MOLECULAR INTERACTIONS OF ARABINOGLACTAN-PROTEINS (AGPs) IN TOBACCO BRIGHT YELLOW-2 CULTURED CELLS AND FUNCTIONAL IDENTIFICATION OF FOUR CLASSICAL AGPs IN *ARABIDOPSIS*

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IDENTIFICATION OF FOUR CLASSICAL AGPs IN ARABIDOPSIS

by

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Abstract

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MOLECULAR INTERACTIONS OF ARABINOGLACTAN-PROTEINS (AGPs) IN TOBACCO BRIGHT YELLOW-2 CULTURED CELLS AND FUNCTIONAL IDENTIFICATION OF FOUR CLASSICAL AGPs IN ARABIDOPSIS (213 pp.)

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Arabinogalactan-proteins (AGPs) are hydroxyproline-rich glycoproteins that are expressed at plant cell surfaces. Two different approaches were employed to study AGPs, (1) perturbation studies in tobacco BY-2 cells to examine molecular interactions of AGPs and (2) reverse genetics studies to elucidate functional roles of AGPs in Arabidopsis. In the first approach, role of a tomato AGP LeAGP-1, as a candidate linker protein between plasma membrane and cytoskeleton was examined. Fluorescent probes used to perform these studies were, green fluorescent protein-microtubule binding domain [(GFP-MBD) which labels cortical microtubules (MTs)], rhodamine-phalloidin (which labels F-actin) and GFP-LeAGP-1 (which labels LeAGP-1 on the cell surface and Hechtian strands). Treatment of BY-2 cells expressing GFP-MBD with β-Yariv reagent (which selectively binds AGPs) resulted in defects in terminal cell expansion accompanied with depolymerization/disorganization of MTs. Similarly, β-Yariv treatment of wild type BY-2 cells resulted in defects in organization of F-actin. Conversely, treating cells expressing GFP-LeAGP-1 with amiprophosmethyl (APM; promotes microtubule depolmerization) and cytochalasin-D (promotes F-actin depolymerization) resulted in relocalization of LeAGP-1 on Hechtian strands and cell surface. These studies indicate a likely role for
glycosylphosphatidylinositol (GPI)-anchored AGPs in cortical MT and F-actin organization and conversely roles for MTs and F-actin in organizing AGPs at cell surface.

In another approach, T-DNA insertion lines for four *Arabidopsis* classical AGPs, AtAGP3, AtAGP4, AtAGP7 and AtAGP9 were examined to identify their functions. T-DNA homozygous mutants of *AtAGP4*, *AtAGP7* and *AtAGP9* were identified (a potential homozygous lethal mutant was identified for *AtAGP3*) and examined for phenotypic aberrations. Expression analyses were performed by northern blotting and reverse transcription-polymerase chain reaction (RT-PCR) studies. These studies indicate high expression for *AtAGP4* and *AtAGP9* in stem and young roots. Real-time quantitative-PCR studies demonstrate absence of RNA transcript in roots of *atagp7*, whereas, decreased transcript levels in *atagp4* and *atagp9(3)* roots and increased transcript levels in *atagp9(5)* roots. Growth-based phenotypic analyses for *atagp9* mutants indicate defects in lateral root development. Germination assays indicate *atagp4*, and *atagp9* mutants show increased tolerance to osmotic and abscisic acid (ABA) stress, thereby enhancing germination whereas *atagp7* shows sensitivity to ABA stress (decreasing germination). Collectively, these studies suggest roles of these classical AGPs in seed germination, stress signaling and root growth and development.

Approved: ____________________________________________

Allan M. Showalter

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List of Abbreviations

ABA: abscisic acid
ABRC: Arabidopsis Biological Resource Center
AFGC: Arabidopsis Functional Genomics Consortium
AG: arabinogalactan
AGP: arabinogalactan-protein
Ala: alanine
APM: amiprophosmethyl
Asn-rich: aspargine-rich
bp: base pair
BY-2: bright yellow-2
cDNA: complementary DNA
CSLM: confocal scanning laser microscope
Cys-rich: cystine-rich
CW: cell wall
d: day
DMSO: dimethylsulfoxide
DRM: detergent-resistant membrane
DSS: disuccinimidyl suberate
DST: disuccinimidyl tartarate
ECM: extracellular matrix
EMS: ethyl methanesulfonate
ER: endoplasmic reticulum
EST: expressed sequence tags
EXT: extensin
FITC: fluorescein isothiocyanate
FLAs: fasciclin-like AGPs
GAGP: gum arabic glycoprotein
GFP: green fluorescent protein
GFP-LeAGP1: tomato arabinogalactan-protein 1
GFP-MBD: GFP-microtubule binding domain
GlcNAc: N-acetyl-glucosamine
GPI: glycosylphosphatidylinositol
GUS: β-glucoronidase
h: hour
His: histidine
HM: homozygous
HZ: heterozygous
HRGP: hydroxyproline-rich glycoprotein
Hyp: hydroxyproline
kDa: kilo dalton
LeAGP: *Lycopersicum esculentum* arabinogalactan-protein
LecRK: lectin receptor kinase
Lys-rich: Lysine-rich
min: minute
MS: Murashige and Skoog
MTs: microtubules
MW: molecular weight
NT-1: Nicotiana tabacum 1
P4H: Proly 4-hydroxylase
PBS: phosphate buffered saline
PCD: programmed cell death
PCR: polymerase chain reaction
PERK: proline-rich extensin-like receptor kinase
PLC: phospholipase C
PLD: phospholipase D
PM: plasma membrane
PMSF: phenylmethanesulphonylfluoride
Pro: proline
PRP: proline-rich protein
rat1: resistance to Agrobacterium transformation
reb1: root epidermal cell bulger 1
RNAi: RNA interference
RT: room temperature
RT-PCR: reverse transcriptase-polymerase chain reaction
SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

Ser: serine

SH: Schenk and Hildebrandt

SIGnAL: Salk Institute Genomics Analysis Laboratory

SOS5: Salt Overly Sensitive 5

TBS: Tris buffered saline

T-DNA: transfer DNA

TE: tracheary element

The: threonine

TTS: transmitting tissue-specific

Tyr: tyrosine

UGE4: UDP-D-glucose-4-epimerase

Val: valine

WAK: wall-associated kinase

WT: wild type
CHAPTER 1: INTRODUCTION
Plant cell wall applications

Plant cell walls are beneficial to humans because they provide both excellent business opportunities and allow us to lead a better life. The material from cell walls is useful in various ways. Commercially, secondary cell walls serve as raw material in the textile, paper, wood and furniture industries; in agriculture, manipulation of cell wall characteristics through biotechnology allows for production of plants with improved characteristics such as low lignin wood, higher disease resistance and fruits and plant products with longer shelf life; in the food industry they serve as a source of dietary fiber and the wall polysaccharides are used as gelling and thickening agents. One such plant product of daily use, plant gums, are valuable and used in the food, pharmaceutical, confectionary, and printing industries where they serve as emulsifiers, stabilizers, and adhesives. An important constituent in the commercially available gum products is arabinogalactan-proteins (AGPs) (Akiyama et al., 1984). AGPs are plant cell surface glycoproteins that belong to a superfamily of hydroxyproline-rich glycoproteins (HRGPs).

Hydroxyproline-rich glycoproteins

HRGPs as the name suggests are rich in hydorxyproline (Hyp) residues (Lamport, 1960; Lamport, 1965; Lamport, 1967) and ubiquitously distributed in the plant kingdom. HRGPs are classified into different sub-classes, proline-rich proteins (PRPs), extensins (EXTs), and AGPs. HRGP represents protein subclasses that are a continuum of
glycoproteins that range from lightly arabinosylated PRPs to moderately arabinosylated extensins and highly arabinosylated AGPs (Kieliszewski and Lamport, 1994).

**Proline-rich proteins (PRPs)**

PRPs are basic and lightly arabinosylated HRGPs that are characterized by Pro-Pro repeats (Marcus et al., 1991; Iter, 1993). Past studies suggest PRPs are classified into two major sub-classes that include the normal plant cell wall PRPs and plant nodulins (Chen and Varner, 1985; Hong et al., 1987; Sheng et al., 1991). The protein backbone of PRPs consists of mostly Hyp and Pro residues in equal amounts (Datta et al., 1989; Kleis-San Francisco and Tierney, 1990). The interesting aspect regarding this class of HRGPs is that either they are devoid of glycan decoration or minimally decorated with oligosaccharides attached on the Hyp residues (Datta et al., 1989). Studies indicate these proteins are characterized by several peptide repeats such as Pro-Pro-X-Y-Lys, Pro-Pro-X-Lys and Pro-Pro-X-Y-Pro-Pro (where X and Y represent either Val, Tyr, His or Glu) (Marcus et al., 1991; Showalter and Rumeau, 1990; Showalter and Varner, 1989).

**Extensins (EXTs)**

Extensins are a class of highly glycosylated HRGPs that are widely distributed across the plant kingdom (Lamport, 1965, Lamport, 1967; Lamport and Catt, 1981). Extensins are characterized by a pentapeptide repetitive motif Ser-Hyp$_4$ and the protein backbones are rich in Hyp and Ser residues to a large extent and Val, Lys, Tyr and His residues to a
smaller extent (Kieliszewski and Lamport, 1987; Kieliszewski et al., 1990; Lamport, 1967). Glycosylation patterns of the extensins are such that Hyp residues are glycosylated with 1 to 4 arabinosyl molecules and a single galactose decorates some serine residues (Lamport, 1967; Lamport et al., 1973; Lamport and Catt, 1981). Extensins are implicated in a number of physiological and developmental processes in plants including pollen fertilization (Wu et al., 2001), abscission and senescence (Markouropoulos and Shirsat, 2003), cell division and differentiation (Ito et al., 1999) and inflorescence stem thickening and height regulation (Roberts and Shirsat, 2005).

**Arabinogalactan-proteins (AGPs)**

AGPs are water soluble and highly glycosylated HRGPs that are ubiquitously found in plants (Bobalek and Johnson, 1983; Clarke et al., 1978; Clarke et al., 1979, Fincher et al., 1983; Showalter and Varner, 1989). AGPs are highly heterogeneous macromolecules with high molecular weights ranging between 60kDa to 300kDa and are composed of 10% protein and 90% carbohydrate (Clarke et al., 1979; Fincher et al., 1983). AGPs are generally acidic in nature and are rich in Hyp, Ser, Ala, Thr and Gly residues (Fincher et al., 1983; Showalter and Varner, 1989).

**AGP structure**

The complete chemical structure of the AGPs is still unknown because of their heterogeneous nature and highly complex structure. Typically, the macromolecular
structure includes the arabinogalactan and arabinoside carbohydrate component (~90%) attached to the Hyp residues on the core protein backbone (~10%) (Bacic et al., 1987; Clarke et al., 1979, Fincher et al., 1983; Pope, 1977; Qi et al., 1991). AGPs are one of the most extensively post-translationally modified proteins in nature. Different types of post-translational modifications in AGPs include cleavage of the N-terminal signal peptide, hydroxylation of proline residues, O-glycosylation and GPI anchor addition and cleavage (Lamport, 1967; Pope, 1977; Qi et al., 1991, Sherrier et al., 1999; Svetek et al., 1999; Youl et al., 1998).

Prolyl 4-hydroxylase (P4H) is an enzyme responsible for conversion of proline (Pro) into 4-hydroxyproline (4-Hyp) during the post-translational modification of AGPs in ER (Cohen et al., 1983; Cooper and Varner, 1983, Schmidt et al., 1991; Serpe and Nothnagel, 1994). Hydroxylation of proline is followed by biosynthesis of carbohydrate portions within the ER and Golgi (Andreae et al., 1988; Gardiner and Chrispeels, 1975; Hayashi and Maclachlan, 1984; Kawasaki, 1981; Mascara and Fincher, 1982; Misawa et al., 1996; Schibeci et al., 1984). Little information is available on the P4Hs and to date, only 2 P4Hs have been cloned in Arabidopsis (AtP4H1 and AtP4H2) and one in tobacco (Hieta and Myllyharju, 2002; Tiainen et al., 2005; Yuasa et al., 2005). AtP4H1 demonstrates specific activity only towards (SPPPV)$_3$, (PPG)$_{10}$, and (APG)$_5$ substrates whereas AtP4H2 is specific only towards (SPPPV)$_3$ peptides (Hieta and Myllharju, 2002; Tiainen et al., 2005). Prolyl hydroxylation also depends on the peptide sequence found in the core protein (Tan et al., 2003).
Classification of AGPs

An earlier classification divided AGPs into two broad classes based on their amino acid composition, classical and non-classical AGPs. Classical AGPs consist of a N-terminal signal peptide domain, an AGP domain that is rich in hydroxyproline (Hyp) residues and a C-terminal glycosylphosphatidylinositol (GPI) anchor (Chen et al., 1994; Du et al., 1994; Gerster et al., 1996; Li and Showalter, 1996; Loopstra and Sederoff 1995; Oxley and Bacic, 1999; Pogson and Davies, 1996, Svetek et al., 1999; Youl et al., 1998.). Non-classical AGPs differ from the classical AGPs in that they have different core compositions such as presence of Cys-rich domains and Asn-rich domains and they lack a C-terminal GPI anchor addition sequence (Chen et al., 1993; Cheung et al., 1993; Du et al., 1996; Mau et al., 1995; Sheng et al., 1991). A more recent classification was suggested by Schultz et al (2002) that identified various AGP classes in Arabidopsis. Based on the annotations available in the Arabidopsis genome and biased amino acid composition, a computer algorithm was designed by Schultz et al (2002), to identify various AGP backbones in Arabidopsis. The different subclasses of AGPs identified in this study include 13 classical AGPs, 10 AG-peptides, 3 lysine-rich AGPs, and 21 fasciclin-like AGPs (Schultz et al., 2002) (Fig. 1.1).
**Figure 1.1** Schematic representation of different classes of AGPs in *Arabidopsis*. The chart shows native AGPs after post-translational modifications. (Adopted and modified from Schultz et al., 2000) The blue branches on native AGPs indicate arabinogalactans and green branches indicate arabinosides. The number in the parenthesis for each class of AGPs indicates their respective number in each class identified in *Arabidopsis*. The zig-zag tail structure at the end terminus of AGPs indicates the glycosylphosphatidyl-inositol (GPI) anchor.

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- **SP**: Signal Peptide
- **GPI**: Glycosylphosphatidylinositol anchor addition sequence
- **AGP**: Arabinogalactan protein domain
- **FLA**: Fasciclin domain
- **Lys**: Lys-rich domain
- **Asn-rich**: Asn-rich domain
Arabinogalactan-peptides (AG)

After their first identification in wheat (Fincher, 1974), studies in Arabidopsis have also identified AG-peptides. In Arabidopsis, AG peptides typically possess all the characteristic features of classical AGPs (Du et al., 1996; Schultz et al., 1998; Schultz et al., 2004). One of the most intriguing aspects regarding the AG peptides is their small size, these peptides are characterized by Pro-rich AGP domains which are approximately 12-13 amino acids in size (Schultz et al., 2000). These AG peptides are a unique class of molecules and are not degradation products of mature classical AGPs. AtAGP24 is one of the best studied AG peptides and is reported to be 17 amino acids in size (Schultz et al., 2002). To date, there are no specific reports on the functions of the AG peptides.

Lysine-rich AGPs (Lys-rich)

Lys-rich AGPs are a small group of homologous AGPs and are characterized by a short (~12 amino acids) Lys-rich domain within the central core AGP domain (Gilson et al., 2001). Similar to AG-peptides, Lys-rich AGPs are variants of classical AGPs. In Arabidopsis, three Lys-AGPs AtAGP17, AtAGP18, and AtAGP19 were identified (Schultz et al., 2000; Sun et al., 2005). Other homologous Lys-rich AGPs found in different plant groups include the tomato LeAGP-1 (Li and Showalter, 1996; Gao et al., 1999a), tobacco NaAGP4 (Gilson et al., 2001), cucumber CsAGP-1 (Park et al., 2003) and pine PtaAGP6 (Zhang et al., 2003). Lys-rich AGPs are one of the best studied group of AGPs in Arabidopsis and other plant groups. A number of mutant studies have been
used to indicate the role of Lys-rich AGPs in plant growth and development. *rat1* (resistance to *Agrobacterium* transformation) is an *Arabidopsis* mutant (Nam et al., 1999, Zhu et al., 2003) that shows reduced levels of *AtAGP17* in the roots (Gaspar et al., 2004). Complementation of this mutant with wild type transcript results in a normal wild type phenotype of successful *Agrobacterium* transformation (Gaspar et al., 2004). This study indicates a role for *AtAGP17* in the first step of *Agrobacterium* infection which allows the tight binding of *Agrobacterium* to the roots. In another study, *AtAGP18* was reported to play a role in female gametogenesis (Acosta-Garcia and Vielle-Calzada, 2004). A study with another T-DNA knockout mutant suggests a role for *AtAGP19* in various aspects of plant growth and development (Yang et al., 2007).

**Fasciclin-like AGPs (FLAs)**

Fasciclin-like AGPs (FLAs) are a special class of AGPs found in *Arabidopsis* that are considered chimeric AGPs because of the presence of putative cell adhesion domains in addition to the AGP domain (Johnson et al., 2003). The fasciclin domain, a highly conserved domain found in Drosophila functions in cell adhesion (Zinn et al., 1988; Elkins et al., 1990). In *Arabidopsis*, FLAs exhibit two highly conserved regions within the fasciclin domains which may indicate that the cell adhesion function is conserved among various FLAs (Johnson et al., 2003). FLAs possess all the other characteristic domains found in a classical AGP. Most of the *Arabidopsis* FLA’s possess two AGP domains and one FLA domain except for a few FLAs in which multiple AGP and FLA
domains are found (Johnson et al., 2003) Based on a study in an *Arabidopsis* mutant, *SOS5* was reported to encode a putative fasciclin-like AGP (FLA-AGP) and function in cell expansion (Shi et al., 2003).

**Glycan structure**

AGPs are characterized by specific arabinosyl and galactosyl linkages. The glycans found in the AGPs include the large type II arabinogalactans and short arabinosides (Pope, 1977; Qi et al., 1991). The type II arabinogalactan is composed of a (1-3)-β-linked galactopyranosyl backbone with (1-6)-β-D linked side chains that include D-galactose and L-arabinose and less frequently L-rhamnose and methylated or non-methylated forms of glucoronopyranose acid (Aspinall, 1970; Defaye and Wong, 1986; Fincher et al., 1983; Gane et al., 1995). One of the well studied AGPs is the *Acacia senegal* gum arabic glycoprotein (GAGP) that shows different types of glycan chains decorating its Hyp residues (Qi et al., 1991).

The carbohydrate-core protein linkage studies suggest that the linkage of the glycans involves *O*-glycosylation (Lamport, 1967). The Hyp-contiguity hypothesis was proposed to explain the *O*-glycosylation pattern of AGPs and this hypothesis is based on the sequence of the core protein (Kieliszewski et al., 1992; Kieliszewski et al., 1995; Kieliszewski and Lamport; 1994). According to the Hyp contiguity hypothesis, arabinogalactan polysaccharides are added only when Hyp residues are non contiguous
and conversely, arabinosides are added only on contiguous Hyp residues (Kieliszewski et al., 1992; Kieliszewski and Lamport, 1994; Kieliszewski et al., 1995). These hypotheses were confirmed further in studies where synthetic genes encoding characteristic HRGP repetitive motifs, Ser-Hyp, Ser-Hyp₂ and Ser-Hyp₄ were expressed in tobacco BY-2 cells (Shpak et al., 1999; Shpak et al., 2001). Analysis of glycosylation pattern confirms the attachment of arabinogalactan on non-contiguous Hyp residues. Similarly, another study by Zhao et al (2002) reported a glycosylation profile for tomato LeAGP-1 that was consistent with the Hyp contiguity hypothesis.

**Glycosylphosphatidylinositol (GPI) lipid anchoring**

One way in which classical AGPs are differentiated from non-classical AGPs is based on the presence or absence of a C-terminal GPI anchor (Fig. 1.2). A number of studies have experimentally shown presence of GPI anchors in AGPs (Oxley and Bacic, 1999; Svetek et al., 1999; Youl et al., 1998). During this post translational modification, the C-terminal hydrophobic domain is cleaved and replaced by a glycosylphosphatidylinositol lipid anchor (GPI) in the ER. GPI anchors in AGPs have been completely biochemically characterized in various studies (Oxley and Bacic, 1999; Sherrier et al., 1999; Svetek et al., 1999; Sun et al, 2004). The GPI lipid anchor allows for attachment of AGPs to the plasma membrane and presumably plays a critical role in biological functions of AGPs (Borner et al., 2003; Oxley and Bacic, 1999; Sun et al., 2004; Svetek et al., 1999; Zhao et al., 2002). Specific types of phospholipases [i.e. phospholipase C (PLC) and
Figure 1.2 Glycosylphosphatidylinositol (GPI) lipid anchor structure of a Lys-rich AGP. (Adapted and modified from Oxley and Bacic, 1999; Svetek et al., 1999; Youl et al., 1998; Sun et al., 2004). The AGP structure (shown on the left side of the figure) shows a GPI anchor attachment site (blue rod; C-terminus) on AGPs. The GPI anchor is embedded in outer leaflet of plasma membrane (PM; light orange shaded box). Also, shown in the figure are the cleavage sites for phosphotidylinositol-specific phospholipase D (PLD) and phospholipase C (PLC). A potential partial β-galactosyl substitution (*) is also shown on the core oligosaccharide. Gal, galactose; GlcN, N-acetylglucosamine; Man, mannose.
phospholipase D (PLD)] likely cleave GPI-anchored AGPs from the plasma membrane to release them into the extracellular matrix (Oxley and Bacic, 1999; Svetek et al., 1999).

**Molecular shape**

Most of the information available on molecular shapes of AGPs was retrieved from rotary shadowing/transmission electron microscopic studies that allowed for *in vitro* visualization of shapes and aggregation. Based on certain models, AGPs were reported to exist either as large globular or medium sized rod-shaped macromolecules. Two models, the “wattle blossom model” and the “twisted hairy rope model” were proposed to explain the macromolecular organization of AGPs. According to the “wattle blossom model”, an overall spheroidal shape of the macromolecules is because of decoration of arabinogalactan polysaccharides on the protein backbone as globular glycans (Fig. 1.3) (Fincher et al., 1983). The AGPs that were reported as spheroidal and are consistent with the “wattle blossom model” include the carrot Hyp-poor AGPs (Baldwin et al., 1993) and tobacco transmitting tissue-specific (TTS) proteins (Cheung et al., 1995). In the “twisted hairy rope” model, the arabinogalactan and arabinosides are attached to the protein backbone as repetitive blocks of wrapped glycans (Fig. 1.3) (Qi et al., 1991). This distribution of glycans generates an elongated macromolecular (rod) shape. The gum arabic AGP was reported as a rod-shaped structure confirming the “twisted hairy rope” model (Qi et al., 1991). Recently, Tan et al (2004) employed nuclear magnetic resonance and sugar analyses techniques to demonstrate for the first time the complete three-
Figure 1.3 Models depicting two possible molecular shapes of AGPs. (Adapted and modified from Showalter, 2001). A. The “wattle blossom” model. The model shows a structure of arabinogalactan polysaccharide (blue balloon) attached to the protein back
bone (red) via a Hyp residue (black) (Fincher et al., 1983). B. The “Twisted hairy rope” model. The model of a gum arabic AGP (GAGP) illustrating arabinogalactan polysaccharides (blue) and short arabinosides. (Qi et al., 1991). C. A structure of Hyp-arabinogalactan polysaccharide of a GFP a fusion protein (Ala-Hyp)$_{51}$ (expressed in tobacco BY-2 cells) based on computer based modeling (Adopted and modified from Tan et al., 2004; Seifert and Roberts, 2007). The peptide region is represented as the red rod and Hyp residue is shown in blue color.
dimensional structure of the arabinogalactan polysaccharide. In this study, a fusion protein (Ala-Hyp)$_{51}$ was expressed in tobacco BY-2 cells and examined for distribution and composition of Hyp-arabinogalactans. This study demonstrated that the twisted hairy rope model was improbable and suggested that arabinogalactan polysaccharides are actually composed of multiple glycosidic branches giving an overall bulky structure to the glycomodules. These models are possible because of the fact that AGPs are known to be heterogeneous in nature and thereby suggesting that either model may hold true for shapes of different types of AGPs found in the plant kingdom

**Probes for AGP functions and interactions**

A number of biochemical and immunohistochemical studies have been performed in the past to probe AGP functions and interactions. The most commonly employed approaches include (1) the use of probes such as Yariv phenylglycosides, (2) antibodies raised against the carbohydrate epitopes/peptide backbone, and (3) forward and reverse genetics analysis.

**Yariv reagent**

β-D-galactosyl and β-D-glucosyl Yariv reagents are used in studies because they selectively bind and precipitate AGPs (Fig. 1.4) (Yariv et al., 1962; Yariv et al., 1967). However, β-D-mannosyl and α-D-galactosyl do not bind AGPs and are often used as
Figure 1.4 Chemical structure of β-D-galactosyl Yariv reagent. (Adapted and modified from Yariv et al., 1962; Yariv et al., 1967; Nothnagel, 1997; Showalter, 2001). The other chemical forms include β-D-glucosyl, β-D-mannosyl and α-D-galactosyl Yariv reagents.
experimental controls (Nothnagel and Lyon, 1986). Previous studies on functional aspects of AGPs were performed in plants and cultured cells using Yariv reagent as an effective probe (Guan and Nothnagel, 2004; Jauh and Lord, 1996; Langan and Nothnagel, 1996; Serpe and Nothnagel, 1994; Willats and Knox, 1996). These studies have shown that Yariv-induced perturbation of AGPs in plants and cell cultures presumably disrupts normal distribution and in vivo function of these glycoproteins, thereby allowing to be used to probe AGP functional roles.

**Antibodies**

Several antibodies generated against either the complex carbohydrate moieties [for example, JIM13 (recognizes β-D-GlcA-(1,3)-α-D-GalA-(1,2)-α-L-Rha), LM2 (recognizes β-linked glucuronic acid), MAC207 (recognizes α-GlcA-(1,3)-α-GalA-(1,2)-α-Rha) (Bradley et al., 1988; Knox et al., 1991; Pennell et al., 1989; Yates and Knox, 1994; Yates et al., 1996)] or the peptide backbones are employed as efficient AGP probes by tracking and/or perturbing AGPs to provide functional insights (Gao et al., 1999). A number of immunolocalization and histochemical studies that have successfully employed these antibodies to identify various AGP carbohydrate epitopes and their roles in plant growth and development have been discussed in the section for AGP functions.
**Genetic approaches**

Recent advanced approaches include forward and reverse genetics that allow probing the specific physiological function of AGPs. One problem associated with this approach has been the functional redundancy in the AGP multi gene family. Based on these three commonly employed approaches, AGPs are implicated in numerous developmental and growth processes at the whole plant, cellular and molecular level which are discussed in the following sections.

**Functions of AGPs at cellular and plant level**

**Cell division**

The earliest evidence for a role of AGPs in the regulation of cell division is based on studies performed in leafy liverworts (Basile, 1990; Basile and Basile, 1993). Antagonists of synthesis of Hyp-containing proteins such as hydroxyproline, 2,2’-dipyridyl, 3,4-dehydroproline were applied to developing liverworts resulting in induction of growth in suppressed primordia (Basile, 1979, Basile, 1980; Basile et al., 1985; Basile, 1990). In another study, Cooper and Varner (1983) demonstrated that application of 3,4-dehydroproline results in inhibition of peptidyl-Pro hydroxylase activity resulting in growth defects. In another study, AGPs were implicated in cell proliferation and cell viability. In this study, it was reported that β-D-glucosyl Yariv reagent treatment of rose suspension cultures (“Paul’s Scarlet”, Rose 57 and Rose 93) results in inhibition of growth, proliferation and viability (Langan and Nothnagel, 1997). Similarly, another
study reported AGPs are involved in regulation of cell proliferation. Treatment of rose suspension cultured cells with β-D-glucosyl Yariv reagent shows dose dependent inhibition of growth (Serpe and Nothnagel, 1994). However, the negative controls, β-D-mannosyl and α-D-galactosyl do not result in growth inhibition. In different studies performed in *Arabidopsis*, tobacco BY-2 cells and rose cultures, treatment with β-D-glucosyl and β-D-galactosyl reagents consistently resulted in growth inhibition and decreased cell viability (Chaves et al., 2002; Guan and Nothnagel, 2004; Langan and Nothnagel, 1997; Serpe and Nothnagel, 1994). Guan and Nothnagel (2004) reported Yariv reagent treatment of *Arabidopsis* cell cultures results in wound-like responses (i.e. increased cytoplasmic vesiculation and callose synthesis) with 50% reduction in cell growth and viability.

**Cell expansion and programmed cell death**

Various studies have indicated roles for AGPs in cell expansion. β-D-glucosyl treatment of lily pollen tubes results in cessation of tip growth, presumably by disruption of AGPs (Jauh and Lord, 1996). Another study in tobacco suspension cultured cells indicates decreased concentration of AGPs and reduced cell expansion in salt adapted cells (Iraki et al., 1989; Zhu et al., 1993). In this study, the concentration of AGPs accumulated in the culture media is reduced in salt adapted cells in comparison to unadapted cells. Similarly, a mutant in *Arabidopsis*, *diminuto*, shows reduced amounts of AGP accompanied by reduced cell elongation in the hypocotyls, petioles, stems and roots (Takahashi et al.,
1995). More convincing evidence for the role of AGPs in cell expansion was provided from a study by Willats and Knox (1996) in Arabidopsis seedlings and carrot suspension cultured cells. This study reported that β-D-glucosyl Yariv treatment of Arabidopsis seedlings results in decreased root length but had no effect on the germination rate. Also, cell elongation in carrot suspension cultured cells was inhibited on treatment with β-D-glucosyl Yariv reagent (Willats and Knox, 1996). This inhibitive growth effect of β-D-glucosyl Yariv reagent was reversible in both Arabidopsis seedlings and carrot suspension cultured cells.

In another study, Ding and Zhu, (1997) demonstrated that β-Yariv reagent treatment of Arabidopsis seedlings lead to epidermal cell bulging and phenocopies a Arabidopsis reb-1 (root epidermal cell bulger) mutant. This Arabidopsis reb-1 mutant demonstrates a root epidermal bulger phenotype and has decreased amounts of AGPs accompanied by defects in root elongation, radial cell expansion in roots and organization of cortical MTs arrays in root epidermal cells (Andème-Onzighi et al., 2002). sos5 (salt overly sensitive5) is an Arabidopsis mutant identified in the presence of excess salt conditions from a pool of ethyl methanesulfonate (EMS) mutants (Shi et al., 2003). This study reported that SOS5 encodes a putative fasciclin-like AGP (FLA-AGP) with a role in cell expansion. The mur1 mutant is an EMS Arabidopsis mutant with altered cell wall monosachharide composition (L-fucose) (van Hengel and Roberts, 2002). This mutant shows a reduced percentage of L-fucose and reduced root elongation in comparison to the WT, indicating a role for fucosylated AGPs in root elongation. (van Hengel and Roberts, 2002). Another
*Arabidopsis* mutant *uge4* demonstrates defects in epidermal cell expansion and root elongation, presumably due to absence of galactosylation of AGPs (Seifert et al., 2002). In a study performed in cell cultures, Yariv reagent treatment also results in the induction of programmed cell death (PCD) in tobacco BY-2 cells and *Arabidopsis* suspension cultured cells (Chaves et al., 2002; Gao and Showalter, 1999b).

As is the case with most of the AGPs reported earlier, *AtAGP19*, a Lys-rich AGP, was shown to play a role in plant growth and development specifically in cell division and expansion (Yang et al., 2007). These physiological roles were reported based on studies conducted with a null T-DNA knockout mutant in *Arabidopsis*. Various phenotypic defects observed in the knockout mutant include: (1) leaves defective in size, shape (i.e. smaller, flatter and rounder) and color (i.e. leaves were pale green color with reduced levels of chlorophyll), (2) decreased number of siliques, lateral roots and seeds, and (3) delayed growth rate. The cells in leaves and hypocotyls also show a defective morphology and overall reduced cell numbers. Overall, this study with the *atagp19* pleiotropic T-DNA knockout mutant indicates a role for this Lys-rich AGP in various developmental processes including leaf and vascular development, lateral root initiation, and hypocotyl and stem growth.
Somatic embryogenesis

Initial reports on the role of AGPs in somatic embryogenesis were based on localization of the JIM4 monoclonal antibody on the surface of the proembryogenic mass from carrot cell cultures (Stacey et al., 1990). Another study demonstrated correlations between localization of epitopes of AGP carbohydrate antibodies and different stages of somatic embryogenesis (Pennell et al., 1992). Convincing evidence for a role of AGPs in somatic embryogenesis was based on studies showing that the addition of exogenous AGPs to the culture media either induces or inhibits somatic embryogenesis depending of the type of AGP added (Egertsdotter and von Arnold, 1995; Kreuger et al., 1995; Kreuger and van Holst, 1993; Kreuger and van Holst, 1995). In a study in carrot cells, it was reported that AGPs treated with endochitinases results in decreased somatic embryogenesis and repurification of AGPs initiates somatic embryogenesis (van Hengel et al., 2001). This study suggests a significant role for AGP glycan structure in regulation of somatic embryogenesis function. A study in carrot immature seeds reported developmental co-regulation of endochitinases and AGPs suggesting their role in somatic embryogenesis (van Hengel et al., 2002).

Pattern formation and vascular development

Based on the cell type expression pattern of AGP epitopes, various immunlocalization studies have indicated a role for AGPs as cell position and identity markers. Initial work supporting this idea was based on studies in leafy liverworts that indicate a role for AGPs
in induction or suppresion of growth in leaf primordia (Basile, 1990; Basile and Basile, 1993). In a study performed by Knox et al (1989), the labeling pattern of JIM4 and MAC207 monoclonal antibodies in carrot roots (specifically, root apical meristem labeling) indicates a role for AGPs as markers of cellular position and not cell type. Additionally, in another study, AGPs were shown to play a role in vascular development (Knox et al., 1991). In this study in the carrot root apex, the JIM13 monoclonal antibody labeling is restricted to root epidermal cells and provascular tissue indicating a role in vascular development (Knox et al., 1991). Similarly, another study in Arabidopsis roots implicates a role for AGPs in xylem development based on JIM13 immunolocalization to the metaxylem initial cell, protoxylem, pericycle and endodermis (Dolan et al., 1995). This labeling pattern is lost during the course of secondary thickening because of lignification of xylem vessels (Dolan and Roberts, 1995). In another research development in the Zinnia elegans cell system, JIM13 is localized to the secondary thickenings of walls of mature tracheary elements (Stacey et al., 1995). Regulated expression studies in maize coleoptiles indicate a relationship between AGP expression and tracheid differentiation (Schindler et al., 1995). Based on observations made on the localization of JIM13 and JIM14 epitopes only on sclerenchyma and tracheid cells, it was reported that AGPs marks specific cells for PCD.

Most of these studies indicate the association of AGPs with different stages of xylem differentiation thereby suggesting a role in vascular patterning. Conclusive evidence for the role of AGP in xylem development was based on studies performed in the Zinnia
*elegans* cell culture system (Motose et al., 2001; Motose et al., 2004). Xylogen is responsible for differentiation of mesophyll cells present at low densities into tracheary elements (TE). Xylogen was identified as a chimeric AGP with roles in cell-cell communication during vascular development (Motose et al., 2001; Motose et al., 2004). Further analysis suggest expression of xylogen mRNA in cambium and immature xylem. A study on double mutants of xylogen in *Arabidopsis* indicates defects in vascular development with defects in the leaf vein connections and tracheary elements (Motose et al., 2004).

RNA-interference (RNAi) studies of *AtAGP18*, a classical Lys-rich AGP in *Arabidopsis*, suggest a role in the initiation of female gametogenesis (Acosta-Garcia and Vielle-Calzada, 2004). In this study, the RNAi silenced T2 lines show a decreased number of seeds and this defect is female-specific. Also, a reduction in *AtAGP18* transcript was observed in the developing gynoecia of RNAi silenced lines accompanied with defects in megaspore development leading to ovule abortion. Further, this study does not elucidate the exact physiological function for *AtAGP18* in female gametogenesis, but suggests a role for the Lys-rich AGP in orchestrating communication between gametophytic and sporophytic cells during early ovule development. In another study, a transposon-insertion mutant *atagp30* was examined and based on ABA-sensitive germination assays and *in vitro* callus root regeneration assays indicated that *AtAGP30* controls the timing of seed germination and root regeneration (van Hengel and Roberts, 2003).
**Reproductive growth and development**

Various investigations on the expression and abundance of AGPs in the reproductive tissues indicate a role for these glycoproteins in reproductive growth and development. One early report suggested a role for AGPs in adhesion of pollen to stigma (Knox et al., 1976). Later, a few reports suggested the presence of MAC207 and JIM8 reactive AGPs in vegetative and reproductive tissues respectively. These studies indicated regulation of AGP epitope expression during flower development. Jauh and Lord (1996) reported localization of the JIM13 antibody exclusively to the epidermal cells found in the transmitting tract of the style. Based on these studies, it was suggested pollen tube adhesion in the transmitting tract is mediated by AGPs and/or pectin. Further studies on stylar transmitting tissue-specific (TTS) proteins allowed purification of a tobacco AGP with a molecular weight of 60kDa-100kDa (Cheung et al., 1995; Wu et al., 1995). TTS protein functions in growth and attraction of pollen tubes by providing an adhesion surface. In this study, deglycosylation of TTS protein by pollen tubes and a gradient of increasing glycosylation were observed within the style compartment of the flower. This deglycosylation of TTS protein presumably serves a nutritional role for the pollen tubes. β-Yariv reagent treatment of the pollen tubes in lily plants results in the cessation of pollen tube growth accompanied by defects in the shape of the pollen tip (bulbous tip) (Jauh and Lord, 1996; Roy et al., 1998).
Specific aims of the research

Currently, most of exciting research on AGPs focuses on examining the functional roles and molecular interactions of this multigene AGP family. Based on these interests, the specific objectives in this dissertation project were twofold:

(a) To examine the molecular interactions of AGPs (specifically LeAGP-1) with cortical MTs and F-actin in tobacco BY-2 cells and,

(b) To identify the functions of classical AGPs, AtAGP3, AtAGP4, AtAGP7 and AtAGP9 in T-DNA insertional mutants of Arabidopsis thaliana (Columbia-0) by employing a reverse genetics approach.

With respect to objective (a), despite the immense progress in understanding plant cell architecture, it remains unclear which molecules/structures/components connect the cell exterior to the interior (Wyatt and Carpita, 1993; Kohorn, 2000; Darley et al., 2001; Martin et al., 2001; Baluska et al., 2003; Gouget et al., 2006). Classical AGPs are one of the potential candidates to facilitate this connection. Tomato AGP, LeAGP-1, a Lys-rich AGP is one of the very well characterized AGPs. Previous studies have demonstrated the expression, localization and biochemical composition of LeAGP-1 (Gao et al., 1999a; Sun et al., 2004; Zhao et al., 2002). In a previous study, GFP-LeAGP-1 was stably expressed in tobacco BY-2 cells and was localized at cell surface and plasma membrane (Zhao et al., 2002). Being a classical AGP, LeAGP-1 includes a C-terminal GPI anchor that presumably allows it to tether onto the outer leaflet of plasma membrane (Zhao et al.,
This cell line can be efficiently used as a system to study the interactions of LeAGP-1 with microtubules and F-actin. The specific aim of this project is to understand the molecular interactions of LeAGP-1 with the microtubules and F-actin. This interest is based on a study performed on an Arabidopsis mutant (reb-1) that suggested a potential connection between AGPs and microtubules (Andème-Onzighi et al., 2002).

Tobacco BY-2 cells provide an excellent system to perform cell biology studies and molecular analysis of cellular level functions. Previous studies have shown that tobacco BY-2 cells are more advantageous than Arabidopsis cell cultures because of their bigger size and well defined intracellular organization. Also, BY-2 cells are more homogeneous and synchronized in the cell cycles in comparison to Arabidopsis cells (Nagata et al., 1992; Samuels et al., 1998; Nagata and Kumagai, 1999). BY-2 cells can be easily manipulated and thereby can be used for microscopic analysis and live imaging of dynamic intra/extracellular architecture (Kost et al., 1998; Nebenführ et al., 1999; Granger and Cyr, 2000). Also, they can be efficiently transformed to produce stable transgenic lines (An, 1985; Klein et al., 1988; Rempel and Nelson, 1995). BY-2 cells because of their well defined cellular architecture serve as fine models for studies on cytoskeletal proteins. Visualization of cortical microtubules and F-actin filaments is well elucidated in the BY-2 cells (Kost et al., 1998; Nebenführ et al., 1999; Granger and Cyr, 2000).
With respect to objective (b), a bioinformatics study by Schultz et al (2002) identified 13 classical AGPs and other classes of AGPs in *Arabidopsis*. For this study, from this list of classical AGPs, *AtAGP3, AtAGP4 AtAGP7* and *AtAGP9* genes were selected for investigating their functional roles in *Arabidopsis* root growth and development. The initial rationale behind selection of these AGPs was their high EST number in the roots (Schultz et al., 2002). Further, the GENEVESTIGATOR® Arabidopsis Microarray database and Analysis Toolbox (Zimmerman et al., 2004) were used to identify root specific and high expressing classical root AGPs. A reverse genetics approach was employed to probe the functions of AGPs because T-DNA insertional mutants for these classical AGPs could be analyzed for phenotypic aberrations and respective functional roles of these AGPs in *Arabidopsis* growth and development. 

In comparison to forward genetics, reverse genetics approaches have made the identification of gene functions a high-throughput process. Targeted mutagenesis and insertional mutagenesis are the most common methods of creating gene-specific mutants (Bouchez and Höfte, 1998; Bouche and Bouchez, 2001; Krysan et al., 1999). Insertion of a foreign DNA sequence into the host genome results in insertional mutagenesis. In *Arabidopsis*, the two most common methods of insertional mutagenesis are transposon-mediated and T-DNA mutagenesis (Krysan et al., 1999; Parinov et al., 1999; Thorneycroft et al., 2001; Valentine, 2003; van Hengel and Roberts, 2003). Generally, T-DNA insertional mutants are preferred over transposon mutants because T-DNA
insertions are more stable through successive generations (Bouche and Bouchez, 2001; Martienssen, 1998; Valentine, 2003; Wisman et al., 1998).

The Salk Institute Genomics Analysis Laboratory (SIGnAL), LaJolla, CA and the Arabidopsis Biological Resource Center (ABRC), Columbus, OH, were responsible for generating and maintaining T-DNA SALK lines for AtAGP3, AtAGP4 AtAGP7 and AtAGP9 genes (Alonso et al., 2003). These SALK lines can be screened for homozygous (HM) mutants by performing a polymerase chain reaction (PCR) based screening (McKinney et al., 1995; Krysan et al., 1996).

*Arabidopsis thaliana* is the first plant to have its genome completely sequenced. This common garden weed serves as an excellent model system for plant researchers. *Arabidopsis* provides numerous advantages over other plants because of its small genome size, short life cycle and overall small size. *Arabidopsis thaliana* (columbia0) growth and developmental stages are well established, and this makes it easy for analysis of mutants with phenotypic aberrations (Boyes et al., 2001). At different stages of growth and development for plants grown on either growth media plates or soil, SALK HM lines can be compared to WT plants to analyze their functional roles with respect to phenotypic aberrations.
CHAPTER 2: MOLECULAR INTERACTIONS OF ARABINO GALACTAN-PROTEINS (AGPs) IN BRIGHT YELLOW-2 TOBACCO CULTURED CELLS
Molecular interactions of arabinogalactan-proteins (AGPs) with cortical microtubules and F-actin in bright yellow-2 (BY-2) tobacco cultured cells

This work has been published in the following two manuscripts.


Abstract

Arabinogalactan-proteins (AGPs), a superfamily of plant hydroxyproline-rich glycoproteins, are present at cell surfaces. Although precise functions of AGPs remain elusive, these glycoproteins are widely implicated in plant growth and development. A well characterized classical tomato AGP (LeAGP-1) containing a glycosylphosphatidylinositol (GPI) plasma membrane anchor sequence was used here to elucidate functional roles of AGPs. Transgenic tobacco BY-2 (Nicotiana tabaccum) 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 WT or expressing GFP-MBD (microtubule binding domain), were treated with β-Yariv reagent and effects on microtubules (MTs) and F-actin were observed. Second, BY-2 cells expressing GFP-LeAGP-1 were treated with amiprophosmethyl (APM) 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, APM 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 GPI-anchored AGPs function to link the plasma membrane to the cytoskeleton.

Introduction

Arabinogalactan-proteins (AGPs) are a class of cell surface plant proteoglycans that may mediate signal transduction at the cell wall-plasma membrane interface (Kjellbom, 1997; Gao and Showalter, 1999a; Showalter, 2001; Kohorn, 2001). AGPs belong to a superfamily of hydroxyproline-rich glycoproteins (HRGPs) 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 in 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 (Carpita and Gibeaut, 1993; Knox, 1992; Roberts, 1994; Wyatt and Carpita; 1993, 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 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 et al., 1967) which selectively bind and perturb AGPs so as to probe their functions (Serpe and Nothnagel, 1994; Willats and
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 (Gaspar et al., 2004; van Hengel and Roberts, 2003; Yang et al., 2007). 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 (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 reb-1* mutant has suggested the connection between AGPs and microtubules (MTs) (Andème-Onzighi et al., 2002). These mutants have a root epidermal bulge phenotype and show decreased amount of AGPs and disorganized cortical MTs arrays (Andème-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 (Piersen 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 AGP, LeAGP-1 is a well characterized lysine-rich AGP and is at our disposal for elucidating molecular interactions of AGPs (Gao et al., 1999a; Gao et al., 2000; Sun et al., 2004; Zhao et al., 2002). A transgenic tobacco (*Nicotiana tabacum*) BY-2 cell line expressing a GFP-LeAGP-1 fusion protein was used to demonstrate that LeAGP-1 is a GPI anchored plasma membrane AGP with potential roles in cell signaling pathways and matrix remodeling (Zhao et al., 2002). Here, we employ this cell line as well as a cell line expressing GFP-MBD [microtubule-binding domain of the microtubule-associated protein 4 (MAP4)] (Marc et al., 1998, Granger and Cyr, 2000) to study the selective perturbation of the cytoskeleton (microtubules and actin) and AGPs in order to demonstrate a cell surface network involving interactions among AGPs, microtubules and F-actin.
Materials and methods

Cell cultures and growth conditions

Two transgenic tobacco BY-2 cell lines (*Nicotiana tobaccum*, Bright Yellow-2) [a cell line expressing GFP-LeAGP-1 (Zhao et al., 2002) and another cell line expressing GFP-MBD] were used to conduct the studies. The BY-2 suspension cell cultures expressing GFP-MBD (Marc et al., 1998, Granger and Cyr, 2000; Supplied by Dr. Richard Cyr, Pennsylvania State University) were maintained in liquid MS media (Murashige and Skoog, 1962) [4.3 g/L Murashige and Skoog salts (Sigma, St. Louis, MO, USA), 30 g/L sucrose, 1 mg/mL thiamine HCl, 100 mg/L myo-inositol, 0.44 mg/L 2,4-dichlorophenoxyacetic acid, pH 5.8] and the BY-2 cell lines expressing GFP-LeAGP-1 were maintained in liquid SH media (Schenk and Hildebrandt, 1972) (3.2 g/L Schenk and Hildebrandt basal salt, Sigma, St. Louis, MO, USA), 1 g/L Schenk and Hildebrandt vitamin powder, 1 mg/mL Kinetin, 1 mg/mL p-chlorophenoxy acetic acid, 1 mg/mL 2,4-dichlorophenoxyacetic acid, 34 g sucrose, 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 10-15 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 (three-day-old i.e. three days after sub culturing) expressing GFP-MBD were washed with fresh NT-1 media (3X) 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 (3d) expressing GFP-LeAGP-1 were washed with fresh SH media (3X) 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, Eugene, OR, USA).

**Amiprophosmethyl (APM) treatment:** Three-day-old tobacco BY-2 cells expressing GFP-MBD and GFP-LeAGP-1 were washed with fresh media (3X) before the treatment. Washed BY-2 cells were treated with 30 μM APM (10mM stock in DMSO, Austratec Pty limited, Victoria, Australia) 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 (3X) and were treated with 20, 25 and 50 μM of cytochalasin-D (10 mM stock in DMSO, Sigma, St Louis, MO, USA) 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 confocal microscopy 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 MS media (2 X 1 min) and attached to poly-L-Lysine (Sigma, St Louis, MO, USA) coated glass slides (1 mg/mL). After 10 min, the cells were fixed with 3.8 % formaldehyde/PBS for 30 min at room temperature and washed with phosphate buffer saline (PBS) (pH 7.4) (3 X 5 min). Cells were permeabilized with 0.1% Triton-X-100/PBS for 10 min and washed again with PBS (3 X 5 min). Cells were then incubated with 1% BSA/PBS for 25 min and labeled with rhodamine-phalloidin/PBS [5 μL of methanolic stock (6.6 μM stock solution) in 200 μL of PBS] for 20 min. After washing (3 X 5 min), the cells were mounted on slides with 50 % glycerol/PBS and observed with a confocal laser scanning microscope.

**Confocal laser scanning microscopy**

Cultured cells were placed on a drop of water on glass slides that were layered with cover slips. The glass slides were positioned onto the inverted platform of a confocal laser-scanning microscope (CLSM 510; Zeiss Corp. Germany) and the cells were imaged using the 488 nm line of an argon laser. Images were recorded with the X10 and X40 objectives (NA 0.75; Zeiss Corp. Germany) and a 488/543-nm dual dichroic excitation mirror with a 510 to 540 nm emission filter. All images were obtained either with a FITC or a Texas
red filter set. All images were processed with Zeiss imaging software and Adobe Photoshop.

**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 GFP-LeAGP-1 (Fig. 2.1A). 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. In the presence of 20 μM cytochalasin-D, an F-actin inhibitor, BY-2 cells showed a non-uniform distribution of GFP-LeAGP-1 at the cell surface and a pronounced accumulation of fluorescence at cell-cell adhesion zones (Fig. 2.1B and 2.1C). 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 non-uniform distribution (Fig. 2.1D). Prolonged treatment of 6 d old cells with β-Yariv reagent (24 h) decreased GFP fluorescence on the cell surface (Fig. 2.1E), but in older cells (13 d) this treatment resulted in GFP-LeAGP-1 localization at end wall regions of adjoining cells (Fig. 2.1F). This pattern can be attributed to inaccessibility of β-Yariv reagent to the cell surfaces of adjoining cells.
Figure 2.1 Confocal laser scanning microscope (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 (6d) 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 non-uniform distribution of GFP-LeAGP-1 fluorescence at the cell surface and enhanced accumulation at the cell-cell adhesion zone (arrow). 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).
Previous studies employing immunolocalization and western analysis demonstrated that LeAGP-1 was localized to the plasma membrane and Hechtian strands (Zhao et al., 2002). These previous results were confirmed using plasmolysis with 4% NaCl to reveal plasma membrane and Hechtian strand localization of GFP-LeAGP-1 (Fig. 2.2A and 2.2B). 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. 2.2, 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 strand (Fig. 2.2D), 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. 2.2E). In contrast, when the transgenic BY-2 cells were treated with amiprophosmethyl (APM) (30 μM for 45 min) followed by plasmolysis, this treatment resulted in a relocalization of GFP-LeAGP-1 from Hechtian strands to a punctate distribution pattern in the periplasmic space (Fig. 2.3). To examine if APM treatment affects the Hechtian strands formation, bright field microscopy was performed to show no effects of the treatment on strand formation. (Fig. 2.4).
Figure 2.2 Treatment of tobacco BY-2 cells (6d) expressing GFP-LeAGP-1 with cytochalasin-D and β-Yariv reagent followed by plasmolysis. A, GFP-LeAGP-1 was localized on the surface of non-plasmolysed BY-2 cells. B, Plasmolysed (4% NaCl for 15 min) BY-2 cells showed GFP-LeAGP-1 was distributed on Hechtian strands (arrow) and plasma membranes. In the following panels, 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 (6d) 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 (13d) 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).
Figure 2.3 Amiprophosmethyl (APM) treatment of tobacco BY-2 cells (6d) expressing GFP-LeAGP-1. A, BY-2 cells plasmolysed with 4% NaCl for 10-15 min showed strands (arrow) connecting the cell wall and the plasma membrane. B and C, Relocalization of GFP-LeAGP-1 from Hechtian strands to a punctate distribution pattern in the periplasmic space was seen in BY-2 cells after 30 μM APM treatment for 1 h followed by plasmolysis with 4% NaCl during the last 15 min of treatment (Arrows mark the punctate distribution pattern in the periplasmic space). All images were single optical sections. CW, cell wall; PM, plasma membrane; HS, Hechtian strands. Bars = 20 μm (A-C).
Figure 2.4 Bright field microscopic image analyzing the formation of Hechtian strands upon APM treatment. Wild type BY-2 cells were treated with 30 μM APM for 1 h followed by plasmolysis with 4% NaCl during the last 15 min of treatment (Arrows indicate the distribution of Hechtian strands in the periplasmic space). CW, cell wall; PM, plasma membrane. Bar = 10 μm
Alterations in cortical MT arrays and cell morphology induced by β-Yariv reagent in BY-2 cells expressing GFP-MBD

In order to visualize cortical MTs, BY-2 cells expressing GFP-MBD were used either directly (unwashed) or washed with fresh NT-1 media prior to treatments with Yariv reagent and APM. Compared to unwashed control cells (Fig. 2.5A), unwashed cells treated with 100 µM Yariv for 24 h exhibited characteristic terminal cell bulger phenotypes (Fig. 2.5, B-D). Optical sectioning of these bulged cells revealed the formation of depolymerization products or “puncta” (Fig. 2.5E). Treatment of unwashed control cells with 100 µM β-Yariv for 5 h resulted in disorganization and depolymerization of cortical MTs (Fig. 2.5F). 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. 2.6, 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. 2.6D). The washing treatment effectively lowers the concentration of Yariv reagent required to elicit cortical MTs 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
Figure 2.5 Confocal images showing depolymerized cortical microtubule arrays and the “terminal bulger” phenotype in unwashed tobacco BY-2 cells (4d) expressing GFP-MBD (microtubule binding domain) 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, C and D). CW, cell
wall; MT, microtubule; N, nucleus; PM, plasma membrane. Bars = 20 μm (A-B, D and F), 10 μm (C) and 5 μm (E).
Figure 2.6 β-Yariv treatment of washed BY-2 cells (4d) 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 microtubules in most (70%) of the cells. E, Treatment with 80 μM β-Yariv reagent for 5 h resulted in disorganization of cortical microtubule 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 amiprophosphomethyl 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; MT, microtubule; N, nucleus. Bars = 5 μm (A, E and F), 10 μm (B-D and H) and 20 μm (G).
disorganization of cortical MTs resulting in terminal cell bulging (Fig. 2.6F) as well as enhanced fluorescent labeling of cortical MTs underlying the end walls of adjacent cells (Fig. 2.6E). A positive control treatment with APM demonstrated extreme depolymerization of cortical MTs with characteristic depolymerization products appearing within 45 min (Fig. 2.6G).

Also, β-Yariv treatment of GFP-MBD expressing BY-2 cells resulted in disruption of cytoplasmic strands (which contain MTs, connect the perinuclear cytoplasm with sites of the cortical cytoplasm) and deposition of 3-4 GFP clumps at the cell surface (Fig. 2.7; unpublished data). In the cells undergoing cell division, it has been reported that the cytoplasmic strands are made up of the actin filaments and MTs (Panteris et al., 2004). These strands are found in the cytoplasm connecting the regions outside the nucleus (perinuclear cytoplasm) and cortical cytoplasm (Panteris et al., 2004).

**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. 2.8A). 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. 2.8B). A positive control
Figure 2.7 β-Yariv treatment of GFP-MBD expressing BY-2 cells results in disruption of cytoplasmic strands and GFP clumping at the cell surface. Cells (4d) washed with fresh NT-1 media were treated with 80 μM β-Yariv reagent for 1 h and imaged with the laser confocal laser scanning microscope. In comparison to the control cells (D) that show cytoplasmic strands (arrow), cells treated with β-Yariv reagent (A, B, C and E) show disrupted cytoplasmic strands and GFP clumping (arrows in B and E) at cell surface. Fluorescent image (A), the phase contrast image (B) and superimposed image of (A) and (B) is shown in image (C). Images are combined projections of multiple optical sections from a confocal Z-series. CW, cell wall; N, nucleus. Bars = 5 μm (A, B and C), 10 μm (D and E).
Figure 2.8 F-actin (labeled with rhodamine-phalloidin) distribution in wild type BY-2 cells (3d) 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-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).
treatment with cytochalasin-D, an F-actin inhibitor, depolymerized F-actin arrays (Fig. 2.8, C-E).
Discussion

Although some AGP mutants in *Arabidopsis* are reported with specific phenotypes, a clear understanding of AGP function, mode of action and molecular interactions remains elusive (Acosta-Garcia and Vielle-Calzada, 2004; Gaspar et al., 2004; Motose et al., 2004; Park et al., 2003; Shi et al., 2003; van Hengel and Roberts, 2003). Consistent with the approach of identifying molecular interactions of AGPs, β-Yariv reagent and cytoskeleton inhibitors were employed to conduct *in vivo* studies at the cellular level to examine molecular interactions between AGPs and the cytoskeleton (MTs and F-actin). Specifically, this study demonstrates that β-Yariv treatment triggers responses in cortical MT and F-actin networks, conversely, cytoskeleton inhibitors relocalize LeAGP-1 in Hechtian 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 et al., 1999b; Guan and Nothnagel, 2004) and plants (Ding and Zhu, 1997; Roy et al., 1998). In non-plasmolyzed 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. 2.1F) and cell-cell adhesion zones (Fig. 2.1B and 2.1C) 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, collagen and laminin via an Arg-Gly-Asp (RGD) 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 RGD 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 (WAKs), endo-1-4-β-D-glucanases and cellulose synthases (Kjellbom et al., 1997; Kohorn, 2000; Kohorn, 2001). A lysine-rich tomato AGP, LeAGP-1, is plasma membrane bound via a C-terminal GPI anchor that tethers the glycoprotein to the plasma membrane (Sun et al., 2004; Zhao et al., 2002). 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 (Fig. 2.2B and 2.3A). More importantly, we show that treatment of BY-2 cells with β-Yariv, a reagent that binds AGPs, relocalizes GFP-LeAGP-1 within the periplasmic space from the Hechtian strands (Fig. 2.2C and 2.2F).

There are conflicting reports on the presence of microfilaments and microtubules within Hechtian strands. Certain studies indicate existence of microfilaments and microtubules within the Hechtian strand (Lang-Pauluzzi, 2000, Lang-Pauluzzi and Gunning, 2000) whereas another study demonstrates their absence (Domozych et al., 2003). Consistent with my hypothesis that cytoskeleton inhibitors would disrupt the localization of LeAGP-1 on Hechtian strands, treatment of BY-2 cells with APM and cytochalasin-D relocalizes GFP-LeAGP-1 in the periplasmic space. APM is a specific depolymerization agent for cortical MTs (Kundelchuk et al., 2002; Granger and Cyr, 2000), 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 relocalizes GFP-LeAGP-1 on Hechtian strands and results in a punctate distribution (Fig. 2.3B and 2.3C). Interestingly, this APM treatment does not disrupt Hechtian strand formation (Fig. 2.4), 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. 2.2D) and in the cytoplasm (Fig. 2.2E). 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 can not 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. In these studies, β-Yariv reagent treatment resulted in inhibition of growth and elongation accompanied with radial expansion of cells. 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 (Andeme-Onzighi et al., 2002). The REB-1 gene encodes a UDP-D-glucose-4-epimerase
(UGE4) which functions in D-galactose synthesis (Seifert et al., 2002). A recent immunocytochemical and biochemical study on reb-1 suggested a role for UGE4 in galactosylation of AGPs and xyloglucans (Nguema-ona et al., 2006).

In these studies reported here, 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. 2.5C-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 microtubules (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 (Williamson et al., 2001; Lane 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 non-terminal 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 microtubules, cell bulging and defects in directional growth (Fig. 2.5) indicate a connection between AGPs, MTs, terminal cell bulging and anisotropic growth.

Yariv reagent depolymerizes and disorganizes cortical MTs in washed or unwashed GFP-MBD expressing cells (Figs. 2.5 and 2.6). In comparison to the unwashed cells (Fig. 2.5), the washed cells (Fig. 2.6) demonstrate no reduction in the fluorescence of GFP-MBD and any differences observed in fluorescence intensity are due either 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. 2.8). Previously, Gao and Showalter, (1999b) reported that β-Yariv treatment of Arabidopsis cells for 72 h results in cytoplasmic shrinkage and nuclear fragmentation, structural changes that are characteristic of programmed cell death (PCD). In another study, treatment of unwashed BY-2 cells with a higher concentration (~80 μM) of Yariv reagent results in PCD within 72 h (Chaves et al., 2002). This study shows that the architectural changes in the cell shape and cytoskeleton take place within 5 h (for washed cells; Fig. 2.6) and 24 h (for unwashed cells; Fig. 2.5) 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-cytoplasmic 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., microtubules and F-actin) mediated by either a direct interaction with transmembrane protein(s) or by an indirect interaction involving lipid rafts (Fig. 2.9). Transmembrane proteins such as WAKs, cellulose synthases, endo-1-4-β-D-glucanases, proline-rich extensin-like receptor kinases (PERKs), formins phospholipase-D, and lectin receptor kinases (LecRKs) (Kohorn, 2000; Kohorn, 2001; Baluska et al., 2003; Nakhamchik et al., 2004; Gouget et al., 2006) may interact directly with AGPs to mediate such cell wall-plasma membrane-cytoskeleton connections. Most of these proteins have either an extracellular or a transmembrane domain that may interact with AGPs to mediate signaling at the plant cell surface. Also, we cannot rule out the possibility of Yariv-induced clustering of cell surface AGPs resulting in cytoskeletal changes. AGPs are abundant plant cell surface proteins and any changes in the distribution of AGPs (crowding/steric effects) may result in changes in distribution of other membrane proteins/components thereby producing a combined mass action that affects the cytoskeleton without any direct interactions.

Another possible mode of interaction involves lipid rafts. Lipid rafts were first discovered in animal cells and are specialized lipid microdomains enriched in cholesterol, glycosphingolipids, GPI anchored proteins and various molecules involved in cell signaling (Zajchowski and Robbins, 2002; van Meer, 2002). Lipid rafts are categorized as specialized centers for signaling cascades because of the identification of a number of
Figure 2.9 A hypothetical cell surface network model involving interactions between LeAGP-1 and the cytoskeleton (i.e., microtubules and F-actin). Glycosylphosphatidylinositol (GPI) anchored LeAGP-1 is localized to lipid rafts, which contain lipids such as glycosphingolipids and sterols (such as stigmasterol, campesterol, and beta-sitosterol), and interacts with microtubules (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: as shown in the figure, (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 a nonglycosylated Lys-rich peptide region.
signaling molecules within these lipid microdomains (Zajchowski and Robbins, 2002). In animals, lipid rafts are implicated in protein sorting, signal transduction, pathogen entry and endocytosis (Ikonen, 2001; Simons and Toomre, 2000; Pike, 2004). A recent study in *Arabidopsis* showed that detergent resistant membranes (DRMs)/lipid rafts are enriched in GPI-anchored proteins, including GPI-anchored AGPs like AtAGP4 (Borner et al., 2005). LeAGP-1 is also a GPI-anchored protein and likely to be associated with lipid rafts. 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 2.9: (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 (shown as B in Fig. 2.9) out of the lipid microdomain leading to activation of intracellular/cytoplasmic kinase. While this model remains to be tested, the studies to date clearly indicate that GPI anchored-AGPs play a role in the plasma membrane-cytoskeleton connections.

A follow-up study to this research focused on examining specific interactions between AGPs and other molecular components using chemical cross-linking approach (Appendix A).
CHAPTER 3: FUNCTIONAL IDENTIFICATION OF FOUR CLASSICAL AGPs (AtAGP3, AtAGP4, AtAGP7, AtAGP9) IN ARABIDOPSIS T-DNA MUTANTS
Abstract

Arabinogalactan proteins (AGPs) are a class of highly glycosylated cell wall hydroxyproline-rich glycoproteins. Various biochemical and immunochemical studies indicate a role for AGPs in different growth and developmental processes, but to date few studies have provided a specific biological mechanism of action for AGPs. In order to elucidate functional roles of AGPs, a reverse genetics approach was adopted to analyze the T-DNA insertional mutants of $AtAGP3$, $AtAGP4$ $AtAGP7$ and $AtAGP9$ in Arabidopsis. Homozygous (HM) T-DNA mutants for $AtAGP4$ $AtAGP7$ and $AtAGP9$ were identified using PCR-based screening. However, isolation of a HM $atagp3$ mutant was unsuccessful. Bioinformatics studies including phylogenetic studies and microarray-based expression analysis indicate the evolutionary clustering and organ-specific expression profiles for these classical AGPs. Northern blotting and RT-PCR studies for $AtAGP4$ and $AtAGP9$ show a high expression of these transcripts in stems and young roots. An $atagp9$ mutant shows a decrease in number of lateral roots and an early flowering phenotype. Also, $atagp4$ and $atagp9(3)$ mutants show suppression of an abscisic acid (ABA)-induced delay in seed germination and increased resistance to ionic (NaCl) and non-ionic (mannitol) osmotic stress during seed germination. However, an $atagp7$ mutant exhibits an ABA suppression of germination only at higher ABA concentration. Quantitative real-time PCR analysis indicates absence of RNA transcript in $atagp7$, a decreased relative transcript level for $atagp4$ and $atagp9(3)$ and increased relative transcript level for $atagp9(5)$. These studies provide information on the roles of these AGPs in seed germination, stress signaling and root growth and development.
Introduction

Mutagenesis approaches play a major role for functional genomics in *Arabidopsis* because this approach allows for the identification of gene function to be a high-throughput process. Various mutant studies have been performed in *Arabidopsis* that suggested direct or indirect roles for AGPs in certain physiological functions. RNA-interference (RNAi) studies of *AtAGP18*, a classical Lys-rich AGP, in *Arabidopsis*, indicate a role for this AGP in initiation of female gametogenesis (Acosta-Garcia and Vielle-Calzada, 2004). *rat1* displays resistance to *Agrobacterium* transformation with reduced levels of *AtAGP17* in the roots (Gaspar et al., 2004). Studies in a transposon-insertion mutant *atatp30*, suggests this AGP has a role in root regeneration and seed germination (van Hengel and Roberts, 2003). A *sos5* (*salt overly sensitive5*) mutant study indicates that a putative fasciclin-like AGP (FLA) plays a role in cell expansion (Shi et al., 2003).

A study on double mutants of xylogen in *Arabidopsis* indicates a role for xylogens in inductive cell-cell communication during vascular development (Motose et al., 2004). These double mutants exhibit problems in vascular development with defects in the leaf vein connections and tracheary elements (Motose et al., 2004). *reb-1* (*root epidermal bulger-1*) mutant displays defects in root elongation, organization of cortical MTs arrays, and root epidermal cell bulging accompanied with decreased amount of AGPs (Andème-Onzighi et al., 2002; Ding and Zhu, 1997). Another *Arabidopsis* mutant, diminuto (dim) has reported defects in steroid biosynthesis accompanied by a deceased percentage of
AGPs (Klahre et al., 1998). *mur1* demonstrates reduced root elongation and a reduced amount of L-fucose in root AGPs indicating a role for highly fucosylated AGPs in root elongation. (van Hengel and Roberts, 2002). A recent study on a T-DNA knockout mutant for a Lys-rich AGP, *AtAGP19*, suggests that this AGP plays a role in plant growth and development specifically in cell division and expansion (Yang et al., 2007).

Based on a bioinformatics study in *Arabidopsis*, different classes of AGPs were identified and included 13 classical AGPs, 10 AG-peptides, 3 lysine-rich AGPs, and 21 fasciclin-like AGPs (Schultz et al., 2002). Functional roles of four classical AGPs, *AtAGP3*, *AtAGP4*, *AtAGP7* and *AtAGP9*, have never been examined in *Arabidopsis*. A previous study by Schultz et al. (2000) reported the expression patterns of *AtAGP3*, *AtAGP4* and *AtAGP9* in roots, leaves, stems and flowers. *AtAGP3* was expressed only in roots, *AtAGP4* was expressed predominantly in roots, leaves and flowers whereas *AtAGP9* was expressed highly in flowers and minimally expressed in roots, stems and leaves (Schultz et al., 2000). In the current study, T-DNA insertional mutants of *AtAGP3*, *AtAGP4*, *AtAGP7* and *AtAGP9* were analyzed for phenotypic aberrations and potential functional roles in *Arabidopsis* growth and development. A PCR-based screening method was employed to isolate HM and heterozygous (HZ) SALK lines. Bioinformatic approaches were employed to analyze evolutionary groupings and tissue-specific expression patterns of *AtAGP3*, *AtAGP4*, *AtAGP7* and *AtAGP9*. Phenotypic analysis of HM lines suggested a role for *AtAGP9* in root development and a role for *AtAGP4* and *AtAGP9* in stress
responses. Quantitative real-time PCR analysis demonstrated the relative transcript levels in different SALK lines.

Material and methods

Identification of T-DNA insertion lines

Based on online resources available at Salk Institute Genomics Analysis Laboratory (SIGnal) and Arabidopsis Biological Resource Center (ABRC), T-DNA insertion lines were identified for AtAGP3 (At4g40090), AtAGP4 (At3g20865), AtAGP7 (At5g65390) and AtAGP9 (At2g14890) using the SIGnal “T-DNA Express” Arabidopsis Mapping Tool. Based on this information retrieved from the web portal “http://signal.salk.edu/cgi-bin/tdnaexpress“, seed stocks (mixture of homozygous, wild type and heterozygous) were ordered from ABRC, Columbus. This collection of Arabidopsis T-DNA lines were made in the Columbia-0 ecotype and received as segregating T3 lines.

PCR-based screening of homozygous lines

WT and SALK lines seeds were sterilized and grown in soil. At the 15d stage, DNA was extracted from a young leaf (0.5-0.7 cm) with a REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, St. Louis, MO). DNA extracted from these plants was used for PCR-based screening for homozygous lines. Gene-specific primers (forward and reverse genomic primers) for WT genes were designed by using the SALK T-DNA verification
primer design tool available at the Salk Institute Genomics Analysis Laboratory web portal ([http://signal.salk.edu/tdnaprimers.2.html](http://signal.salk.edu/tdnaprimers.2.html)). A model for the primer design is shown in Figure 3.1. Information for T-DNA left border primer was retrieved from same site. The left border T-DNA primer was LBa1 (from pBIN-pROK2) primer (5’ TGG TTC ACG TAG TGG GCC ATC G 3’). Gene-specific primers used for PCR of different T-DNA mutant SALK lines were as follows, for SALK_024865 (AtAGP4), forward primer (FP) 5’ AGG AGG AGT TGC GAC TGG AGG 3’; reverse primer (RP) 5’ TTT GCC AAA ATA TAT ACC ATA AA 3’. For AtAGP9, five different T-DNA SALK lines were available. For simplification in screening of SALK lines, they were numbered 9(1-5) corresponding to different sites of insertion (Table. 3.1). For SALK_010915 [AtAGP9(1)], FP 5’ TTC CCC TCC TGC TCA AGT TCC 3’; RP 5’ TGG AAT GAG AAA TGA AGC CAC AA 3’. For SALK_060744 [AtAGP9(2)], FP 5’ TTC CCC TCC TGC TCA AGT TCC 3’; RP 5’ TGG AAT GAG AAA TGA AGC CAC AA 3’. For SALK_005873 [AtAGP9(3)], FP 5’ TTC CCC TCC TGC TCA AGT TCC 3’; RP 5’ TGG AAT GAG AAA TGA AGC CAC AA 3’. For SALK_149384 [AtAGP9(4)], FP 5’ GCT TTG TAA CTT TGT TTG TAA GGC A 3’; RP 5’ GTG GTG GTG GTG GAT TTG CTG GAG 3’. For SALK_058644 [AtAGP9(5)], FP 5’ GCT TTG TAA CTT TGT TTG TAA GGC A 3’; RP 5’ TGC TGG TGG AGG AGA AGC CAC AA 3’. For the screening of T-DNA mutant SALK_147517 (AtAGP3), FP 5’ TCT GTT TAT CGT TAC AAA GTT CGG 3’; RP 5’ CCA TGT ATA TCT CTA TGT GCC TGC 3’.
Figure 3.1 A schematic model showing the different sets of primers used in PCR-based screening of SALK T-DNA mutant lines. A combination of FP+RP was used to amplify a WT band whereas a Lba1+RP was used to amplify a mutant allele band. For heterozygous (HZ) lines, a combination of FP+RP+Lba1 amplifies a WT band and a mutant allele band. For homozygous (HM) lines, this same combination of primers results in only the mutant allele band.
Table 3.1 List of classical *Arabidopsis* AGPs showing the available T-DNA insertion lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>ABRC</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AtAGP3</em></td>
<td>At4g40090</td>
<td>SALK_147517</td>
<td>Exon</td>
</tr>
<tr>
<td><em>AtAGP4</em></td>
<td>At5g10430</td>
<td>SALK_024865</td>
<td>1000-Promotor</td>
</tr>
<tr>
<td><em>AtAGP7</em></td>
<td>At5g65390</td>
<td>SALK_039285</td>
<td>Exon</td>
</tr>
<tr>
<td><em>At</em>AGP9(1)</td>
<td>At2g14890</td>
<td>SALK_010915</td>
<td>Intron</td>
</tr>
<tr>
<td><em>At</em>AGP9(2)</td>
<td></td>
<td>SALK_060744</td>
<td>Intron</td>
</tr>
<tr>
<td><em>At</em>AGP9(3)</td>
<td></td>
<td>SALK_005873</td>
<td>Intron</td>
</tr>
<tr>
<td><em>At</em>AGP9(4)</td>
<td></td>
<td>SALK_149384</td>
<td>1000-Promotor</td>
</tr>
<tr>
<td><em>At</em>AGP9(5)</td>
<td></td>
<td>SALK_058644</td>
<td>1000-Promotor</td>
</tr>
</tbody>
</table>

This list of T-DNA insertion lines was generated from the *Arabidopsis* Biological Resource Center (ABRC) database by using the SIGnAL “T-DNA Express” *Arabidopsis* Mapping Tool.
For SALK_039285 (\textit{AtAGP7}), 5’ GAT CTC AGA TCC GGA TTC CAG 3’; 5’TTC ACT TAA ACG TTT CTG CTA TTT G 3’. For each screening of the HM lines, four possible combinations of primers were used; FP+RP, FP+LBa1, RP+LBa1 and FP+RP+LBa1. PCR was performed in a Gene Amp\textsuperscript{®} PCR System 9700 (Applied Biosystems, Foster City, CA). PCR amplification conditions for screening all the SALK lines were, 95°C for 3 min; 35 cycles of 95°C for 30 s, X°C for 1 min, and 72°C for 1 min; final exte X°C) used for different SALK lines were, 50°C for SALK_024865 (\textit{AtAGP4}), SALK_010915 [\textit{AtAGP9(1)}], SALK_060744 [\textit{AtAGP9(2)}], SALK_005873 [\textit{AtAGP9(3)}], and SALK_149384 [\textit{AtAGP9(4)}]; 55°C for SALK_058644 [\textit{AtAGP9(5)}]; 48°C for SALK_147517 (\textit{AtAGP3}) and SALK_039285 (\textit{AtAGP7}).

\textbf{Phylogenetic analysis}

AGP sequences were retrieved from NCBI database and converted into FASTA format. Sequences for classical AGPs, lysine-rich AGPs, fasciclin-rich AGPs and AG peptides were used for this analysis. The FASTA files were aligned by Clustal W software. These aligned sequences were used to generate a phylogenetic tree (100/1000 bootstrap trials) using an in-house (Dr. Ahmed Faik, Department of Environmental and Plant Biology, Ohio University) developed java-based Tree View software (Faik et al., 2006). This software was a combination of PHYLIP package, PHYML algorithm and TREE VIEW analyzer which was developed by Dr. Ahmed Faik’s research group at the Department of Environmental and Plant Biology, Ohio University.
**Microarray based genomic resource**

Microarray-based expression profiles from the Genevestigator Gene Atlas ([https://www.genevestigator.ethz.ch/](https://www.genevestigator.ethz.ch/)) were generated by using the locus ID of *AtAGP3, AtAGP4, AtAGP7 and AtAGP9*. The criteria selected for generation of organ-specific expression profiles was “chip type: ATH1 (22k full genome *Arabidopsis* Affymetrix Gene Chip)”.

**RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)**

At different growth stages total RNA was extracted from various tissues with the RNeasy plant kit as described in Sun (2004). Soil grown plants (3-4 weeks in age) were used to harvest stems, rosette leaves and flowers, whereas 1 week old plate-grown plants were used for harvesting young roots and 2 week old plants were used to harvest old roots. Plant tissue was ground in liquid nitrogen with mortar and pestle that was DEPC treated and autoclaved. RNA extraction was performed with the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) and followed by RT-PCR. The one-step RT-PCR Kit (QIAGEN, Valencia, CA) was used to perform the RT-PCR. Primers designed for *AtAGP4* were, forward primer 5’ TAT TCG CCA CTT CAG CAC TCG CT 3’ and reverse primer 5’ GGC GAA GAA AGC CTT GTT GGA GAA 3’. The primers for *AtAGP9* were forward primer 5' AGT CAC AAC ATC TCC TCC TCC AGT 3' and reverse primer 5’ GCT CCA TTC TGG TCG TTC ACA TCA 3’. Actin was used as the
loading control. RT-PCR was performed in Gene Amp® PCR System 9700 (Applied Biosystems, Foster City, CA). RT-PCR conditions were, Pre-PCR step 50°C for 30 min, 95°C for 15 min; followed by 29 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; final extension at 72°C for 10 min followed by 4°C storage.

**Northern blotting**

Northern blotting procedures were performed as described by Sun et al. (2005) and Yang et al. (2007) with minor modifications. Total RNA was extracted with RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) and separated on a 1.2% denaturing agarose-formaldehyde gel. RNA was transferred onto Zeta-Probe Genomic Tested Blotting Membrane (Bio-Rad, Hercules, CA). Gene-specific probes for *AtAGP4* and *AtAGP9* were synthesized and radio-labeled with α-32P-dCTP according to the manufacturer’s instructions described in Prime-a-Gene Labeling System (Promega-Madison, WI). The membrane was hybridized overnight at 65°C to the probe in the hybridization solution (1M Na2HPO4, 10% SDS, pH 7.2). After washing the membrane with hybridization buffer, membrane was wrapped in saran wrap and exposed to Kodax Biomax MS film placed in a Bio-Rad cassette. The exposure was performed at -80°C for 24h-48h depending on the optimum intensity required. The exposed film was developed and fixed with KODAK GBX reagents. Actin was used as the internal loading control. The actin primers used were 5’ GTG CTC GAC TCT GGA GAT GGT GTG 3’ and 5’ CGG CGA TTC CAG GGA ACA TTG TGG 3’.
**Real-time quantitative polymerase chain reaction (qPCR)**

*RNA extraction:* Wild type (WT) and SALK mutants were grown on MS growth media for 15d and harvested for RNA. Tissue was grounded in liquid nitrogen and total RNA extracted with an RNeasy plant kit (QIAGEN, Valencia, CA).

*Deoxyribonuclease (DNase) treatment:* Total extracted RNA (~1µg) was treated with DNase I, amplification grade (Sigma, St. Louis, MO) as instructed in the manufacturer’s manual. DNase treated samples were used for first strand cDNA synthesis.

* cDNA synthesis:* cDNA synthesis was performed using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Carlsbad, CA).

*qPCR:* SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen, Carlsbad, CA) was used to perform qPCR. qPCR was performed with a Stratagene Mx3000P™ Real-Time PCR System (Stratagene Inc. La Jolla, CA).

**Promoter::GUS expressing transgenic plants**

Primers were designed for the promoter regions of *AtAGP3*, *AtAGP4*, *AtAGP7* and *AtAGP9*. The promoter primers were *AtAGP3* 5’ CGG CTT AAT TAA CTT CAT TGC AGC TTC TCC AAC C 3’ and 5’ AAA AGG CGC GCC TTC TCT CAG TTT CAA TGT CTT TCG 3’. *AtAGP4* primers were 5’ GGC CTT AAT TAA GGC TTC CCG CCA
AAT ATT ATA GCG 3’ and 5’ AAA AGG CGC GCC TTC TCT CTC TTT CTC TTT GGA 3’. *AtAGP7* primers were 5’ CGG CTT AAT TAA GGC CCA AAA CTC TAT AGG CCC A 3’ and 5’ AAT TGG CGC GCC TTT TTC CAC TCT CTC TAA AGA TTT G 3’. *AtAGP9* primers were 5’ GGC CTT AAT TAA CAG ATT AAC CGG TCG ACT AAC TCC and 5’ AAA AGG CGC GCC TTT TGC TTT TGC TTT TTC TCT CTG 3’. Promoter sequences were amplified by PCR and cloned into the pMDC164 binary vector (Curtis and Grossniklaus, 2003). *P*<sub>AtAGP3::GUS</sub>, *P*<sub>AtAGP4::GUS</sub>, *P*<sub>AtAGP7::GUS</sub> and *P*<sub>AtAGP9::GUS</sub> fusion constructs in binary vectors were transformed into *Agrobacterium* (LBA4404 strain) by using GENE PULSER II electroporation system (Bio-Rad, Hercules, CA). *Agrobacterium* cells were screened on LB plates supplemented with streptomycin (25µg/ml) and kanamycin (25µg/ml) antibiotics. Four week old *Arabidopsis* WT plants were transformed with *Agrobacterium* stocks using the floral dip method (Clough and Bent, 1998). Seeds collected from the transformed WT plants were sterilized (30% bleach solution and 0.1% triton-X-100) and screened on MS plates supplemented with hygromycin (20µg/ml). MS plates were cold stratified for 3d at 4°C followed by dark treatment for 3-5d. Transformed seedlings were transferred to fresh MS plates (no hygromycin) for 8-10d and finally potted in soil.

**Plate-based phenotypic analysis**

Wild type and mutants seeds were surface sterilized prior to growing them on the growth plates. Seeds were treated with 70% ethanol for a minute. This was followed by treatment
with 30% bleach in 0.1% triton-X-100 with constant agitation on a rotary shaker (80 rpm) for 10 min. After discarding the bleach/triton-X-100 solution, seeds were washed (~5 times) with sterile, double distilled (dd) H₂O. The entire procedure of seed sterilization and phenotypic analyses was performed as described by Boyes et al. (2001), but with minor modifications. Surface-sterilized seeds were transferred onto growth media plates (pH 5.8) made with MS media (Murashige and Skoog, 1962) [4.3 g/L Murashige and Skoog salts (Caisson labs, North Logan, UT)] and 1% sucrose supplemented with 0.6% phytagel (Phytotechnology labs, Shawnee Mission, KS). Plates were cold stratified at 4°C for 72h and transferred to the tissue culture room [22°C with long day conditions (16h photoperiod)]. Phenotypic analyses was performed daily starting from day 1 and was terminated after 15d (after >50% seedlings had primary root lengths of >6cm).

Different aspects of early growth were recorded during the 2 week period of the Arabidopsis development such as, seed germination, emergence of radicle, hypocotyl and cotyledon, leaf development, primary root length and lateral roots numbers. Primary root length (from 7d-12d growth stage) was measured with a metric ruler under a dissection scope. Lateral roots were counted either by scanning the plates under EPSON PERFECTION 1450U color scanner or by counting the numbers under the dissecting scope. Measurements were made on the scanned images with IMAGE-PRO software (Media Cybernetics Inc.). Pictures of plants at different growth stages were taken either with Sony digital camera or Nikon proshot digital camera and images were processed with Adobe Photoshop (Version. 7.0).
Germination assay

Wild type and mutant plant seeds (~150-200) to be examined for germination assay were sown on *Fisherbrand* P5 filter paper (Fisher Scientific, Hampton, NH) placed in petri plates and saturated with either distilled water (control) or ionic/non-ionic osmotic reagents or abscisic acid (ABA). The concentrations of ionic osmotic reagents used for the assay were 100mM NaCl and 175mM NaCl. The concentrations used for the non-ionic osmotic reagents were 200mM mannitol and 350mM mannitol. The concentrations of ABA (0.5µM, 1µM, 2µM and 20µM) used for the assay were made from a 5mM stock. Seeds were cold stratified for 48h at 4°C and later placed at 22°C under long day conditions (16h photoperiod). For ionic and non-ionic reagent treatments, seeds were scored for germination based on emergence of the radicle, whereas for ABA treatments seeds were scored for emergence of the hypocotyl and cotyledons. Using a dissecting microscope, seeds were examined for emergence of radicles, hypocotyls and cotyledons for four days. Each assay was replicated 3-4 times. Data from these experiments were placed in an MS excel sheet and mean and standard error were calculated to plot a graph.
Results

Basic molecular information of four classical genes

AtAGP3, AtAGP4, AtAGP7 and AtAGP9 are typical classical AGPs genes with an N-terminal signal peptide sequence, a AGP domain rich in hydroxyproline and a C-terminal GPI-anchor addition sequence. The basic molecular information for AtAGP3, AtAGP4, AtAGP7 and AtAGP9 is provided in Table. 3.2. AtAGP3, AtAGP4 and AtAGP7 show a single exon in their sequences, whereas AtAGP9 includes two exons and an intron. The earlier rationale behind selecting only AtAGP4 and AtAGP9 for functional analyses was their high number of ESTs in the root. Previous studies on expression sequence tags (ESTs) with the AFGC (Arabidopsis Functional Genomics Consortium) array at MSU (Michigan State University, East Lansing) identified 18 ESTs for AtAGP4 and 26 ESTs for AtAGP9 in the root region (Schultz et al., 2002). Similarly, AtAGP3 and AtAGP7 were also selected for functional analyses because of their microarray-based expression profiles indicating that AtAGP3 is root-specific whereas AtAGP7 shows high expression in roots.

Phylogenetic analyses demonstrates evolutionary clustering pattern of AtAGP3, AtAGP4, AtAGP7 and AtAGP9

FASTA files of classical AGPs in Arabidopsis including the 14 classical AGPs, 10 AG peptides and 3 Lys-rich AGPs were retrieved and analyzed with TREE VIEW software. AtAGP3, AtAGP4, and AtAGP7 cluster and branch closely suggesting their close-
Table 3.2 Basic molecular information on *AtAGP3, AtAGP4, AtAGP7* and *AtAGP9*.

<table>
<thead>
<tr>
<th></th>
<th><em>AtAGP3</em></th>
<th><em>AtAGP4</em></th>
<th><em>AtAGP7</em></th>
<th><em>AtAGP9</em></th>
</tr>
</thead>
<tbody>
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<td>79-486</td>
<td>64-456</td>
<td>59-1135</td>
</tr>
<tr>
<td><strong>5'UTR</strong></td>
<td>1-68 bp</td>
<td>1-78 bp</td>
<td>1-63 bp</td>
<td>1-58 bp</td>
</tr>
<tr>
<td><strong>Coding region</strong></td>
<td>69-332</td>
<td>79-486</td>
<td>64-456</td>
<td>59-568;1070-1135</td>
</tr>
<tr>
<td><strong>Exon</strong></td>
<td>1-729</td>
<td>1-782</td>
<td>1-755</td>
<td>1-568; 1070-1380</td>
</tr>
<tr>
<td><strong>Intron</strong></td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>569-1069</td>
</tr>
<tr>
<td><strong>3'UTR</strong></td>
<td>333-729</td>
<td>487-782</td>
<td>457-755</td>
<td>1136-1380</td>
</tr>
</tbody>
</table>

The molecular information for the genes was retrieved from [http://www.arabidopsis.org](http://www.arabidopsis.org).

bp, base pair; ORF, open reading frame; UTR, untranslated region.
relatedness and similarity in evolution (Fig. 3.2). AtAGP9 branches with AtAGP19, a lysine-rich AGP. A recent study has shown that, AtAGP9 and AtAGP19 are closely related and have high degree of similarity at the amino acid level but do not show any similarity in the Lys-rich region (Yang, 2006). Although, previous studies have suggested a connection between evolutionary clustering and functional similarity (Faik et al., 2006), it remains to be shown if any of the closely branched AGPs can duplicate their functional properties.

**Identification of SALK lines and isolation of homozygous T-DNA mutants**

To determine the role of *AtAGP3, AtAGP4, AtAGP7* and *AtAGP9* in *Arabidopsis*, T-DNA insertion lines were identified via the SIGnAL T-DNA Express *Arabidopsis* Gene Mapping Tool. The location of the T-DNA insertion in the four classical AGPs is detailed in Table. 3.1. PCR-based screening method was used to identify homozygous lines for each of the four classical AGP genes. The exact locations of T-DNA insertion in the each of the SALK lines were identified based on DNA sequencing using gene-specific primers (Appendix B). Although the T-DNA lines have a kanamycin-resistance marker, this trait was not used for screening of the HM lines because of the possibility for loss of the marker gene in successive generations. For *AtAGP4*, SALK_024865 was available with an insertion within the promoter region (467 bp upstream of start site and a HM mutant was successfully isolated (Fig. 3.3). T-DNA insertion was identified For *AtAGP9*, five,
**Figure 3.2** Phylogenetic analyses of AG-peptides, classical and Lys-rich AGPs from *Arabidopsis*. Protein sequences retrieved from NCBI database were converted into FASTA format and aligned by Clustal W. The aligned sequences were used to generate a
phylogenetic tree (100 bootstrap trials) using an in-house developed java-based Tree View software. The classical AGPs studied in this project are highlighted with an asterisk in the tree. The last letter in the name of the proteins is used to designate the different classes of Arabidopsis AGPs: C, classical; P, peptides; K, lysine-rich. ‘At#’ indicates the Arabidopsis thaliana locus identification number for the AGPs.
Figure 3.3 Gene structure of *AtAGP4* and PCR based screening for a homozygous SALK_024865 line. A, Schematic representation of the gene structure of *AtAGP4* based on DNA sequencing indicating the location of T-DNA insertion (467 bp upstream of ATG site) in the promoter region. B, PCR based screening of homozygous SALK_024865 line shows the presence of wild type (WT), heterozygous (HZ) and homozygous (HM) lines. Lane 1 indicates the WT control; lanes 2-3, 5-8 indicate the HZ lines and lanes 4 and 9 indicate the HM lines. Arrows indicate the gene specific forward and reverse primers. M, 50 base-pair DNA marker.
different SALK lines were available with T-DNA insertions in different positions in the
gene sequence [numbered 9(1-5) corresponding to the different sites of insertion] (Table.
3.1). For SALK_010915 [AtAGP9(1)] (Fig. 3.4B) and SALK_149384 [AtAGP9(4)] (Fig.
3.4E) isolation of HM mutants was unsuccessful and only HZ lines were identified. This
is not due to HM lethality of the mutant but because of absence of HM lines in the pool
of plants used for screening. For SALK_060744 [AtAGP9(2)] (Fig. 3.4C), SALK_005873
[AtAGP9(3)] (Fig. 3.4D) and SALK_058644 [AtAGP9(5)] (Fig. 3.4F) several HM plants
were identified for each of the SALK lines and the seeds stocks for each independent HM
plant were maintained separately. The T-DNA insertions for AtAGP9(3) and AtAGP9(5)
were located in intron (861 bp downstream of ATG site) and promoter (306 bp upstream
of ATG site) (Fig. 3.4A). HM lines for AtAGP9(2) were not sequenced for identification
of T-DNA insertion. Screening of SALK_147517 (AtAGP3) was unsuccessful and only
HZ lines were identified (Fig. 3.5B). Selfing of the HZ did not show a typical Mendelian
genetics ratio of 1:2:1 (WT:HM:HZ) in the F2 generation. Surprisingly, only WT and HZ
appeared upon PCR-based screening and no HM lines were identified (Fig. 3.5C). A total
of 40 plants were grown on the MS media and from this pool of plants 5 was identified as
WT and 3 were identified as HZ lines. One plausible explanation for not obtaining the
HM mutant is that the HM atagp3 knock out mutant is a lethal mutation. To confirm if
the only plants surviving during the screening process were WT and HZ, a plate-based
growth screening was performed. This growth method suggested that only WT and HZ
survived on the plates and potentially all HM lines that were embryo-lethal failed to
Figure 3.4 Gene structure of *AtAGP9(3)* and *AtAGP9(5)* and identification of homozygous SALK lines in *AtAGP9* based on PCR screening.

A, Location of T-DNA insertions in *AtAGP9(3)* in the intron region (861 bp downstream of the ATG site) and *AtAGP9(5)* in the promoter region (306 bp upstream of ATG site) based on DNA sequencing. Also, the schematic used here is a general representation for the insertion lines of *AtAGP9(1)*, 9(2) and 9(3) in the intron and *AtAGP9(4)* and 9(5) in the promoter region respectively. The red arrows represent gene-specific FP and RP for *AtAGP9(5)* and the black arrows represent the gene-specific FP and RP for *AtAGP9(3).*
B, PCR based screening of SALK_010915 \([AtAGP9(1)]\) line shows presence of only wild type (WT) and heterozygous (HZ) lines. Lane 1 indicates the WT control, lanes 2, 4 indicate the WT line and lane 3 indicates the HZ line. M, 100 base-pair DNA marker.

C, PCR based screening of homozygous SALK_060744 \([AtAGP9(2)]\) line shows presence of WT, HZ and homozygous (HM) lines. Lane 1 indicates the WT control, lane 2 indicates the WT line, lane 3 indicates the HM line and lane 4 indicates the HZ line. M, 100 base-pair DNA marker.

D, PCR based screening of homozygous SALK_005873 \([AtAGP9(3)]\) line. Lane 1 indicates the WT control, lanes 2-5 indicate the WT lines and lane 6 indicates the HM line. M, 100 base-pair DNA marker.

E, PCR based screening of homozygous SALK_149384 \([AtAGP9(4)]\) line. Lane 1 indicates the WT control, lane 2 indicates the WT line and lane 3 indicates the HZ line. M, 100 base-pair DNA marker.

F, PCR based screening of homozygous SALK_058644 \([AtAGP9(5)]\) line. Lane 1 indicates the WT control, lanes 2-3 indicate the WT lines and lanes 5-6 indicate the HM lines. M, 100 base-pair DNA marker.
**Figure 3.5** Gene structure of *AtAGP3* and PCR based screening of SALK_147517.

A, Schematic representation of the gene structure of *AtAGP3* indicating the location of T-DNA insertion in the exon region (146 bp downstream of ATG site) based on DNA sequencing. The black arrows represent the gene specific FP and RP.

B, PCR based screening of SALK_147517 (*AtAGP3*) line shows presence of only wild type (WT) and heterozygous (HZ) lines. Lane 1 indicates the WT control, lanes 2, 5 indicate the WT lines and lane 6 indicates the HZ line. Lanes 3-4 do not show any bands. M, 100 base-pair DNA marker.

C, PCR based analysis of F2 generation of selfed HZ SALK_147517 (*AtAGP3*) lines. Lane 1 indicates the WT control, lanes 3, 5, 7, 9 indicate the WT lines and lanes 2, 4, 6, 8, 10 indicate the HZ lines. M, 100 base-pair DNA marker.
germinate (Fig. 3.6A). These HZ plants grow slower than WT until 3 weeks and ultimately grow to the same size as WT after flowering (Fig. 3.6B). In order to examine if the HZ lines were embryo lethal, siliques from the selfed HZ lines were analyzed for ovule abortion, but no defects in ovule abortion were observed (Fig 3.6C). The T-DNA SALK_039285 line for AtAGP7 was screened to identify multiple HM lines (Fig. 3.7). Again, seeds stocks for independently identified HM lines were maintained separately so that they could be used for independent phenotypic analysis and thus insure the use of only those seeds stocks that show a replicable mutant phenotype.

**Organ-specific expression analysis of AtAGP3, AtAGP4, AtAGP7 and AtAGP9**

Information from the public database (Genevestigator Gene Atlas) was retrieved to analyze the organ-specific expression pattern of AtAGP3, AtAGP4, AtAGP7 and AtAGP9. These expression profiles suggest that AtAGP3 is specifically expressed only in lateral roots and the elongation zone of the root (Fig. 3.8); AtAGP7 is moderately expressed in the seedling stage (hypocotyls and radicle), stem and petiole and highly expressed in the roots (lateral roots and elongation zone) (Fig. 3.9). AtAGP4 is highly expressed in the roots, stems and node region (Fig. 3.10); AtAGP9 is moderately expressed throughout various tissues during development but highly expressed in the stems (Fig. 3.11).
Figure 3.6 Germination defects and phenotype in the F2 generation of selfed heterozygous SALK_147517 (AtAGP3) lines. 80% of the F2 generation seeds failed to germinate, suggesting lethality of the \textit{atagp3} T-DNA mutant (A). At the 13d stage \textit{AtAGP3} HZ lines are smaller and show a stunted root growth in comparison to the WT (B). Ovules from WT and \textit{AtAGP3} HZ plants (5 week old) observed under a dissecting scope.
Figure 3.7 Gene structure of *AtAGP7* and PCR based screening for homozygous SALK_039285 lines.

A, Schematic representation of the gene structure of *AtAGP7* indicating the location of the T-DNA insertion in the exon region (134bp downstream of ATG site) based on DNA sequencing. The black arrows represent the gene specific FP and RP.

B, PCR based screening for homozygous SALK_039285 (*AtAGP7*) lines. Lane 1 indicates the WT control and lanes 3, 4 and 6 indicate the HM line. Lanes 2 and 5 do not show any bands. M, 100 base-pair DNA marker.
Figure 3.8 Microarray-based expression profiles for *AtAGP3* from the Genevestigator Gene Atlas. Profiles were retrieved from the webportal (https://www.genevestigator.ethz.ch/) and shows the relative expression intensities of *AtAGP3* in different organs of *Arabidopsis* (Columbia-0 ecotype).
Figure 3.9 Microarray-based expression profiles for *AtAGP7* from the Genevestigator Gene Atlas (https://www.genevestigator.ethz.ch/). Profiles show the relative expression intensities of *AtAGP7* in different organs of *Arabidopsis* (Columbia-0 ecotype).
Figure 3.10 Microarray-based expression profiles for AtAGP4 from the Genevestigator Gene Atlas (https://www.genevestigator.ethz.ch/). Profiles show the relative expression intensities of AtAGP4 in different organs of Arabidopsis (Columbia-0 ecotype).
Figure 3.11 Microarray-based expression profiles for *AtAGP9* from the Genevestigator Gene Atlas (https://www.genevestigator.ethz.ch/). Profiles show the relative expression intensities of *AtAGP9* in different organs of *Arabidopsis* (Columbia-0 ecotype).
To confirm the profiles observed in the microarray-based database, RT-PCR and northern blotting analysis were performed with RNA extracted at various stages of *Arabidopsis* development. RT-PCR analysis indicates *AtAGP4* is highly expressed in stems, roots, rosette leaves and seedlings and less expressed in flowers (Fig. 3.12A). The RNA transcript levels from all organs were normalized with actin for RT-PCR and northern blotting and plotted as a graph showing relative mRNA abundance as fold change with reference to normalized actin. The microarray data for *AtAGP4* and *AtAGP9* was consistent by the northern blotting analysis and shows a similarity in expression profiles. Quantification via northern blotting indicates high expression of *AtAGP4* in stem and young roots, low expression in seedlings and no expression in flowers (Fig. 3.12B). Similarly, RT-PCR analysis of *AtAGP9* shows uniform expression in stems, roots, flowers, rosette leaves and seedlings (Fig. 3.13A). However, northern analysis indicates high expression only in the stem, young roots and seedlings, low expression in old roots and flowers and no expression in rosette leaves (Fig. 3.13B). Cross referencing of RT-PCR and northern analysis results with the Genevestigator Gene Atlas confirms the organ-specific expression pattern and also indicates a similarity in expression for *AtAGP4* and *AtAGP9*. In order to investigate tissue-specific expression pattern of *AtAGP3, AtAGP4, AtAGP7* and *AtAGP9, promoter::GUS (β-glucoronidase)* fusions were constructed. WT plants were successfully transformed with *promoter::GUS* fusions and seeds from transformed WT plants (*AtAGP3* and *AtAGP7*) were collected. Screening of *GUS* transformants on MS plates supplemented with hygromycin (20µg/ml) was
Figure 3.12 Organ-specific expression profile of AtAGP4 in Arabidopsis.

A, Reverse-transcription (RT)-PCR analysis indicates the presence of AtAGP4 transcript abundantly in roots, stems, rosette leaves and seedlings and less abundantly in flowers. Ethidium bromide staining of actin control shows equal loading of RNA samples. RNA transcript levels from all organs were normalized with actin and plotted as a graph showing relative mRNA abundance. RT, roots; ST, stem; RL, rosette leaves; FL, flower, SL, seedling; M, 50 bp DNA marker.

B, Northern blot analysis shows the abundance of AtAGP4 transcript only in stems and young roots and low expression in seedlings. Actin was used as a loading control. Probes were labeled with [α-32P]dCTP in using the Prime-a-gene labeling system. RNA transcript levels from all organs were normalized with actin and plotted as a graph.
showing relative mRNA abundance. ST, stem; RL, rosette leaves; FL, flower, SL, seedling, YR, young root; C, control.

RNA samples for both RT-PCR and northern blot analysis were extracted from either seedlings/young roots (14d) or different organs of 3-4 week old *Arabidopsis* plants.
Figure 3.13 Organ-specific expression profile of *AtAGP9* in *Arabidopsis*.

A, Reverse-transcription (RT)-PCR analysis indicates the presence of *AtAGP9* transcript in all the examined organs. RNA transcript levels from all organs were normalized with actin and plotted as a graph showing relative mRNA abundance. Ethidium bromide staining of the actin control shows equal loading of RNA samples. RT, roots; ST, stem; RL, rosette leaves; FL, flower, SL, seedling.

B, Northern blot analysis shows abundance of *AtAGP9* transcript only in stems, young roots and seedlings. Actin was used as a loading control. Probes were labeled with [α-32P]dCTP using the Prime-a-gene labeling system. RNA transcript levels from all organs were normalized with actin and plotted as a graph showing relative mRNA abundance. ST, stem; RL, rosette leaves; FL, flower, SL, seedling, YR, young root; OR, old root; C, control.
RNA samples for both RT-PCR and northern blot analysis were extracted from either seedlings/young roots (14d) or different organs of 3-4 week old *Arabidopsis* plants.
unsuccessful. Transformants were selected on hygromycin plates but most of the transformants did not survive when transferred to fresh MS plates without hygromycin. Only three transformants from \( P_{\text{AtAGP3}}::\text{GUS} \) and one for \( P_{\text{AtAGP7}}::\text{GUS} \) lines were successfully screened and transferred to a fresh MS plate without hygromycin. However, later when transferred to soil, all four transformants died.

**Lateral root development in homozygous mutant lines**

A plate-based phenotype analysis was performed to identify phenotypic aberrations in HM lines in comparison to the WT. The focus of the phenotypic analysis was on roots because all four classical AGP genes under study were highly expressed in roots. *Arabidopsis* plants grown on transparent growth media plates were conveniently analyzed under the dissecting microscope for any root phenotypes. atagp4 showed no defects in root and shoot development. In 14d old plants, atagp9(3) plants had decreased numbers of lateral roots in comparison to the WT (Fig. 3.14A-B). This phenotype was not observed in the other T-DNA insertion lines found in *AtAGP9*. Moreover, the primary root length was unaffected in the all atagp9 HM lines (Fig. 3.14A). atagp7 primary root analyses in 7d, 8d, 10d and 12d old plants did not show any differences in the primary root length (Fig. 3.15A).
Figure 3.14 Root phenotype analyses in wild type, *atagp4* and *atagp9* T-DNA mutant. A, In 14d old seedlings, the number of lateral roots in *atagp9(3)* mutants were decreased compared to wild type (WT). B, 14d old WT and *atagp9(3)* *Arabidopsis* seedlings showing decreased lateral roots. A close-up image for lateral roots for both WT and *atagp9(3)* are shown in the inset box. C, Average primary root lengths of 8d and 14d old WT, *atagp4* and *atagp9* seedlings. No major differences were observed in the primary root lengths of the mutant lines. Error bars in A and C indicate SE (*n* > 40).
Figure 3.15 Root phenotype analyses in wild type and the atagp7 T-DNA mutant. A, Average primary root lengths of 7d, 8d, 10d and 12d old WT and mutant seedlings. Error bars indicate SE (n > 50). B, In 13d and 14d old seedlings, the number of lateral roots in atagp7 mutants are similar compared to wild type (WT). C, 18d old WT and atagp7 Arabidopsis seedlings grown on MS plates (supplemented with 1% sucrose) showing lateral roots. Enlarged images of inset boxes showing lateral roots in WT and atagp7.
Above ground phenotype for *atagp9*(5) and *atagp7*

Soil-based phenotype analysis was performed to examine the above ground phenotypic aberrations in HM lines. In a phenotypic analysis done in 4 week old plants, *atagp9*(5) showed an early flowering phenotype in comparison to WT (Fig. 3.16A). The average flowering times for WT and *atagp9*(5) were 30.09 ± 1.1 d and 27.06 ± 0.8 d respectively. Examination of 3 week old plants in, *atagp7* revealed that the HM line grew at a much slower rate but eventually reached same size as that of WT after the flowering stage (Fig 3.16B).

**ABA sensitivity of *atagp7, atagp4, and atagp9*(3)**

ABA is a plant growth hormone that regulates seed dormancy in plants. Germination rates were analyzed in WT and HM lines in presence and absence of ABA. Residual dormancy in seeds was removed by cold stratification of seeds in refrigerated condition to maintain synchronous germination. WT and HM seeds were saturated either with water (control) or different concentrations of ABA. In the control treatment, all the seeds including WT and HM lines completely germinated within 24-48 h. In ABA treated seeds, germination was significantly reduced in *atagp7* only at a high concentration of ABA (20µM) (Fig. 3.17A-E). While no difference in germination rates was observed at the 1d stage, seeds with 4d of treatment with 20µM ABA showed that ~70% germination
Figure 3.16 Above ground-phenotypes of *atagp9* and *atagp7* T-DNA mutants. A, Wild type (WT) and *atagp9*(5) plants with first flower opening times. *atagp9*(5) shows an early flowering phenotype in comparison to the WT. B, 3-week old WT and *atagp7* plants.
Figure 3.17 Germination assay of wild type and atagp7 seeds in response to abscisic acid (ABA) treatment. At the 2d stage (after 3d cold stratification), germinating seedlings of WT and atagp7 showed different percentages of germination in 2µM ABA (A-B). C, A close-up view of WT and atagp7 germinating seedlings (2d-old) in response to 2µM ABA. D, Germination percentages of WT and atagp7 at 1d (D) and 4d (E) in response to different concentrations of ABA (0.5µM, 1µM, 2µM and 20µM). In comparison to the WT, ABA suppresses atagp7 germination to a higher extent. Seeds were scored for germination based on emergence of hypocotyls and cotyledons. Seeds treated with double distilled water (control) show 100% germination within 24-30 h. Error bars indicate SE.
(n > 200). A two-tailed students t-test indicates P=0.36 (1d) and P=0.08 (4d) suggesting the 4d ABA treatment is marginally significant (α=0.05).
occurred in WT compared to only 35% in *atagp7* (Fig. 3.17E). Consequently, these studies indicated that *atagp7* germinating seeds are overly sensitive to ABA treatment with respect to germination in comparison to WT.

However, ABA treatment of *atagp4* and *atagp9(3)* displayed a contrasting effect on the germination rate compared to *atagp7*. *atagp4* and *atagp9(3)* seedlings exhibited increased resistance to ABA stress by suppression of the ABA-induced delay in seed germination (Fig. 3.18C). In seeds treated with 1.5 µM ABA for 3d, ~100% germination was observed for *atagp4* and *atagp9(3)*, whereas the WT shows only 75% germination. In the control treatment (dd H2O), a germination rate of 100% was achieved at similar stage in WT and HM lines.

**atagp4 and atagp9(3) response to ionic and non-ionic osmotic stress**

To examine the response of WT, *atagp4*, *atagp9(3)* and *atagp9(5)* lines to ionic and non-ionic osmotic stress, germination assays were performed. WT and HM seeds were germinated in the presence and absence of ionic (NaCl) and non-ionic (mannitol) osmotic reagents. WT and HM seeds grown in water show 100% germination within 24-48 h (after cold stratification). In seeds treated with 175mM NaCl or 350 mM mannitol for 3d, germination was 20% and 50% respectively, whereas, *atagp4* and *atagp9(3)* showed close to 100% germination in both osmotic reagents (Fig. 3.18A-B). Clearly, *atagp4* and *atagp9(3)* showed an increased tolerance to ionic and non-ionic osmotic stress conditions.
A

Germination (%)

Day

WT

agp

agp

B

Germination (%)

Day

WT

agp

agp

C

Germination (%)

ABA (µM)

WT

agp

agp
**Figure 3.18** Germination assay of wild type, *atagp4* and *atagp9(3)* seeds in response to osmotic and abscisic acid (ABA) stress. *atagp4* and *atagp9(3)* mutants showed increased resistance to both ionic, i.e. 175mM NaCl (A) and non-ionic, i.e. 375mM mannitol (B) osmotic stress. C, *atagp4* and *atagp9* seedlings treated with ABA (3d) exhibited increased resistance to ABA stress. Seeds were sown on filter paper saturated with water/NaCl/mannitol/ABA at indicated concentrations. After 24h, 48h and 96h seeds (~150 seeds) were scored for germination (emergence of radicle). Seeds treated with double distilled water (control) show 100% germination within 24-48h. Error bars indicate SE (*n* > 150). A two-tailed students t-test indicates *atagp4* (*P*=0.1) and *atagp9(3)* (*P*=0.14) response to NaCl treatment are marginally significant. For mannitol treatment, *atagp4* (*P*=0.4) and *atagp9(3)* (*P*=0.4) are insignificant (*α*=0.05). ABA treatment (3d) of *atagp4* (*P*=0.03) and *atagp9(3)* (*P*=0.03) indicates response is significant (*α*=0.05). Closed triangles, WT; closed squares, *atagp9(3)*; closed diamond, *atagp4*.
Quantitative real-time PCR analysis of *atagp4*, *atagp7*, *atagp9(3)* and *atagp9(5)*

To analyze the relative expression levels (fold change) of RNA transcripts in WT and HM lines, quantitative real-time PCR analysis was performed with RNA extracted from 15d roots. *atagp4* showed a decreased transcript level (0.2 fold) in comparison to the WT expression level (Fig. 3.19A). Analysis of the relative expression level in *atagp7* indicates a total absence of transcript in comparison to the WT (Fig. 3.19b). However, in *atagp9(3)*, the transcript level is decreased (0.7 fold) whereas in *atagp9(5)* the transcript level is increased (2.5 fold) indicating variable expression levels in different insertion lines (Fig. 3.19C).
Figure 3.19 Quantitative real-time PCR analysis of WT and homozygous mutant lines. RNA from WT and atagp4 (A), atagp7 (B), atagp9(3) and atagp9(5) (C) were analyzed for relative transcript abundance. Total RNA was harvested from WT and HM roots at the 15d growth stage. The transcript levels were normalized to actin levels and the values were plotted as relative expression levels (fold change) of WT and HM plants. Each data value is a representative of mean ± standard error of three independent technical replicates.
**Discussion**

Screening of SALK_147517 (*AtAGP3*) was narrowed down to identification of only heterozygous lines (Fig. 3.5). Identification of HM lines was unsuccessful even after repeated attempts of growing selfed HZ lines in high humidity conditions. Initially, it was assumed the seeds grown on soil require high humidity to survive the initial germination phase. Surprisingly, even on repeated PCR-based screenings with plants grown in different humidity conditions, only WT and HZ survived (Fig. 3.5B-C). Isolation of HM mutants was unsuccessful most likely because the T-DNA insertion in *atagp3* results in an embryo-lethal mutation (Fig. 3.5C). In order to confirm the Mendelian ratio of 1:2:1 (WT:HZ:HM) in F2 generation of selfed HZ plants, a plate-based phenotype screening was performed to examine the phenotype of HZ plants (Fig. 3.6A-B). This analysis suggested only WT and HZ plants survived and HM lines failed to germinate (Fig. 3.6A). Surprisingly, a ratio of 1:2:1 was not observed in the F2 generation of selfed plants and out of the 40 seeds tested by this method, a high percentage of seeds (80%) did not germinate. Normally, selfing of a HZ results in a typical 1:2:1 ratio with 25% WT, 50% HZ and 25% HM, but the results from selfing of this Salk line does not confirm this ratio. Visual observation of the phenotype of *AtAGP3* HZ lines indicates plate grown plants are smaller in size with reduced primary root length until 3 week stage and later on grow to the same size as the WT after flowering (Fig. 3.6B). Microarray-based expression profiles for *AtAGP3* indicate it is exclusively and highly expressed in the roots (especially the lateral root and elongation zone) (Fig. 3.8). Accordingly, this expression pattern suggests that any potential disruption in the biological function of *AtAGP3* may
result in the severe effects in the growth physiology of the HM line. Another possible approach for examining if the HM line is embryo-lethal was by examining for aborted ovules in the siliques of F2 generation of the selfed HZ lines (Constan et al., 2004; Yang et al., 2007). This examination was performed but no aborted ovules were observed in HZ line (Fig. 3.6C). This indicates that ovule development in the selfed HZ line is not defective and the lethality of HM line only becomes apparent during seed germination.

Plate based phenotypic analysis allows convenient examination of root growth and development to identify any phenotypic aberrations in the mutants. Expression profiles of AtAGP3, AtAGP4, AtAGP7 and AtAGP9 indicate these classical AGPs are highly expressed in roots (Fig. 3.8-Fig. 3.13). Thus, the reasoning behind close observation of potential root phenotypes in these mutants is that dysfunction of any of these genes may show an abnormal phenotype in roots, especially in the tissues showing high expression levels (lateral roots and elongation zone). Initially, WT and HM plants were grown separately on different sets of growth media plates for phenotypic analysis. Later, both WT and HM were grown and analyzed on the same plates to avoid any variations in the growth media and environmental conditions. In this analysis, only atagp9(3) with a T-DNA insertion in the intron region displays defects in lateral root development (i.e. decreased number of lateral roots) (Fig. 3.14A), whereas other HM lines including atagp9(2)(data not shown) and atagp9(5) (Fig. 3.14) do not display this defect. Although, it was expected for the T-DNA insertion lines to exhibit a similarity in any phenotypic defect, this was not the case for the atagp9 HM lines. This can be further explained based
on the fact that RNA transcript levels in \textit{atagp9(3)} HM line and \textit{atagp9(5)} are different potentially relating to difference in physiological roles in root development (Fig. 3.19C).

The HM T-DNA mutant for \textit{AtAGP4} (which is highly expressed in roots), does not exhibit any problems in root structure or development (Fig. 3.14C). This is potentially due to the fact that RNA transcript level in \textit{atagp4} mutant is reduced only by 20% in comparison to WT (Fig. 3.19A). Similarly, \textit{atagp7} HM mutant plants grown on plates do not display defects in primary root length or lateral root development (Fig. 3.15). This is surprising because the RNA transcript in the \textit{atagp7} is completely lost and the mutant still does not display any defects in root growth and development (Fig. 3.19B). However, germination assays (Fig. 3.17) and above ground phenotype (Fig. 3.16B) studies indicates a delayed growth phenotype in soil. This study indicates the \textit{atagp7} mutant displays problems with initial growth resulting in an overall slower growth phenotype. Most of the previous studies with various mutants including \textit{rat1}, \textit{atagp30}, \textit{sos5}, \textit{reb-1}, \textit{mur1 atagp19} have implicated a role for AGPs different aspects of root structure and development, however information on the exact biological mechanism of action is still lacking (Andème-Onzighi et al., 2002; Gaspar et al., 2004; Shi et al., 2003; van Hengel and Roberts, 2002; van Hengel and Roberts, 2003; Yang et al., 2007).

Flowering time in plants is dependent on a number of environmental cues. The most significant of them include light and vernalization. Within the plant, internal cues that control flowering times are maintained by the synergistic action of two transcription
factors *FRIGIDA (FRI)* and *FLOWERING LOCUS C (FLC)* (Grennan, 2006; Gazzani et al., 2003; Michaels et al., 2003; Sung and Amasino, 2005). With the current knowledge on aspects related to flowering time, it is unclear where AGPs fit into this pathway for regulation of flowering time. Phenotypic analysis of HM lines for *atagp9* revealed an early flowering phenotype in *atagp9(5)* plants (Fig. 3.16A). This phenotype was consistently displayed when grown in *Arabidopsis* growth rooms. This display of phenotype may be explained based on the fact that RNA transcript is actually increased (2.5 fold) in *atagp9(5)* in comparison to the *atagp9(3)* and WT plants (Fig. 3.19C). This suggests a correlation between the observed phenotype and overexpression of the *AtAGP9* gene in *atagp9(5)* line. However, transfer of plants to growth chambers (i.e. controlled temperature conditions) inconsistently showed the early flowering phenotype. Only a small percentage of *atagp9(5)* plants showed an early flowering phenotype. None of the other *AtAGP9* HM lines displayed any of the growth chamber phenotype problems. In the expression studies performed with this gene, RT-PCR and Northern analysis with Microarray-based expression studies confirm the high expression of *AtAGP9* in flowers (Figs. 3.13 and 3.15). However, the exact role of *AtAGP9* with reference to early flowering phenotype is still unclear.

During abiotic stress, plant cells are constantly bombarded with various stress signals. For most of stress signals, it is still not clear what the primary receptors are on the cell surface. In response to abiotic stress, various signaling cascades including ABA, ethylene, calcium, phospholipids and reactive oxygen species are initiated within the cell
(Bray, 1997, Hasegawa et al., 2000; Knight and Knight, 2001; Pastori and Foyer, 2002, Perruc et al., 2004; Xiong et al., 2002; Xiong and Zhu, 2002). In the stress studies, \textit{atagp4} and \textit{atagp9(3)} display abnormal physiological responses to abiotic stress, i.e suppression of ABA-induced delay in seed germination and increased tolerance to ionic and non-ionic osmotic reagents (Fig. 3.18). These physiological responses can be correlated with the relative RNA transcript levels of \textit{AtAGP4} and \textit{AtAGP9} in HM lines (Fig. 3.19A and C). The transcript levels in \textit{atagp4} and \textit{atagp9(3)} are decreased in comparison to the WT plants (Fig. 3.19A and C). Although, a specific role for AGPs in stress signaling is not clearly elucidated, a previous study in \textit{Arabidopsis} reported a role for \textit{AtAGP30}, a bonafide AGP, in seed germination and stress response. A transposon-insertion mutant \textit{atagp30} was isolated in this previous study and based on ABA-sensitive germination assays conducted with this mutant it was suggested that \textit{AtAGP30} functions in prevention of seed germination. Similar to \textit{atagp30} mutant, \textit{atagp4} and \textit{atagp9(3)} also display a suppression of ABA-induced germination inhibition. In the same study on \textit{AtAGP30}, AGPs were implicated in cellular signaling with roles in ABA and stress signaling. AGPs coated with polysaccharides are abundantly distributed on the cell surface and can potentially act as receptors of ABA and various stress signals. However, it still needs to be demonstrated what the exact role of AGPs is in stress and ABA signaling.

Quantitative real-time PCR studies indicate a connection between transcript levels of AGP genes in HM lines and stress response. These studies show that in \textit{atagp4} and
*atagp9(3)*, the relative transcript level is decreased in comparison to WT (Fig. 3.19A and C) and this transcript level may correspond to the abnormality in physiological abiotic stress response during seed germination. In WT plants, these AGPs may function as repressors in stress signaling pathway preventing the seeds from germination in abiotic stress conditions, whereas in the HM lines, knockdown of these AGP genes results in loss of repressor activity, thereby providing the seed germination process increased resistance to abiotic stress.
CHAPTER 4: CONCLUSIONS
Conclusions

Molecular interactions of AGPs

Although considerable progress was made to understand the role of AGPs in plant growth and development, their exact functional roles and the molecular mechanisms underlying their interactions with either intra- or extra-cellular molecules was unknown. This research reports potential role of AGPs in organization of the cytoskeleton [microtubules, (MTs) and F-actin] in tobacco BY-2 cells and conversely the role of MTs and F-actin in organization of AGPs at cell surface but the mechanism of action of these biomolecules still remains unclear. Perturbation studies were conducted in tobacco BY-2 cells to analyze the effects of an AGP inhibitor (β-Yariv reagent) on the organization of microtubules [labeled by GFP-MBD (green fluorescent protein-microtubule binding domain)] and F-actin (labeled by rhodamine-phalloidin) and conversely to analyze the effects of a microtubule inhibitor (amiprophosmethyl) and an F-actin inhibitor (cytochalasin-D) on the localization of GPI-anchored GFP-LeAGP-1. These studies implicated a role for GPI-anchored LeAGP-1 in mediating the cell wall-plasma membrane-cytoskeleton connection.

My studies, recently published in Plant Physiology and Plant Signaling and Behavior, were a step forward in understanding these connections at the molecular level and more specifically the role of a GPI-anchored tomato AGP (LeAGP-1) as a linker connecting
the cell wall and the cytoskeleton (microtubules and F-actin) (Sardar et al., 2006; Sardar and Showalter; 2007). These interactions were investigated in tobacco BY-2 cells by employing fluorescent probes such as GFP-MBD, rhodamine phalloidin and GFP-LeAGP-1. Initially, the localization of GFP-LeAGP-1 was determined in untreated BY-2 cells and its localization was compared after treatment with microtubule (APM) and F-actin (cytochalasin-D) inhibitors. These inhibitors disrupt the uniform distribution of GFP-LeAGP-1 on the cell wall, plasma membrane and Hechtian strands. Conversely, \( \beta \)-Yariv reagent treatment effectively disrupts the distribution and organization of cortical microtubules and F-actin. \( \beta \)-Yariv treatment also results in defects in cell morphology such as terminal cell bulging, and this phenotype partially phenocopies a previously reported \textit{Arabidopsis reb}-1 (root epidermal bulger) mutant that shows a reduction in the total amount of AGPs (Andème-Onzighi et al., 2002). Based on these results, a hypothetical cell surface networking model was presented involving direct or indirect interactions among GPI-anchored AGPs, cortical microtubules and F-actin.

In this model, a few possible modes of interactions between the cell surface AGPs and the cytoskeleton were suggested including: (a) direct interactions via a transmembrane protein, (b) indirect interactions via the lipid rafts/detergent resistant membrane, and (c) a mass action involving \( \beta \)-Yariv-induced disruption of distribution of cell surface AGPs and various other wall/membrane components. Formins include an extracellular extensin-like domain and actin binding domains making them one of the most suitable candidates for associations with cell surface AGPs (Keller, 1993; Cvrckova, 2000; Deeks et al.,
Apart from transmembrane proteins discussed in the recent manuscript, other proteins such as callose synthases and class VIII myosin can also potentiate these direct interactions (Baluska et al., 2003). GPI-anchored AGPs may structurally interact with transmembrane callose synthases that are found at sites of active callose deposition. Previous studies suggest that myosin VIII linked to F-actin at the plasma membrane interface is enriched at sites of callose deposition (Baluska et al., 2000; Baluska et al., 2003; Reichelt et al., 1999). Thus any changes in the distribution or structure of AGPs can produce downstream effects in the F-actin organization via the callose synthase-myosin VIII connections. Another emerging link between the extracellular AGPs and the cytoskeleton is via the lipid rafts/detergent resistant membranes. This link is interesting because it relates to the fact that lipid rafts can act as centers of signaling cascades. In animals, various studies have implicated roles of lipid rafts in signaling processes (Ikonen, 2001; Pike, 2004; Simons and Toomre, 2000; Zajchowski and Robbins, 2002). In both animals and plants, GPI-anchored proteins have emerged as efficient markers for lipid rafts because of their high affinity for the lipid microdomains (De Angelis et al., 1998; Mayor et al., 1998; Nichols et al., 2001; Borner et al., 2005). These lipid microdomains/detergent resistant membranes in plants are enriched with sterols such as stigmasterol, campesterol, and β-sitosterol and also include a GPI-anchored AGP, namely AtAGP4 (Borner et al., 2005). It remains to be demonstrated whether most or all GPI-anchored AGPs are localized to these lipid microdomains and play significant roles in signaling. Nevertheless, these rafts can potentially transduce signals via GPI-anchored AGPs to the cytoskeleton, thereby controlling cytoskeleton organization. Recently, an
animal lipid raft associated protein, CLIPR-59, was shown to affect microtubule dynamics by reducing the elongation of microtubules (Lallemand-Breitenbach et al., 2004). Also, other proteins such as RhoA and endostatin have been shown to associate with lipid rafts and play a role in the regulation of the actin cytoskeleton (Nobes et al., 1995; Giancotti and Ruoslahti, 1999; Wickström et al., 2003). These studies indicate that in plants, lipid rafts may potentially perceive signals via some cell surface receptors (GPI-anchored AGPs) and transduce them downstream to the cytoskeleton via various signaling molecules.

Apart from the peptide backbone and GPI anchor, the carbohydrate linkages and oligosaccharide may contribute to potential molecular interactions and physiological functions of AGPs. Structurally, the glycan structure of AGPs represents O-glycosylation of Hyp residues with type II AG molecules (Lamport, 1967; Pope, 1977; Qi et al., 1991). The type of linkages that are found include the (1-3)-β-linked galactopyranosyl backbone and (1-6)-β-D linked side chains (Aspinall, 1970; Defaye and Wong, 1986; Fincher et al., 1983; Gane et al., 1995). Certain studies have suggested that the negatively charged AGPs may interact with the pectic (1→4)-β-galacturonan and hemicellulosic (1→4)-β-glucans based on their sequential co-extraction studies (O’Neill and Selvendran, 1985; Carpita, 1989; Iraki et al., 1989; Shea et al., 1989; Serpe and Nothnagel, 1994). In a specific study performed with carrot cell walls, Baldwin et al (1993) reported that AGPs interact with pectin in presence of divalent calcium ion and this specific binding was calcium dependent. These specific molecular interactions with pectic components were
reported either during co-precipitation or extraction experiments *in vitro* conditions and still remain to be clearly demonstrated in planta. In a study in carrot suspension cell cultures, Immerzeel et al., (2006) reported that AGP fractions isolated from these cultures were rich in galacturonic acid and susceptible to pectin methylesterase and phosphogalacturonase indicating a close connection with pectin. A recent report in salt-adapted tobacco BY-2 cells demonstrated that sonic disruption of 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 this report, it was suggested that AGPs function as ‘pectic plasticizer’ being present in the pectic network in low amounts and contribute to overall porosity and may allow control of hydrostatic pressures and cell expansion. Similar to extensins (Brady and Fry, 1997; Fry, 1982; Lamport, 1965; Lamport 1980; Qi et al., 1995), AGPs may oxidatively cross-link with each other to produce large complexes. Kjelbom et al., (1997) reported that oxidative cross-linking among AGPs is enhanced in the presence of hydrogen peroxide and catalase. Although, the arabinogalactan polysaccharide distribution is quite similar among various plant groups (Akiyama and Kato, 1981; Defaye and Wong, 1986; Zhao et al., 2002) their exact functional roles are still unknown. These polysaccharides may serve a protective role as mucilage (Tautyvydas, 1978) or in the form of gum exudates (Qi et al., 1991) in different plant groups.
Functional identification of *AtAGP3*, *AtAGP4*, *AtAGP7* and *AtAGP9*

Reverse genetics approaches were adopted to elucidate functional roles of AGPs in T-DNA insertional mutants of *AtAGP3*, *AtAGP4*, *AtAGP7* and *AtAGP9* in *Arabidopsis*. Initially, T-DNA insertion lines for each of the classical genes under study were identified and ordered via the SIGnAL T-DNA Express *Arabidopsis* Gene Mapping Tool. Based on PCR-based screening WT, HM and HZ lines were identified. Isolation of a HM mutant for *AtAGP3* was unsuccessful and only HZ lines were identified potentially due to an embryo lethal mutation. However, T-DNA mutants for *AtAGP4*, *AtAGP7*, and *AtAGP9* were successfully identified.

Once the HM lines were isolated, progeny of the HM lines were subsequently examined for phenotypic aberrations by employing both plate-based and soil-based phenotypic analysis. These classical AGPs were selected for functional analysis because of their high expression in roots. Root development in *Arabidopsis* is well established and all four of these classical genes are highly expressed in the roots, especially in the root elongation region and lateral roots. Based on this information, it was hypothesized that dysfunction of any of these classical genes as a result of T-DNA insertion may result in abnormal root growth and development. A summary of progress made with the T-DNA mutant research is presented in the Table 4.1

Phylogenetic analyses *AtAGP3*, *AtAGP4*, *AtAGP7* and *AtAGP9* with other AGPs from *Arabidopsis* including AG-peptides, Lys-rich AGPs and classical AGPs indicates close
grouping and branching for all four classical AGPs. AtAGP3, AtAGP4, AtAGP7 cluster closely, whereas AtAGP9 branches and groups with a Lys-rich AGP, AtAGP19. This type of clustering suggests close-relatedness and similarity in evolution indicating gene functions may be duplicated or compensated by closely related groups. A microarray-based expression analysis was retrieved from the Genevestigator Gene Atlas to establish the organ-specific expression pattern of these genes. This micro-array data was confirmed by performing RT-PCR and northern analysis. Northern blotting and RT-PCR analysis for AtAGP4 and AtAGP9 show a high expression of these transcripts in stems and young roots. RT-PCR analysis indicates AtAGP4 is highly expressed in stems, roots, rosette leaves and seedlings and less expressed in flowers (Fig. 3.12A) whereas, AtAGP9 shows uniform expression in stems, roots, flowers, rosette leaves and seedlings (Fig. 3.13A). Northern blotting analysis indicates high expression of AtAGP4 in stem and young roots, low expression in seedlings and no expression in flowers (Fig. 3.12B) however, AtAGP9 indicates high expression only in the stem, young roots and seedlings, low expression in old roots and flowers and no expression in rosette leaves (Fig. 3.13B). Microarray-based expression profiles suggest AtAGP3 is specifically expressed in roots whereas AtAGP7 shows high expression in both stem and roots. Analysis of T-DNA mutants reveals interesting growth and phenotypic aberrations. T-DNA mutants for both AtAGP7 and AtAGP9 show defects in lateral root development. Also, atagp4 and atagp9 T-DNA mutants show abnormal responses to abiotic stress treatment.
Table 4.1 Summary of research on T-DNA mutants of four classical AGPs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>T-DNA insertion</th>
<th>ABRC Salk lines</th>
<th>Mutants</th>
<th>Phenotypes</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtAGP4</td>
<td>1000 bp promoter (467bp upstream ATG)</td>
<td>SALK_024865 (1000-P)</td>
<td>HM available</td>
<td>on plate: no root phenotype on soil: no phenotype G.assay: ionic, non-ionic stress resistant ABA stress resistant</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RT, ST, FL, RL, SL</td>
</tr>
<tr>
<td>AtAGP9(1)</td>
<td>Intron (ND)</td>
<td>SALK_010915 (intron)</td>
<td>HM n/a</td>
<td>Not analyzed</td>
<td>ND</td>
</tr>
<tr>
<td>AtAGP9(2)</td>
<td>Intron (ND)</td>
<td>SALK_060744 (intron)</td>
<td>HM available</td>
<td>on plate: No root phenotype</td>
<td>ND</td>
</tr>
<tr>
<td>AtAGP9(3)</td>
<td>Intron (851bp down-) (-stream ATG site)</td>
<td>SALK_008873 (intron)</td>
<td>HM available</td>
<td>on plate: decreased lateral roots G.assay: ionic, non-ionic stress resistant ABA stress resistant</td>
<td>ND</td>
</tr>
<tr>
<td>AtAGP9(4)</td>
<td>1000 bp promoter (ND)</td>
<td>SALK_149384 (1000-P)</td>
<td>HM n/a</td>
<td>Not analyzed</td>
<td>ND</td>
</tr>
<tr>
<td>AtAGP9(5)</td>
<td>1000 bp promoter (306bp upstream ATG site)</td>
<td>SALK_058644 (1000-P)</td>
<td>HM available</td>
<td>on plate: no root phenotype on soil: early flowering</td>
<td>ND</td>
</tr>
<tr>
<td>AtAGP7</td>
<td>Exon (146bp downstream ATG site)</td>
<td>SALK_147517 (Exon)</td>
<td>only HZ</td>
<td>Stunted growth</td>
<td>T1 lines available</td>
</tr>
<tr>
<td>AtAGP7</td>
<td>Exon (134bp downstream ATG site)</td>
<td>SALK_039285 (Exon)</td>
<td>HM available</td>
<td>on plate: germination problems on soil: delayed growth G.assay: ABA sensitive</td>
<td>T1 lines available</td>
</tr>
</tbody>
</table>

ABA, abscisic acid; bp: base pair; G.assay, germination assay; HM, homozygous; HZ, heterozygous; plate, HMqPCR: homozygous mutant quantitative polymerase chain reaction; ND, not determined; n/a: not applicable; P_{AtAGP::GUS} (promoter::GUS fusion); RT: root; ST: stem; FL: flower; RL: rosette leaves; SL: seedling; YR: young root; OR: old root.
In general, AtAGP4, AtAGP7 and AtAGP9 may play roles as cell surface receptors in abiotic stress signaling. Based on these studies various potential functional roles of AGPs may be drawn, roles of AtAGP4, and AtAGP9 in ABA and ionic and non-ionic stress signaling and seed germination; role for AtAGP7 in ABA stress signaling; role for AtAGP3 in overall root development; and a role for AtAGP9 in lateral root growth and development.
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Appendix A: Chemical cross-linking studies to examine interactions of arabinogalactan proteins (AGPs) with molecular components in the cell wall, plasma membrane and cytoskeleton
Abstract

Although AGPs are one of the most abundantly distributed glycoproteins on the plant cell surface, little information is available regarding their potential interactors and their mechanism of interaction. My dissertation work indicated possible molecular interactions of AGPs with cortical MTs and F-actin in BY-2 cells [Sardar HS, Yang J and Showalter AM. Plant Physiology 142: 1469-1479 (2006); Sardar HS and Showalter AM. Plant Signaling and Behavior 2(1): 8-9 (2007)], but direct evidence of such interaction or any other interactions is still lacking. Thus, this study was performed to examine specific interactions between AGPs and other molecular components using chemical cross-linking approach. Two homobifunctional cross-linkers, membrane permeable and impermeable cross-linkers, disuccinimidyl suberate (DSS) and disuccinimidyl tartarate (DST) were used to cross-link AGPs with other neighboring molecular components. Tobacco BY-2 cells were treated with DSS and DST for 30 min and total protein was extracted. Extracted proteins were separated on a SDS-PAGE and stained with coomassie blue or β-Yariv reagent. Based on the coomassie staining, the general migration pattern for protein samples from cells treated with DSS (but not with DST) showed a difference when compared to the untreated cells. However, β-glucosyl Yariv staining did not show a difference in size of the protein band but indicated a decrease in the staining intensity. These results indicate that some AGPs may be cross-linked into complexes too large to enter the gel, thereby preventing their detection. Overall, these cross-linking studies may help identify the molecular candidates that interact with AGPs.
Introduction

Currently, there are no simple and reliable *in vivo* methodologies to search for molecules interacting with AGPs due mostly to the complex nature of these glycoproteins. In the past, our studies have focused on investigating AGPs interactions with other molecules by using either fluorescent (GFP) or biochemical (Yariv) probes (Sardar et al., 2006). The assumption is that AGPs highly decorated with negatively charged O-glycans are likely to interact with positively charged components such as pectin. However, only a few studies have addressed such potential connections between pectin and AGPs (Baldwin et al., 1993; Carpita, 1989; O’Neill and Selvendran, 1985; Iraki et al., 1989; Serpe and Nothnagel, 1994; Shea et al., 1989).

Elucidation of AGP interactions and the interacting partners is an importance step toward understanding their role in plant growth and development. For example, an *in vitro* study on AGPs extracted from sugar beets showed that AGPs cross-link to themselves in the presence of hydrogen peroxide and catalase suggesting a role for plasma membrane localized AGPs in cell wall integrity (Kjellbom et al., 1997). One promising approach for elucidating AGP interactions involves using an *in vitro* chemical cross-linking strategy. Although several studies have shown general advantages in using homobifunctional cross-linkers to identify components of many multi-protein interaction complexes (Akita et al., 1997; Cox et al., 1990; Farries and Atkinson, 1989), no studies were carried out using this strategy to identify candidate AGP interactors.
A general principle is that reversibly cleavable homobifunctional cross-linkers are used to identify closely distributed macromolecules conjugated to their interacting partners. For example, a study in plants has successfully employed DSS and DST homobifunctional cross-linkers to identify interacting protein complexes involved in the import machinery of the chloroplast membranes (Akita et al., 1997). In animal studies, the principle behind using cross-linkers is that cross-linking results in conformational changes in the structure of the macromolecule thereby producing defects in biological activity (Cox et al., 1990; Knoller et al., 1991; Park et al., 1986).

DSP and DST have been used extensively in the past to perform cross-linking studies as membrane permeable cross-linkers (Ji, 1983; Staros and Anjaneyulu, 1989). To date, homobifunctional cross-linkers haven’t been used to perform studies with AGPs. Therefore, the rationale for using these homobifunctional cross-linkers was to allow cross-linking of primary amine groups and thereby facilitating protein-protein interaction studies between AGPs and other closely associated polypeptides. Also, the presence of the non-glycosylated Lys-rich region of the LeAGP-1 exposes the free amine groups of the Lysine residues and increases the potential of cross-linking via this region. In addition, the capacity of the glycan epitopes to interact with β-glucosyl Yariv reagent represents an additional advantage that may help in the success of the cross-linking strategy in investigating AGP interactions. Yariv reagent will not only selectively bind to carbohydrate epitopes of AGPs, but also can be a useful probe in addition to anti-AGP antibodies. In these studies, cross-linking of tobacco BY-2 cells in the presence of DSS
and DST homobifunctional cross-linkers resulted in a decrease in the number and the intensity of protein bands on the SDS-polyacrylamide gel electrophoresis (PAGE).

Material and methods

Cell cultures and growth conditions

Wild type tobacco BY-2 (Nicotiana tobaccum, Bright Yellow-2) and Arabidopsis cell lines were used to conduct the cross-linking studies. Cell cultures were grown in liquid MS media (Murashige and Skoog, 1962) [4.3 g/L Murashige and Skoog salts (Sigma, St. Louis, MO, USA), 30 g/L sucrose, 1 mg/mL thiamine HCl, 100 mg/L myo-inositol, 0.44 mg/L 2,4-dichlorophenoxyacetic acid, pH 5.8] in the tissue culture room (Porter 507). These cells were maintained at a temperature of 24°C on a rotary shaker (120 rpm) and subcultured weekly (1:10) into fresh culture media.

Total protein extraction

BY-2 cells (5d) were homogenized in an extraction buffer [50 mM Tris-HCl pH 7, 10mM potassium chloride, 1 mM EDTA, 0.1 mM magnesium chloride, 8% (w/v) sucrose and 1mM phenylmethanesulphonylfluoride (PMSF) that was added to the buffer just before grinding the cells]. The cells were ground using a polytron (model PTA 20S; Brinkmann, Westburg, NY) by giving three bursts of 15-20s. The homogenized samples were centrifuged for 5 min (1000 rpm, 4°C) to remove the cell debris. The soluble fraction was used for SDS-PAGE analysis.
**Chemical cross-linking reactions**

Cross-linking reactions were performed according to manufacturer’s instructions (Pierce Biotechnology, Inc. Rockford, IL) and also based on discussions with Dr. Ahmed Faik (Department of Environmental and Plant Biology, Ohio University). BY-2 cells (5-day old) were suspended in fresh NT-1 media. Cells were washed two times with fresh NT-1 media to remove cell debris and excess AGPs secreted into the culture media. Cells were suspended in PBS and washed two times with ice-cold PBS (pH 7.5). Cross-linkers prepared in dimethyl sulfoxide (DMSO) were incubated with BY-2 cells at a final concentration of 5%. A final concentration of 1mM or 3mM cross-linker solution was used for the reaction by adding 250μl (1mM or 3mM stock) to 2.25 ml of BY-2 cells (~25 x 106 cells/ml) in the reaction buffer (1 M PBS, pH 7.5 for disuccinimidyl tartarate and 1 M PBS, pH 8.0 for disuccinimidyl suberate). These reactions were incubated at room temperature (RT) and after 0 and 30 min incubation time, quenching buffer (1 M Tris/HCl pH7.5) was added to stop the reaction (RT). These cells were immediately used for total protein extraction. The cross-linking reagents, disuccinimidyl suberate (DSS) and disuccinimidyl tartarate (DST) were purchased from PIERCE (Pierce Biotechnology, Inc. Rockford, IL).

**SDS-PAGE**

SDS-PAGE was performed as described by Laemlli (1970) and Sambrook and Russel (2001) with minor modifications. Protein extracts were suspended in 2X Laemmli buffer (50μl of β-mercaptoethanol was added to 950 μl of Laemmli buffer to achieve a final
concentration of 5%) and then boiled for 15 min to denature the proteins. Boiled samples were then centrifuged at 3000 rpm for 2 min to remove insoluble materials and the supernatant was directly loaded onto 10% SDS-PAGE gels. Electrophoresis was performed using the Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad, Hercules, CA). Approximately 15 μg of protein sample per lane were loaded on SDS-PAGE gels. After protein separation, bands were visualized using coomassie staining and their sizes compared to the Precision Plus Protein All Blue Standards and low range Prestained Marker Standards (Bio-Rad, Hercules, CA).

Coomassie staining

SDS-PAGE gels were transferred to the petri plate containing 0.25 % of Coomassie brilliant blue R-250/100 ml of methanol acetic acid solution.(9:1): Staining was performed on a rotary shaker (80 rpm) with approximately 5 volumes of Coomassie stain for 4-8 h. Destaining was performed in 30% methanol, 10% acetic acid solution for 8-12 h changing the destaining solution 3-4 times. Gels were scanned on a EPSON PERFECTION 1450U color scanner and the images were processed using Adobe Photoshop (version 7.0).

Yariv staining

Protein gels were stained in 200 μM β-glucosyl Yariv solution containing 1% NaCl. The staining was performed overnight on a rotary shaker (80 rpm) at room temperature. The stained gels were washed with PBS (pH 7.5) for 24-28 h to remove excess β-Yariv
reagent and gels were scanned on a EPSON PERFECTION 1450U color scanner and the images were processed using Adobe Photoshop (version 7.0).

Results and Discussion

The working hypothesis was that AGPs interact with other molecular components located in the cell wall and/or at the plasma membrane. To identify potential interactors, chemical cross-linking was carried out on tobacco BY-2 suspension cultured cells using two homobifunctional chemical cross-linkers, DSS and DST. Both DSS and DST are water-insoluble with two N-hydroxysuccinimide ester (NHS esters) groups on both ends (homobifunctional) separated by spacer arm lengths of 11.4 Å and 6.4 Å respectively. These cross-linkers react with the primary amine groups found either on the N-terminus or on the side chains of amino acid residues such as Lys to form stable amide bonds. Absence of charged groups in these water insoluble cross-linkers make them lipophillic, thereby making them excellent cross-linkers to attach AGPs to intracellular or intramembrane interactors. The rationale behind using these cross-linkers is that most of the AGPs have lysine residues that can be sites for cross-linking AGPs to other proteins, thereby increasing the possibilities of identifying interacting partners. However, another possibility is that these Lys residues may be involved in a molecular interaction and not available for cross-linking to chemical cross-linkers.

In preliminary experiments, coomassie staining of proteins extracted from BY-2 cells treated with 1mM or 3mM DSS indicated decreased number bands and thereby
suggesting a difference in the migration pattern of proteins when compared to untreated control. However, DST, which has a short spacer arm (6.4 Å), did not show this difference in the migration pattern. Coomasie blue staining does not stain AGPs well, so β-glucosyl Yariv staining was employed to specifically monitor the migration pattern of AGPs after the cross-linking process. The protein extracts from tobacco BY-2 cells that were treated with DSS or DST showed no change in the size, but a marked decrease in the intensity of the stained band (~100kD), suggesting that less AGPs entered the gel (Fig. 2.10 and 2.11A). There are several possible explanations for the decrease in Yariv staining: 1) incomplete homogenization of cells may have resulted in less AGP extraction. This possibility is less likely to occur because the control cells were treated in a similar way and gave good results. 2) the cross-linking was successful thereby generating large size complexes that could not enter the gel during SDS-PAGE. It may be easy to verify this possibility by precipitating the complex with Yariv reagent and monitoring the purified samples on protein gel. 3) DSS and DST did cross-link AGPs to certain insoluble polymer/protein interactor in the cell wall resulting in loss of AGPs with the debris during centrifugation step. To remediate this problem, cell wall polymers may be treated with hydrolases (cellulase and pectinase) that may release “AGPs-interacting molecules” complexes that can be analyzed by staining on gels or in solution. Thus, more experimentation is required to confirm any of above suggested scenarios. Nevertheless, these preliminary results represent a starting point for optimization of the cross-linking strategy. These optimizations may include, but are not limited to, the following: The use
**Figure 1.** Coomassie-stained SDS-PAGE gel showing proteins extracted from 1 mM DSS and DST treated BY-2 cells. Tobacco BY-2 cells treated with 1 mM DSS and DST and separated on a 10% SDS-PAGE gel followed by staining with coomassie brilliant blue. Protein samples were extracted from cross-linked BY-2 cells at 0 (I₀, II₀, III₀) and 30 (I₃₀, II₃₀, III₃₀) min. I₀ and I₃₀ represents proteins from the DMSO treated control treatment. II₀ and II₃₀ represents proteins extracted from cells treated with 1 mM DSS. III₀ and III₃₀ represent the proteins extracted from cells treated with 1 mM DST. M, Marker lane shows the low range prestained SDS-PAGE standards.
Figure 2. SDS-PAGE gels showing proteins extracted from 3 mM DSS and DST treated BY-2 cells. Tobacco BY-2 cells treated with 3 mM DSS and DST and separated on a 10% SDS-PAGE gel followed by staining with either coomassie brilliant blue (A) or β-Yariv reagent (B). Cross-linked protein samples were extracted from BY-2 cells at 0 (I₀, II₀,
III₀) and 30 (I₃₀, II₃₀, III₃₀) min. I₀ and I₃₀ represents proteins from the DMSO treated control treatment. II₀ and II₃₀ represents proteins extracted from cells treated with 3 mM DSS. III₀ and III₃₀ represent the proteins extracted from cells treated with 3 mM DST. M, Marker lane shows the low range prestained SDS-PAGE standards.
of plasma membrane-enriched fractions instead of whole cells is one such optimization. This approach may provide further information regarding the membrane-associated partners. The use of protoplasts may also provide information on AGP interactions with intracellular molecules.

In addition, in order to avoid the problem associated with identification of distinct AGP bands and their size in protein gels stained with either β-Yariv reagent and coomassie stain, protein gel blots of plasma membrane extracts may be probed with carbohydrate AGP-specific monoclonal antibodies, JIM13 (recognizes β-D-GlcA-(1,3)-α-D-GalA-(1,2)-α-L-Rha), and MAC207 (recognizes α-GlcA-(1,3)-α-GalA-(1,2)-α-Rha) (Bradley et al., 1988; Knox et al., 1991; Pennell et al., 1989; Yates and Knox, 1994; Yates et al., 1996). Furthermore, to specifically probe the interactions of LeAGP-1, extracts from transgenic BY-2 cell lines expressing GFP-LeAGP-1 may be used for analysis. Homobifunctional chemical cross-linkers can be used to cross-link membrane and detergent-resistant membrane (DRM) fractions from this transgenic cell line. Studies have shown that DRMs are enriched in GPI-anchored proteins such as AGPs (Borner et al., 2005; Mongrand et al., 2004). Since LeAGP-1 is a GPI-anchored protein, its association with lipid rafts may be examined. Proteins gel blots could also be analyzed by using PAP and anti-GFP antibodies. Any difference observed in the banding patterns (decrease or increase in protein size) may be recorded and the protein sample can be trypsin digested and sent for LC-MS/MS analysis to identify the identity of components in the cross-linked protein complex.
Appendix B: Sequence information for \textit{AtAGP3}, \textit{AtAGP4}, \textit{AtAGP7} and \textit{AtAGP9}
**AtAGP3** (At4g40090)

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ATGGCTCTTAAAGACATTTGCAAGCTCTGATCTTTCTTGGTCTATTTGCGGCTCAAGCTCCGGCTCCGGCACCTATCACGTTTCTCCCTCCGGTAGAGTGCTCCTTCTCCA
GTTGTTACACAAACTGCAGAACCACCGGCTCGTTGCTTTCCAACCCCTATTTCCAGCCAACGA
ACCCACTCCGGTTCCAACAAGTCCTCCACCACCGTCTCAACCCCGGACCACGTGCCCAACACTCCCTGTCGTCTTCTCCTCC
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**Figure.1** Gene sequence of *AtAGP3* based on DNA sequencing. The T-DNA insertion site (146 bp downstream of ATG site) found in the HM line is highlighted here with red color and is underlined. The start site (ATG) and stop site (TAA) are also shown in blue color.
**AtAGP4** (At5g10430)

TTATGTAAACGTTAATCCATGATTATGGATTTATACCTAAAGAAAGCGACATATATAATAATT
ACATGTAAAGACTTCATCCGCCTAATAAGTGTATTTTTACTTTCCATATGTAACACTATAAT
TTTATAGGAATCTTGTTTTTTAAATTCTCACATATTCCATATTTTATATGCTGAGCTGCTGCA
CTACACTGTCCTCTGATGTTGGCTCTATTCGCCACTTCAGCACTCGCTCAAGCCCTG
CTCCTACTCCACCGCCACTCTCTCTCCGCAACTCCCCCTCCAGTCGCAACTCTCCCTCTC
CAGTGCTGCTACACCACTGCTACACCACTGCTACACCACTGCTACACCACTGCTACACCA
CCAGTCTCTCCACCGCCACTCGTCATCGTCTCTTCTCCTGCTGTATGCGGATGATGATGCG
GTTGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGATG

**Figure 2** Gene sequence of AtAGP4 based on DNA sequencing. The T-DNA insertion site (467 bp upstream of ATG site) found in the HM line is highlighted here with red color and is underlined. The start site (ATG) and stop site (TAA) are also shown in blue color.
**AtAGP7** (At5g65390)

\[
\begin{align*}
\text{ATG} & \quad \text{AATTCGAAGATCATCGAAGCTTTCTTCATCGTTGCTCTTTCACTACCTCATGCCT} \\
& \quad \text{CGCTCAAGCTCCAGCTCTCTTCTCCAACCACCACCGTCACTCTCTTCCGCCCTGCCTC}
\end{align*}
\]

\[
\begin{align*}
& \quad \text{CACCACCCCCGCGCAACCTCCCGCTCCGACCCACCTCACTCCACCACCTGCGTTCTCCTCCCTGC}
\end{align*}
\]

\[
\begin{align*}
& \quad \text{GCCACCCTCTTCCTCCGCTTCAGCTCTTTTCCGCCCTGCCTGTCCGATGCCTACCGCTTC}
\end{align*}
\]

\[
\begin{align*}
& \quad \text{TCCACCTGCAACCAGGAGTGCCCGAGTTAGCCCCGGCGAAGCTAGCCGGCGACACCTTCCG}
\end{align*}
\]

\[
\begin{align*}
& \quad \text{CTTCTGCCGCTCCTCCACCTAAGCCCGCTTTAACCACAAAGCTTTTCGTCGGGTCA}
\end{align*}
\]

\[
\begin{align*}
& \quad \text{CTTGTCCGCCCGATTATTTACGGCCTGTCTTGGTCTTAG}
\end{align*}
\]

**Figure.3** Gene sequence of *AtAGP7* based on DNA sequencing. The T-DNA insertion site (134 bp downstream of ATG site) found in the HM line is highlighted here with red color and is underlined. The start site (ATG) and stop site (TAA) are also shown in blue color.
**AtAGP9** (At2g14890)

AGCACGGCAAACCTCTCACCATGCCATGCGATTATTATTATTATACACACTTTACTAAAACCACTTCAATACGACGCATGTATCCGCGTTTTGATAATAATAGCTTCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

TCGTCCTCATCGCCGGCGTTACTGGTCAAGCTCCGACTTCACCACCACCCGCTACACCAGCTCCACCAACTCCACCAGCAGCAACTCCTCCTTCTGGTCTCGAAGCAGCAAGTAAAAATGCTCGATCTTTCGCTATTGCTGTGATCTGTA

Figure 4 Gene sequence of AtAGP9 based on DNA sequencing. The T-DNA insertion site for *atagp9(3)* and *atagp9(5)* mutant lines are highlighted here with red color (underlined) and green color (underlined) respectively. The start site (ATG) and stop site (TAA) are also shown in blue color.
Figure 5 Gene structures of atagp7, atagp4 and atagp9 showing the T-DNA insertional sites and primers used for quantitative real-time PCR (qRT PCR) analysis of WT and homozygous mutant lines. RNA from WT and atagp7 (A), atagp4 (B), atagp9(3) and atagp9(5) (C) were analyzed for relative transcript abundance. Arrows represent the forward and reverse primers used for qRT PCR analysis.
**AtAGP3** (139 aa)

1 MALKTLQALI FLGLFAASC L AQPAPAPIT FLPPVESSPS P VVTPTAEPPA
51 PVASPPIPAN EPTPVPTTPP TVSPPTTSPT TSPVASPPKP YALAPGPSGP
101 TPAPAPAPRA DGPDVAD **SALT NKAFLVSTVI AGALYAVLA**

**AtAGP4** (135 aa)

1 MGSKIVQVF L MLALFATSAL AQAPAPTPTA TPPPATPPPV ATPPPVATPP
51 PAATPA SATP PPAATPAPAT TPPSVAPSPA DVPAPAPAP EGPAPVSPSSA
101 PGPSD **ASPAP SAAFSNKAFF AGTAFAMY AA IVLA**

**AtAGP7** (130 aa)

1 MNSKIIEAFF IVALFTTSC L AQPAPSPTT TVTPPPVPATP PPAATPAPT
51 TPPPAVSPAP TSSPPSSAPS PSSDAPTASP PAEGPGVSP GELAPTSADA
101 SAPPPN **AALT NKAFVVGSLV AAI YAVLA**

**AtAGP9** (191 aa)

1 MARSFAIAVI CIVLIAGVTG QAPTSPPTAT PAPPTTTTPP PAATPPPVSA
51 PPPVTTISPPP VTTAPPANP PPPSVSSPPA SPPATPPPV ASPPPPVASP
101 PPPATPPPVAT PPPAPPLASPF AQVPAAPPTT KPDPSPSFS SPPPLFSSDA
151 PGPSTDSPSAP APTDVNDQ N **GASKMVSSL VFGSVLVWFMI**

**Figure. 6** Protein sequences of AtAGP3, AtAGP4, AtAGP7 and AtAGP9. The N-terminal signal peptide is represented in bold and C-terminal glycosylphosphatidylinositol anchor addition sequence is shown as italicized. Sequence data was retrieved from www.arabopsis.org.