washed with fresh SH media or NT-1 media and treated with 4% NaCl solution for 10 to 15 min at the end of respective pharmacological treatments.

**Pharmacological Treatments**

**β-Yariv Reagent Treatment**

Washed and unwashed BY-2 cells were subjected to Yariv treatments. BY-2 cells (3 d) expressing GFP-MBD were washed with fresh NT-1 media (three times) and treated with different concentrations of β-Yariv reagent for different time periods. Also, unwashed BY-2 cells expressing GFP-MBD were treated with 100 μM β-Yariv for 24 h. In another treatment, BY-2 cells (3 d) expressing GFP-LeAGP-1 were washed with fresh SH media (three times) and treated with 80 μM β-Yariv reagent for 1 h. After the inhibitor treatment, cells were fixed for cortical F-actin using rhodamine-phalloidin (Molecular Probes).

**APM Treatment**

Three-day-old tobacco BY-2 cells expressing GFP-MBD and GFP-LeAGP-1 were washed with fresh media (three times) before the treatment. Washed BY-2 cells were treated with 30 μM APM (10 mM stock in dimethyl sulfoxide; Austratec Pty) for 1 h.

**Cytochalasin-D Treatment**

Three-day-old wild-type tobacco BY-2 cells and tobacco cells expressing GFP-LeAGP-1 were washed with fresh media (three times) and were treated with 20, 25, and 50 μM of cytochalasin-D (10 min stock in dimethyl sulfoxide; Sigma) for different time periods. Following the inhibitor-drug treatment, BY-2 cells were fixed for cortical F-actin using rhodamine-phalloidin.

After the pharmacological treatments, CLSM was conducted to examine the distribution and localization of fluorescence in respective cell lines.

**Rhodamine-Phalloidin Staining**

Wild-type BY-2 cells were washed with fresh Murashige and Skoog media (2 × 1 min) and attached to poly-L-lys (Sigma)-coated glass slides (1 mg/mL). After 10 min, the cells were fixed with 3.8% formaldehyde/phosphate-buffered saline (PBS) for 30 min at room temperature and washed with PBS, pH 7.4 (3 × 5 min). Cells were permeabilized with 0.1% Triton-X-100/PBS for 10 min and washed again with PBS (3 × 5 min). Cells were then incubated with 1% bovine serum albumin/PBS for 25 min and labeled with rhodamine-phalloidin/PBS (5 μL of methanolic stock; 6.6 μM) in 200 μL of PBS) for 20 min. After washing (3 × 5 min); the cells were mounted on slides with 50% glycerol/PBS and observed under CLSM.

**CLSM**

Cultured cells were placed on a drop of water on glass slides that were layered with coverslips. The glass slides were positioned onto the inverted platform of a CLSM (510; Zeiss) and the cells were imaged using the 488-nm line of argon laser. Images were recorded with ×10 and ×40 objectives (NA 0.75; Zeiss) and a 488-543 nm dual dichroic excitation mirror with a 510- to 540 nm emission filter. All images were obtained either with a fluorescein isothiocyanate or a Texas red filter set. All images were processed with Zeiss imaging software and Adobe Photoshop.

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**LITERATURE CITED**


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