FUNCTIONAL IDENTIFICATION OF THREE LYSINE-RICH ARABINOGLACTAN-PROTEINS (AGPS) IN ARABIDOPSIS

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Abstract

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FUNCTIONAL IDENTIFICATION OF THREE LYSINE-RICH ARABINOGLACTAN-PROTEINS (AGPS) IN ARABIDOPSIS (317 pp.)

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Arabinogalactan-proteins are a family of hydroxyproline-rich glycoproteins present on cell surfaces and function in plant growth and development. AtAGP17, 18 and 19 comprise the lysine-rich AGP subfamily in Arabidopsis and consist of an N-terminal signal peptide, a classical AGP domain interrupted by a small Lys-rich region and a C-terminal glycosylphosphatidylinositol anchor addition sequence. AtAGP17, 18 and 19 were examined in terms of expression, subcellular localization and function. AtAGP17, 18 and 19 have organ-specific expression patterns, as revealed by Northern blotting. Similarly, AtAGP18 and 19 promoter controlled β-glucuronidase activities were high in the vasculature and styles as well as young expanding organs. Microarray and massively parallel signature sequencing data showed largely consistent transcription profiles of these three AGP genes. On the protein level, these AGPs were most abundant in roots and flowers, moderate in stems, seedlings and siliques and low in rosette leaves. Using green fluorescence protein-AtAGP18/19 fusion proteins, AtAGP18 and 19 were localized to the plasma membrane and Hechtian strands in transgenic tobacco cultured cells. A null T-DNA knockout mutant of AtAGP19 was obtained and examined. Compared to wild
type plants, the \textit{atagp19} mutant had: 1) smaller, rounder and flatter rosette leaves, 2) lighter green color, 3) delayed growth and flowering, 4) fewer lateral roots, 5) shorter hypocotyls, 6) shorter and thinner inflorescence stems, 7) reduced secondary growth and 8) less seed production. Several abnormalities in cell size, number, shape and packing were also observed in the mutant. Complementation of this mutant with the wild type \textit{AtAGP19} gene restored the wild type phenotype and confirmed that \textit{AtAGP19} plays important roles in cell division and expansion, leaf formation, lateral root initiation, stem growth, vascular development and reproduction.

Approved:

Allan M. Showalter

Professor of Environmental and Plant Biology
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Molecular phenotypes

grew slower and had fewer lateral roots

Hypocotyl cell length in atagp19 was reduced

had reduced pigmentation

had altered leaf morphology

had shorter petioles

had normal venation

Delayed flowering in atagp19

Secondary growth was highly reduced in atagp19

showed distinctive stem phenotypes

Compromised fertility in atagp19

was more resistant to drought stress

Salt treatment

was more resistant to ABA

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List of Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

35S CaMV promoter: 35S cauliflower mosaic virus promoter

ABA: abscisic acid

ABRC: Arabidopsis Biological Resource Center

AG: arabinogalactan

AGP: arabinogalactan-protein

BAP: 6-benzylaminopurine

bp: base pair

BY-2: bright yellow-2

CSHL: Cold Spring Harbor Laboratory

d: day

DIC: differential interference contrast

dsRNA: double stranded RNA

ECM: extracellular matrix

EGFP: enhanced green fluorescence protein

EXT: extensin

FLAs: fasciclin-like AGPs

GA: gibberellin

GFP: green fluorescence protein
GPI: glycosylphosphatidylinositol

GT: glycosyltransferase

GUS: β-glucuronidase

h: hour

hpRNA: hairpin RNA

HRGP: hydroxyproline-rich proteins

Hyp-protein: hydroxyproline-containing protein

IAA: indole-3-acetic acid

LB medium: Luria broth medium

min: minute

miRNA: microRNA

MPSS: massively parallel signature sequencing

MS medium: Murashige and Skoog medium

MW: molecular weight

NAA: naphthalene acetic acid

NASC: Nottingham Arabidopsis Stock Centre

NOS terminator: nopaline synthase terminator

nt: nucleotide

PBS: phosphate buffered saline

PCD: programmed cell death
PCR: polymerase chain reaction
PM: plasma membrane
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
SEM: scanning electron microscopy
SH: Schenk and Hildebrandt medium
siRNA: short interfering RNA
TBS: Tris buffered saline
T-DNA: transfer DNA
TE: tracheary element
TPM: transcripts per million
TTS: transmitting tissue-specific
WAK: wall-associated kinase
WT: wild type
Chapter 1 INTRODUCTION
Hydroxyproline-rich glycoproteins (HRGPs)

HRGPs are a superfamily of proteins rich in proline (Pro), or more precisely, hydroxyproline (Hyp), the post-translationally hydroxylated form of Pro. Since their discovery (Lamport and Northcote, 1960; Lamport, 1967), HRGPs have been regarded as ubiquitous components of plant cell walls in addition to the complex network of polysaccharides. No animal homologs are available for structural and functional study of plant HRGPs, implying that Hyp-O-glycosylation of HRGPs may serve a specialized function in the plant kingdom (Kieliszewski and Shpak, 2001). Indeed, HRGPs are implicated in many aspects of plant growth and development (Showalter, 1993).

The plant HRGP superfamily can be divided into three major groups: proline-rich proteins (PRPs), extensins (EXTs) and arabinogalactan-proteins (AGPs). These families are not always distinct from one another; there have been various reports of HRGPs having characteristics of two families, such as hybrid AGPs (Kieliszewski et al., 1992b; Lind et al., 1994; Goodrum et al., 2000; Schultz et al., 2002). Instead, the HRGP superfamily should be viewed as a continuum of molecules with repetitive peptides and O-glycosylation. PRPs are basic and minimally glycosylated, while AGPs are acidic and highly glycosylated (Kieliszewski and Lamport, 1994; Sommer-Knudsen et al., 1998).

EXTs are basic glycoproteins containing characteristic repeat sequences, such as Ser-Hyp-Hyp-Hyp-Hyp. EXTs are highly glycosylated with short arabinosyl chains
(containing 1 to 4 arabinose) attached to the Hyp residues. Single galactosyl units can also be added to Ser residues (Kieliszewski and Lamport, 1994).

PRPs are characterized by peptide motifs such as Pro-Pro-X-Y-Lys, Pro-Pro-X-Lys or Pro-Pro-X-Y-Pro-Pro, where X and Y can be valine (Val), tyrosine (Tyr), histidine (His) and glutamic acid (Glu). PRPs contain approximately equal amounts of Pro and Hyp and are only minimally glycosylated with short arabinose side chains attached to some of the Hyp residues. Hydroxylation never occurs to the first Pro of a PRP repeat (i.e., Pro-Hyp-X-Y-Lys) proceeded by a Lys residue (Showalter, 1993; Sommer-Knudsen et al., 1998).

AGPs are the most heavily glycosylated members of the HRGP superfamily and the focus of this dissertation. AGPs are broadly distributed in the plant kingdom, from bryophytes to angiosperms (Fincher et al., 1983; Basile and Basile, 1987; Nothnagel, 1997). They play important roles in plant growth and development (Majewska-Sawka and Nothnagel, 2000; Showalter, 2001). AGPs also have commercial applications in industry as they are the major functional components of a number of plant gums or exudates that are used as additives in food, candy and stamp industries. In addition, AGPs have economical importance in medicine, because they can stimulate immune systems (Serpe and Nothnagel, 1999; Yamada and Kiyohara, 1999; Showalter, 2001).
Structure of AGPs

Protein backbones

AGPs usually contain only 1-10% protein moiety by weight, with the rest being carbohydrates. AGPs have biased amino acid compositions and divergent sequences. Protein backbones of AGPs are typically rich in Hyp/Pro, alanine (Ala), serine (Ser) and threonine (Thr). Ala-Hyp, Hyp-Ala and Ser-Hyp are common dipeptide motif repeats in AGPs (Showalter, 2001).

Classes of AGPs

AGPs are traditionally divided into two classes: classical and nonclassical AGPs (Figure 1.1), based on their protein moieties (Mau et al., 1995; Du et al., 1996). Classical AGPs are 85 to 151 amino acids in length and consist of an N-terminal signal peptide, a central AGP domain and a C-terminal hydrophobic domain (Gaspar et al., 2001; Showalter, 2001). The hydrophobic domain can be post-translationally replaced by a glycosylphosphatidylinositol (GPI) lipid anchor (Schultz et al., 1998; Youl et al., 1998). Lysine-rich AGPs constitute a sub-class of the classical AGPs and have a small Lys-rich region (containing approximately twelve amino acids) within the classical AGP domain.
Figure 1.1 Protein backbones of different classes of AGPs. Not drawn to scale.

(a) A classical AGP.
(b) A Lys-rich classical AGP.
(c) A non-classical AGP.
(d) An AG peptide.
(e) A FLA. This FLA has a fasciclin domain and an AGP region. FLAs can also contain two fasciclin domains or two AGP regions.
Seven Lys-rich AGPs from different plant species have been identified to date: LeAGP1 in tomato *Lycopersicon esculentum* (Li and Showalter, 1996; Gao *et al*., 1999), NaAGP4 in *Nicotiana alata* (Gilson *et al*., 2001), AtAGP17, 18 and 19 in *Arabidopsis thaliana* (Schultz *et al*., 2002; Sun *et al*., 2005), CsAGP1 in cucumber *Cucumis sativus* (Park *et al*., 2003) and PtaAGP6 in pine *Pinus taeda* (Zhang *et al*., 2003).

Nonclassical AGPs contain AGP domains as well as domains that are atypical of AGPs, such as Asn-rich (Mau *et al*., 1995), Cys-rich (Du *et al*., 1996) and His-rich (Kieliszewski *et al*., 1992b) domains. A Hyp-deficient AGP was also isolated from carrot *Daucus carota* (Baldwin *et al*., 1993). Nonclassical AGPs have signal peptide sequences, but not C-terminal hydrophobic domains; therefore, they are not predicted to be GPI-anchored.

AG peptides and fasciclin-like AGPs (FLAs) represent recent additions to the AGP classification system. AG peptides are distinguished by their small AGP domains, which are typically only 10-15 amino acids in length; these AGP domains are flanked by a signal peptide and in many cases by a GPI-anchor addition sequence. FLAs contain fasciclin-like domain(s) in addition to AGP domain(s), and in most cases, a GPI anchor (Schultz *et al*., 2000; Borner *et al*., 2002; Borner *et al*., 2003; Johnson *et al*., 2003). Among the 50 putative *Arabidopsis* AGPs identified to date, there are 14 classical AGPs, 3 Lys-rich AGPs, 12 AG peptides and 21 FLAs (Schultz *et al*., 2002; Borner *et al*., 2003).

Besides FLAs, other chimeric AGPs, composed of small AG glycomodules (small
Pro-, Ala-, Ser- and Thr-rich units) in addition to other protein domains, also exist (Schultz et al., 2002; Borner et al., 2003). Xylogens in Zinnia elegans and Arabidopsis contain both an AGP domain and a nonspecific lipid transfer protein domain (Motose et al., 2004). In rice, two chimeric AGPs, an early nodulin-like protein and a lipid transfer-like protein, were recently identified (Mashiguchi et al., 2004). Finally, there are also hybrid AGPs containing AGP motifs and other HRGP (e.g., extensin) motifs (Qi et al., 1991; Schultz et al., 2002).

Molecular shape of AGPs

Two models, the “wattle blossom” model (Fincher et al., 1983) and the “twisted hairy rope” model (Qi et al., 1991), describe the molecular shape of AGPs (Figure 1.2). The “wattle blossom” model proposes that AGPs are spheroidal with large arabinogalactan (AG) polysaccharides folding into globular units and decorating the core protein (Fincher et al., 1983). In contrast, in the ‘twisted hairy rope’ model, AG chains and oligoarabinoses wrap around the protein backbone, generating a rod-like shape. Both models were based upon electron microscopic imaging of AGP molecules and were supported by experimental evidence. For example, a Hyp-poor carrot AGP and a tobacco Nicotiana tabacum transmitting tissue-specific (TTS) protein, which was identified as an AGP, had the “wattle-blossom” structure (Baldwin et al., 1993; Cheung et al., 1995). On
Figure 1.2 Two models for molecular shape of AGPs.
(From Showalter, 2001).
Top, the “wattle blossom” model.
Bottom, the “twisted hair” model.
the other hand, the gum arabic glycoprotein (GAGP) from *Acacia senegal* displayed a rod-like shape, supporting the “twisted hairy rope” model (Qi *et al.*, 1991).

AGPs observed by electron microscopy had a strong tendency to polymerize into large aggregates (Baldwin *et al.*, 1993; Cheung *et al.*, 1995). This aggregation of AGPs is consistent with their adhesiveness.

**Post-translational modifications of AGPs**

AGPs undergo complex and extensive modifications during and after their translation, including signal peptide removal, Pro hydroxylation, Hyp glycosylation and GPI anchor addition (Gaspar *et al.*, 2001).

AGPs, like other secreted proteins, contain N-terminal signal peptides. After the signal peptides are synthesized on ribosomes, AGPs are co-translationally inserted into the endoplasmic reticulum (ER) and have the signal peptides cleaved off.

*Prolyl hydroxylation*

During AGP biosynthesis, Pro residues are hydroxylated to form Hyp residues. This post-translational modification of AGPs occurs in the ER after processing of the signal peptide.

Not all prolines are modified to hydroxyprolines; in most classical AGPs, 80 to 90%
of Pro residues are hydroxylated. For example, in LeAGP1 and AtAGP17, 95% and 83% of Pro residues are hydroxylated, respectively (Zhao et al., 2002; Sun et al., 2005).

With detailed analysis of known extensins and other HRGPs, hydroxylation predictions can be made by examining neighbouring amino acids of Pro residues. Motifs in which Pro residues are always hydroxylated include Ala-Pro, Pro-Ala, Pro-Pro, Pro-Val and Ser-Pro. There are also motifs in which prolines are not hydroxylated, such as Lys-Pro, Tyr-Pro and Phe-Pro (Kieliszewski and Lamport, 1994). These rules attempt to provide a simple solution for prediction of Pro hydroxylation sites, and exceptions to the rules have been reported. For example, when synthetic repetitive sequences (Ser-Pro-Pro-Pro)n and (Val-Pro-Val-Pro)n were expressed in tobacco suspension-cultured bright yellow-2 (BY-2) cells, various degrees of incomplete prolyl hydroxylation were observed, suggesting that side chains of flanking amino acid might influence Pro hydroxylation, and that tobacco prolyl hydroxylase has low affinity for tripeptidyl prolines (Shpak et al., 2001; Tan et al., 2003).

3,4-dehydroproline (DPro) is a proline analog and an inhibitor of prolyl hydroxylase (Cooper and Varner, 1983). It has been used to elucidate the functional significance of HRGPs by blocking proline hydroxylation of HRGPs (Fowden et al., 1963; Cooper, 1984; Basile et al., 1985; Schmidt et al., 1991).
Glycosylation

Carbohydrate moiety

AGPs are the most highly glycosylated proteins in nature; carbohydrates compose up to 99% of AGPs by weight. Arabinose and galactose are the two major carbohydrate components in AGPs, along with other sugars of less abundance. Type II AG polysaccharides (ranging from 30 to 150 sugar residues) constitute the major carbohydrate moiety in AGPs and consist of a highly branched β-D-(1-3)-galactopyranose backbone with β-D-(1-6)-galactan side chains containing arabinose, rhamnose, mannose, xylose, glucose, fucose, glucosamine, glucuronic acids and galacturonic acid (Showalter, 2001). The first structure of a Hyp-AG polysaccharide was experimentally determined (Tan et al., 2004). Besides AG polysaccharides, short arabinose oligosaccharides also decorate some Hyp residues in AGPs (Showalter, 2001).

O-glycosylation

Hyp residues of AGPs are O-glycosylated, and O-glycosylation is likely to initiate in the ER and finish in the Golgi (Gaspar et al., 2001). The Hyp-contiguity hypothesis predicts amino acid sequence-driven glycosylations of AGPs (Kieliszewski et al., 1992a;
Kieliszewski and Lamport, 1994; Kieliszewski et al., 1995). In this hypothesis, AG polysaccharides are attached to clustered and non-contiguous Hyp residues, and arabinose oligosaccharides decorate contiguous Hyp residues.

Sugar analyses of naturally occurring AGPs as well as synthetic AGP gene products have supported this hypothesis (Kieliszewski et al., 1995; Shpak et al., 1999; Kieliszewski and Shpak, 2001; Shpak et al., 2001; Zhao et al., 2002; Tan et al., 2003; Sun et al., 2005). Enhanced green fluorescence protein (EGFP) was fused to several AGPs to facilitate selection of transgenic tobacco cell cultures and detection and purification of the fusion proteins (Shpak et al., 1999; Shpak et al., 2001). Synthetic genes composed of single simple repetitive glycomodules were useful for examining sequence-based glycosylation. The first test of the Hyp contiguity hypothesis was with the (Ser-Pro)$_n$ motif; all non-contiguous Hyp residues turned out to be decorated with AG polysaccharides (Shpak et al., 1999). A related test with (Ser-Hyp-Hyp)$_n$ and (Ser-Hyp-Hyp-Hyp-Hyp)$_n$ exhibited exclusive arabinosylation to contiguous Hyp residues (Shpak et al., 2001). Hyp glycoside profiles from two purified Lys-rich AGPs, namely LeAGP1 and AtAGP17, were also consistent with predictions based on the Hyp contiguity hypothesis (Zhao et al., 2002; Sun et al., 2005). Tan et al. (2003) showed that (Ala-Pro-Ala-Pro)$_n$ was 100% hydroxylated and arabinogalactosylated. In the same study, (Thr-Pro-Thr-Pro)$_n$ and (Val-Pro-Val-Pro)$_n$ had 20% to 30% Pro residues that were not hydroxylated; approximately 40% to 50% of the Hyp residues remained not glycosylated.
or only arabinosylated. These results indicated that Pro hydroxylation and Hyp glycosylation were influenced by flanking amino acids. Arabinosylation or non-glycosylation of non-contiguous Hyp was reported earlier (Kieliszewski et al., 1995).

Refining the Hyp contiguity code involves defining both noncontiguous Hyp clusters (how many intervening residues permit arabinogalactosylation) and the influence of flanking residues on hydroxylation and subsequent glycosylation (Tan et al., 2003). The distance between two non-contiguous Hyp residues that allows for addition of AG polysaccharides was found to be 5 amino acids in LeAGP1 (Zhao et al., 2002).

N-glycosylation

Unlike O-glycosylation, the consensus sequence for N-glycosylation is defined: Asn-X-Ser/Thr (X stands for any amino acid). However, since AGPs are extensively O-glycosylated, little attention has been given to N-glycosylation in AGPs. Nonetheless, N-glycosylation exists in some AGPs, such as tobacco TTS1 and TTS2 as well as GaRSGP (Cheung et al., 1993; Sommer-Knudsen et al., 1996). Other AGPs, PtX14A9 (Loopstra and Sederoff, 1995), LeAGP1 (Li and Showalter, 1996) and CsAGP1 (Park et al., 2003) also contain potential N-glycosylation sites. N-glycosylation in these and other AGPs remains to be demonstrated.
GPI anchor processing

GPI-anchored proteins have been identified in animals, protozoa and yeast (McConville and Ferguson, 1993; Udenfriend and Kodukula, 1995). In plants, many classical AGPs, AG peptides and FLAs are potentially GPI-anchored (Borner et al., 2002; Borner et al., 2003; Schultz et al., 2002). For example, among 248 predicted GPI-anchored proteins in *Arabidopsis*, 29 (12%) are classical AGPs or AG peptides, and 14 (6%) are FLAs. Overall, 100 (40%) GPI-anchored proteins in *Arabidopsis* contain AG glycomodules (Borner et al., 2003).

Possession of a GPI anchor by some AGPs are experimentally confirmed by biochemical analysis (Youl et al., 1998; Oxley and Bacic, 1999; Sherrier et al., 1999; Svetek et al., 1999; Borner et al., 2003; Sun et al., 2004b). In particular, the GPI anchor addition site was determined for several AGPs, such as NaAGP1 from *Nicotiana alata*, PcAGP1 from pear *Pyrus communis* (Youl et al., 1998) and AtAGP10 from *Arabidopsis* (Schultz et al., 2000).

The GPI anchor core structure is conserved in animals, yeast, protozoa and plants, as revealed by the structural analysis of the GPI anchor in PcAGP1 (Oxley and Bacic, 1999). This GPI anchor has a minimal core oligosaccharide that is consistently found in animal, protozoa and yeast: D-Manα(1-2)-D-Manα(1-6)-D-Manα(1-4)-D-GlcN-inositol (Figure
1.3). Approximately 50% of PcAGP1 GPI anchors are substituted with $\beta$-(1-4)-galactose at the third mannose, which is distinct from GPI anchors in animals, yeast and protozoa. The ceramide lipid of the PcAGP1 GPI anchor is embedded in the PM and is composed primarily of phytosphingosine and tetracosanoic acid, which is also found in yeast and rose AGPs (Oxley and Bacic, 1999; Svetek et al., 1999).

GPI anchor processing probably takes place in the ER (Youl et al., 1998). The AGP is anchored to the luminal face of the ER membrane and then the Golgi membrane. Presumably, GPI-anchored AGPs are secreted to the outer leaflet of the plasma membrane (PM) via vesicular transport.

A PM-bound AGP can be further processed by endogenous phospholipase C or D (PLC or PLD) and hence released from the PM to cell wall or extracellular destinations (Figure 1.3b). Interestingly, in some AGPs (e.g., LeAGP1, NaAGP4 and AtAGP17, 18 and 19), an entire exon encodes the consensus region for GPI anchor addition (Li and Showalter, 1996; Gilson et al., 2001; Schultz et al., 2002), indicating this may be an important functional domain (Showalter, 2001).

The C-terminal hydrophobic region serves as the signal for GPI anchor addition. When the predicted GPI anchor addition sequence of LeAGP1 was fused to an endoglucanase reporter protein, the protein was targeted to the cell surface; PLC treatment released the endoglucanase from the cell surface, confirming the presence of a GPI anchor in the engineered protein. On the other hand, when the predicted cleavage site
Figure 1.3 GPI anchor structure.
(From Oxley and Bacic, 1999).
(a) Structure of GPI anchors from animals, protozoa and yeast.
R groups indicate possible substituents. R1 is usually α-Man, R2 is ethanolamine phosphate, R3 is mono-, oligo-, poly-saccharides, or ethanolamine phosphate, and R4 is palmitate.
(b) Structure of the GPI anchor from PcAGP1. The ethanolamine-phosphate and the core oligosaccharide structure of this GPI anchor are common to all GPI anchors. The GPI anchor has a partial galactosyl substitution of the third mannose and includes a phosphoceramide lipid. Potential sites of cleavage by phospholipase C (PLC) and phospholipase D (PLD) are indicated by arrows.
for LeAGP1 GPI anchor processing was missing, the fused endoglucanase was not GPI-anchored (Takos et al., 2000).

In general, the C-terminal GPI anchor addition sequence (15-31 residues) contains small aliphatic amino acids at \( \omega, \omega+1 \) and \( \omega+2 \) sites, followed by a short spacer region (7-10 residues, rich in hydrophilic amino acid) and ends with a hydrophobic region (8-20 amino acids) (Figure 1.4). The protein C-terminus, after the \( \omega \) residue, is removed. The \( \omega \) residue, the site of GPI anchor addition, is usually Ser, Asn, Ala, Gly, Asp, or Cys, whereas the \( \omega+2 \) residue is Ala, Gly, Thr, or Ser. The \( \omega+1 \) residue is less critical (Udenfriend and Kodukula, 1995; Showalter, 2001). Several computer programs allow for prediction of GPI anchor attachment sites in proteins, such as PSORT (Nakai and Horton, 1999) and big-PI (Eisenhaber et al., 2003).

**AGP probes**

Two types of AGP probes, Yariv phenylglycosides and antibodies, are most commonly used, with which, scientists have accumulated many data on distribution and biological function of AGPs.

Synthetic Yariv reagent reacts specifically with AGPs to form a reddish-brown color (Yariv et al., 1962; Yariv et al., 1967). Operationally, AGPs can be defined as Yariv-reactive molecules (Kreuger and van Holst, 1996; Mashiguchi et al., 2004),
Figure 1.4 GPI anchor processing.
(From Showalter, 2001).
Consensus sequence in the C-terminus of AGPs directs addition of a GPI anchor. The amino acid residue designated $\omega$ is the site of GPI anchor addition, and the remaining C-terminal residues are removed. A spacer region follows before the terminal hydrophobic tail.
although a small proportion of AGPs are unreactive with Yariv (Lind et al., 1994; Smallwood et al., 1996). The three terminal sugars on Yariv are critical for AGP binding. Certain sugars, such as (β-D-galactosyl)$_3$ and (β-D-glucosyl)$_3$, allow for AGP binding, while (α-D-galactosyl)$_3$ and (β-D-mannosyl)$_3$ do not. AGP binding to Yariv reagents can be employed to detect AGP distribution in plant extracts or tissue sections, to precipitate AGPs for purification and quantification and to perturb their function. Yariv reagents that do not react with AGPs typically serve as negative controls in function-probing experiments.

Important progress in characterizing AGPs has also been made with AGP antibodies. Most antibodies recognize the carbohydrate epitopes on AGPs; the JIM and MAC series are examples of the commonly used monoclonal antibodies. These antibodies are extremely useful for probing organ-, tissue- and cell-specific expression and glycosylation of AGPs sharing common epitopes, but they are not able to discriminate among individual AGPs (Gao et al., 1999). Another drawback about these antibodies is that they may cross-react with other macromolecules bearing the same sugar epitopes, such as pectin (Kreuger and van Holst, 1996; Showalter, 2001).

A limited number of antibodies against AGP protein backbones are also available, allowing for the analysis of individual AGPs. PAP and anti-AGPB are antibodies against the Lys-rich regions of LeAGP1 and PtaAGP6, respectively. These antibodies are feasible because the Lys-rich region, unlike the extensively glycosylated AGP domain, is not
glycosylated (Gao et al., 1999; Gao and Showalter, 2000; Zhang et al., 2003). Other protein-specific antibodies are directed against FLA domains (Shi et al., 2003; Ito et al., 2005), deglycosylated core proteins (Lind et al., 1994; Sommer-Knudsen et al., 1996) or bacterially expressed core protein (Wang et al., 1993). Summarization of various antibody probes for AGPs and their epitopes is presented in Showalter (2001).

**Distribution of AGPs**

Yariv reagent, AGP antibodies, biochemical purification, molecular cloning, hybridization, RT-PCR, expressed sequence tag (EST), and more recently, microarray and massively parallel signature sequencing (MPSS) analyses represent the major tools to examine AGPs from the plant level to the cell level. These tools are employed either individually or in combinations. For instance, on light microscopy and electron microscopy levels, the JIM series of antibodies as well as the PAP and anti-AGPB (the antibodies against the Lys-rich regions of LeAGP1 and PtaAGP6, respectively) antibodies localized AGPs in developing xylem elements (see next section). Besides immunolocalization, several AGP cDNAs were cloned from differentiating xylem of loblolly pine (*Pinus taeda*) and characterized. Most of these AGP genes were highly and preferentially expressed in differentiating xylem, contributing evidence to support the hypothesis that AGPs are important for xylem development (Loopstra and Sederoff, 1995;
AGPs are present in all lower and higher plants studied to date (Jermyn and Yeow, 1975; Fincher et al., 1983; Basile and Basile, 1987; Nothnagel, 1997; Lee et al., 2005). They exist on the PM (Komalavilas et al., 1991; Sherrier et al., 1999; Borner et al., 2003), in periplasm (Zhao et al., 2002; Sun et al., 2004b; Sun et al., 2005; Lamport et al., 2006), in cell walls (Jermyn and Yeow, 1975; Basile and Basile, 1987; Schopfer, 1990; Li et al., 1992), in the extracellular secretions (Clarke et al., 1979b; Gleeson and Clarke, 1980; Wu et al., 2001) and intracellular vesicles (Herman and Lamb, 1992). They also accumulate in the media of cultured cells (Komalavilas et al., 1991; Smallwood et al., 1996; Gao et al., 1999; Lamport et al., 2006). Subcellular localization of AGPs determined by immunolocalization was confirmed by subcellular fractionation as well as isolation and characterization of AGPs (Sherrier et al., 1999; Borner et al., 2003; Sun et al., 2004b).

AGPs are expressed in different organs including seedlings, leaves, stems, roots, flowers and seeds (Clarke et al., 1979a; Fincher et al., 1983; Nothnagel, 1997). On the tissue level, AGPs are particularly abundant and well documented in xylem, style and tissue cultures.

The expression and glycosylation of AGPs is organ- and tissue-specific and developmentally controlled. For example, LeAGP1 is preferentially expressed in young organs (Pogson and Davies, 1995; Li and Showalter, 1996). Different sets of AGPs are found in different organs and tissues (Kreuger and van Holst, 1996; Majewska-Sawka
and Nothnagel, 2000) at different developmental stages (Kreuger and van Holst, 1993; van Hengel et al., 2002; Qin and Zhao, 2006); they can also have specific glycosylation patterns (Wu et al., 1995; Gao and Showalter, 2000) and stage-dependent expression levels (Lu et al., 2001; Qin and Zhao, 2006). The temporally- and spatially-controlled presence of AGPs can presage and hence are implicated in developmental changes and pattern formation (Knox et al., 1989; Pennell and Roberts, 1990; Knox et al., 1991).

More expression patterns of AGPs are discussed below in the context of their inferred function. Organ- and tissue-specific and developmental expression of AGPs revealed by antibodies and RNA gel blot is reviewed by Showalter (2001).

With increasing availability of AGP genetic sequences, RT-PCR (Pereira et al., 2006), Northern blotting (Schultz et al., 2000), in situ hybridization (Acosta-Garcia and Vielle-Calzada, 2004; Motose et al., 2004; Ito et al., 2005; Dahiya et al., 2006) and microarray experiments are often utilized to reveal AGP expression on the cell, tissue and organ levels in WT and mutant plants. Without bench work, expression profiles of AGPs can also be obtained by searching public EST, microarray and MPSS databases in Arabidopsis as well as a few other plant species (Schultz et al., 2002; Zhang et al., 2003; Zimmermann et al., 2004; Faik et al., 2006; Nakano et al., 2006).

In addition, AGP expression is responsive to biotic and abiotic stress, including wounding, pathogen invasion, heat shock and phytohormones. Some AGPs, including LeAGP1, NaAGP4, the carrot Hyp-poor AGP, PvPRP1 and gum arabic, change their
expression levels upon wounding and/or pathogen infection and/or heat shock (Clarke et al., 1979a; Sheng et al., 1991; Pogson and Davies, 1995; Li and Showalter, 1996; Baldwin et al., 2000; Gilson et al., 2001). Moreover, LeAGP1 is upregulated by cytokinin (Sun et al., 2004a); whereas CsAGP1 is upregulated to gibberellin (GA) and auxin (Park et al., 2003).

**Function of AGPs**

Emphasis in AGP research has shifted increasingly from characterizing their biochemistry to deciphering their function. Widespread expression, abundance and heterogeneity of AGPs suggest that AGPs have vital and multiple roles *in planta.* Extensive research and accumulating evidence implicate AGPs in various aspects in plant growth and development, such as morphogenesis, xylem differentiation, programmed cell death, somatic embryogenesis, reproduction, cell proliferation, cell expansion, cell adhesion and signaling. However, their exact roles and mode of action is still elusive. Six most widely suggested roles of AGPs are elaborated in the following.

Several major tools have been used to elucidate AGP function. Initially, function of AGPs was largely inferred from tissue-specific and developmentally-controlled localization of AGP epitopes, either collectively or individually. A correlation, instead of a causality relationship, between expression and function was obtained in these types of
studies. Cloning, biochemical isolation and characterization of AGPs (i.e., from specialized plant structures or as active components of physiological processes) also contributed to functional identification of AGPs. A third research approach utilizes Yariv phenylglycosides that selectively bind AGPs and perturb their function, such as β-D-glucosyl and β-D-galactosyl Yariv reagents. Exposing plants and suspension-cultured cells to these Yariv induces morphological and behavioral changes in plants and cultured cells and sheds light on the function of AGPs. Yariv phenylglycosides that do not interact with AGPs, such as α-D-galactosyl and β-D-mannosyl, are often included in such treatments as negative controls. Notably, this type of analysis is based on the assumption that effects of Yariv reagent derive from its functional disturbance of AGPs; moreover, such effects cannot be attributed to individual AGPs but rather to sets of expressed AGPs. Over the past few years, AGP mutants have been generated with reverse genetics techniques and represent a powerful and direct means to assign function to AGPs.

*Pattern formation and vascular development*

AGPs were proposed to be cell position markers and function in vascular development, based on their temporally- and spatially-regulated expression patterns correlated with pattern formation and xylem differentiation. For example, the JIM4
antibody bound to provascular tissues in the carrot embryo (Stacey et al., 1990) and vascular tissues in carrot seedlings and mature plants. In the carrot root apex, the JIM4 epitope specifically reacted with two segments in the vascular cylinder, reflecting the future pattern of vascular differentiation (Knox et al., 1989). In oilseed rape Brassica napus, the JIM8 epitope was expressed in prelignified xylem vessels and 13 reproduction-related cell types. It was speculated that transient expression of the JIM8 epitope in xylem vessels after wall thickening but before lignification might be related to vasculature patterning (Pennell et al., 1991). Another monoclonal antibody, JIM13, labeled cells of the future xylem axis in the carrot root apex as well as developing xylem cells (Knox et al., 1991; Casero et al., 1998). The JIM13 epitope was present in xylem vessel elements at the earliest stages of differentiation in Arabidopsis roots (Dolan and Roberts, 1995); in contrast, it spread from protoxylem to metaxylem in radish roots, corresponding to xylem maturation (Casero et al., 1998). In maize Zea mays coleoptiles, the JIM13 and JIM14 epitopes were restricted to future sclerenchyma and tracheid cells undergoing secondary cell wall thickening and programmed cell death (PCD), a process tightly coupled to tracheary element (TE) differentiation (Schindler et al., 1995; Fukuda, 1997). Other evidence suggesting the involvement of AGPs in PCD included decreased viability or induced PCD of suspension-cultured cells exposed to Yariv (Langan and Nothnagel, 1997; Gao and Showalter, 1999; Chaves et al., 2002).

The above studies rendered valuable information on expression of groups of AGPs
sharing same carbohydrate epitopes; specialized localization of individual AGPs was
analyzed with antibodies designed against AGP protein backbones (such as unique
Lys-rich regions or fasciclin-like domains). LeAGP1 was localized to differentiating
metaxylem and secondary xylem TEs in tomato roots and stems from the initiation of cell
wall thickening to the end of differentiation (Gao et al., 1999; Gao and Showalter, 2000).
Likewise, PtaAGP6 was expressed in pine stem tracheids immediately before and during
secondary cell wall thickening (Zhang et al., 2003). Besides PtaAGP6, several other
AGPs were cloned from differentiating xylem of loblolly pine, suggesting developmental
roles for these pine AGPs in wood formation (Loopstra and Sederoff, 1995; Loopstra et
al., 2000; Zhang et al., 2000).

With the recent discovery of FLAs, evidence connecting FLAs to differentiating
xylem is also accumulating. AtFLA11 was identified as the major transcript in
Arabidopsis inflorescence stems; its genetic and protein expression was specific in the
stem and silique sclerenchyma cells, suggesting a role in secondary cell wall formation
(Ito et al., 2005). Two other studies highlighted AtFLA11 as a gene co-regulated with
secondary cell wall cellulose synthase genes, also implicating it in secondary wall
formation (Brown et al., 2005; Persson et al., 2005). A possible interaction between
AtFLA11 and the cellulose synthase complex in the context of lipid rafts was then put
forward, consistent with the report that Yariv inhibited cellulose deposition on cultured
tobacco protoplasts (Vissenberg et al., 2001). More interestingly, ZeFLA11, a homologue
of AtFLA11, was specifically expressed in differentiating xylem elements with reticulate type wall thickenings but not spiral thickenings in Zinnia stems (Dahiya et al., 2006).

Xylem-specific or -preferential localization of AGPs only correlates with vascular development; however, xylogen, a chimeric AGP, directly promoted vascular differentiation. Xylogen mediates local intercellular communication necessary for TE differentiation of Zinnia mesophyll cells. When Zinnia mesophyll cells were embedded in microbeads of agarose gels at a low- or high-density, and microbeads of both cell densities were cultured in the same liquid medium, TE differentiation was suppressed remarkably in the low-density microbeads compared to high-density microbeads. Addition of conditioned medium from Zinnia cells undergoing TE differentiation greatly increased the frequency of TE differentiation in low-density microbeads. Xylogen was purified as the active ingredient from the conditioned medium and characterized to be an AGP (Motose et al., 2001). In Zinnia stems, xylogen was expressed in procambium and immature xylem cells. In particular, it was directionally localized to the apical side of differentiating treachery elements, possibly bringing neighboring cells into differentiation and a vascular network. Furthermore, a double xylogen mutant in Arabidopsis (atxyp1 atxyp2) had discontinuous leaf venation patterns (Motose et al., 2004).
Somatic embryogenesis

Somatic embryogenesis is the process in which somatic cells in tissue culture become embryogenic and develop complete embryos. It is established that AGPs are required for somatic embryogenesis.

Initially, it was found that carrot suspension-cultured cells secreted different sets of AGPs, depending on the age of the culture. Moreover, addition of mature carrot seed AGPs reinitiated embryogenic cell formation in a weakly embryogenic line. It was hence suggested that AGPs directed cell development during somatic embryogenesis (Kreuger and van Holst, 1993). When AGP fractions were isolated from carrot and tomato seeds using monoclonal antibodies ZUM15 and ZUM18, ZUM15 AGP fractions prevented embryogenesis, while ZUM18 fractions enhanced formation of embryos. On the other hand, AGPs containing both ZUM15 and ZUM18 epitopes had no effect on embryogenesis, leading to the speculation that biological activities of AGPs were determined by the presence of embryogenesis-promoting and -inhibiting epitopes, which can be present on separate or the same AGPs (Kreuger and van Holst, 1995). When the monoclonal antibody JIM8 was used to label AGPs in cell walls of carrot suspension-cultured cells, a correlation emerged between cell embryogenic potential and presence of the JIM8 epitope. JIM8(+) cells were embryogenic, and JIM8(-) were not able to form somatic embryos on their own. However, addition of JIM8(+) cells or
conditioned medium of JIM8(+) cells or only soluble signal molecules released by JIM8(+) culture could induce somatic embryo formation in JIM8(-) cells (McCabe et al., 1997).

Furthermore, carrot AGPs contained glucosamine and N-acetylglucosamine and were substrates of endochitinases. Endochitinase-mediated cleavage of AGPs appeared important for the function, as chitinase-treated AGPs were more active in inducing embryo formation (van Hengel et al., 2001). In a subsequent study, it was established that AGP epitopes expressed in immature carrot seeds were developmentally regulated, the capacities of AGPs to promote somatic embryogenesis are dependent on developmental regulation of AGP composition (van Hengel et al., 2002). The requirement of AGPs for embryo development has also been reported for other plant organisms, such as spruce (Egertsdotter and von Arnold, 1995), Cichorium (Chapman et al., 2000) and rape (Tang et al., 2006).

Reproductive growth

AGPs were first suggested to aid in plant reproduction as an adhesive base on stigma surface for pollen attachment and recognition, because they are abundant in the style and stigma exudates, and they are sticky (Clarke et al., 1979b; Gleeson and Clarke, 1980). A series of immunolocalization studies demonstrated that expression of specific AGP
epitopes were precisely regulated during plant development. In pea, the MAC207 antibody reacted with all cells in vegetative meristems, primordia and organs as well as throughout undeveloped flower buds, but not with certain cells in developing stamens and carpels. Therefore, variations in the MAC207 epitope expression presaged sexual development (Pennell and Roberts, 1990). An opposite expression pattern was obtained with the PM JIM8 epitope in oilseed rape, which was almost exclusively expressed in the early embryo and certain cell types in the floral sexual organs, including the endothecium and sperm cells, the nucellar epidermis, synergid cells and the egg cell (Pennell et al., 1991). These observations suggested that AGPs might serve as cell surface markers or regulate differentiation of certain tissue systems or control developmental changes.

AGPs have long been known to be important for pollen tube tip growth. MAC207 and JIM8 monoclonal antibodies labeled AGPs in ungerminated *Arabidopsis* pollen grains (van Aelst and van Went, 1992). AGPs were detected at pollen tube tips of lily *Lilium longiflorum* and *Annona cherimola*, but not tomato *Lycopersicon pimpinellifolium*, *Aquilegia eximia* or tobacco. The immunolocalization data corresponded to inhibition of pollen tube growth by Yariv reagent; Yariv stained pollen tube tips and only inhibited pollen tube growth of lily and *Annona cherimola*, but not the other three species. Removal of Yariv reagent allowed new tip growth from the flanks of arrested tips, which could be predicted by the site of AGP secretion that might initiate polarized growth of the new tip (Jauh and Lord, 1996; Mollet et al., 2002). Although virtually no AGPs were
detected in tobacco pollen tube tips (Li et al., 1992; Li et al., 1995; Mollet et al., 2002), AGP epitopes were found along the callosic walls of pollen tubes.

Pollen grains germinate on the stigma, and the tubes grow in the extracellular matrix (ECM) along the transmitting tract of the style to fertilize the egg in the ovary. In addition to the presence of AGPs in pollen, AGPs constitute a predominant class of proteins in the ECM of the stylar transmitting tract of many plant species, possibly to aid in pollen-pistil interactions (Hoggart and Clarke, 1984; Cheung and Wu, 1999). TTS proteins represent such AGPs enriched in the ECM of the tobacco stylar transmitting tissues they could attract, stimulate and nourish pollen tube growth (Cheung et al., 1995; Wu et al., 1995). Intriguingly, TTS proteins exhibited a gradient of increasing glycosylation from the stigma to the ovary, coincident with directional growth of pollen tubes in the style and suggesting a guidance role for TTS proteins in pollen tubes (Wu et al., 1995). AGPs virtually identical to tobacco TTS proteins were isolated from Nicotiana alata stylar transmitting tissues. TTS proteins have an extremely broad spectrum of glycosylation levels, and sugar modifications significantly affected their biochemical properties (Wu et al., 2000).

Aside from the above aspects in plant reproductive growth, AGPs are also implicated in female gametogenesis, fertilization, zygotic division and early embryogenesis. AtAGP18, a Lys-rich AGP, appears essential for female gametogenesis, as functional megaspores in AtAGP18 RNA interference (RNAi) mutants failed to enlarge
and divide, resulting in ovule abortion and reduced seed set (Acosta-Garcia and Vielle-Calzada, 2004). By using the JIM13 antibody and Yariv reagent on tobacco in vitro zygote and ovary culture systems, AGPs seemed to be required for fertilization of egg cells. After fertilization, AGPs were asymmetrically distributed in the zygote and likely to establish and maintain a polarity that is crucial for asymmetric zygotic division (Qin and Zhao, 2006).

The last part of plant reproductive growth involves shredding of floral organs, siliques and seeds. Recently, an Arabidopsis AG peptide, AtAGP24, was functionally associated with abscission of Arabidopsis floral organs and was regulated by an upstream signal IDA (INFLORESCENCE DEFICIENT IN ABSCISSION). It was suggested that this AG peptide could either function as a signaling molecule or indirectly regulate breakdown and remodeling of abscission zone cell walls through xyloglucan endotransglycosylase/hydrolase activities (Stenvik et al., 2006).

Cell division

Multiple lines of evidence suggest that AGPs regulate cell division, starting from a series of investigations on morphogenesis in leafy liverworts, which indicated that AGPs suppressed cell proliferation under normal conditions. Antagonizing normal Hyp-containing protein (Hyp-protein) biosynthesis in liverworts induced growth of
otherwise suppressed primordia and hence increased leaf and branch development (Basile, 1979; Basile and Basile, 1980; Basile et al., 1985; Basile, 1990). A morphoregulatory role of Hyp-proteins was proposed (Basile, 1980), but the nature of the Hyp-protein responsible for these changes was unknown. Subsequent studies narrowed down to AGPs as the candidates with altered Hyp content and restricting cell proliferation under normal conditions (Basile and Basile, 1990). Reinforcing morphoregulatory roles of AGPs, cesium chloride gradient analysis of AGPs extracted from cell division-suppressed or -desuppressed liverwort plants revealed that desuppressed plants released high buoyant density AGPs into the culture medium (Basile and Basile, 1993).

Opposite to the above studies, others reported inhibition of plant and cell growth upon disturbing synthesis or function of AGPs. For example, prevention of prolyl hydroxylase activity (Cooper and Varner, 1983) impaired growth of mung-bean radicles (Fowden et al., 1963) and a soybean Glycine max cell culture (Schmidt et al., 1991).

Dr. Nothnagel’s group provided more evidence for AGPs’ involvement in cell division with β-D-glucosyl Yariv phenylglycoside that specifically binds AGPs (Serpe and Nothnagel, 1994; Langan and Nothnagel, 1997). These investigators found that Yariv reagent inhibited growth of “Paul’s Scarlet” rose (Rosa sp.) cultured cells in a dose-dependent manner, with complete inhibition achieved at 50 µM. Negative controls, α-D-galatosyl and β-D-mannosyl Yariv phenylglycosides that do not bind AGPs, had no inhibitory effects on cell growth. Inhibition of cell growth was caused by suppression of
cell division, but not cell expansion or cell viability (Serpe and Nothnagel, 1994).

Analyses of two “Paul’s Scarlet” rose suspension culture lines, Rosa 57 and Rosa 93, correlated their different responses to β-glucosyl Yariv with their different AGP complements. Rosa 57 cells accumulated more AGPs in the conditioned culture medium, while Rosa 93 cells accumulated more cell wall-associated AGPs. Although the growth of both cell lines was inhibited by β-glucosyl Yariv reagent, only viability of Rosa 93 was severely compromised. A cell cycle analysis of Rosa 57 cells upon Yariv perturbation showed that these cells were not preferentially arrested in the S, G2 and M phases, leaving G1 and random points in the cell cycle as two possible sites for cell division arrestment (Langan and Nothnagel, 1997).

When somatic embryogenesis of carrot cultured cells was studied, a more direct although subtle piece of evidence for AGPs’s involvement in cell division was discovered. AGPs promoted cell division in a population of non-dividing carrot protoplasts by two to three fold (van Hengel et al., 2001).

**Cell expansion**

Zhu et al. (1993) compared levels of PM and extracellular AGPs in salt-adapted and unadapted tobacco cultured cells and found that salt-adapted cells accumulated less of both PM and secreted AGPs relative to normal cells. Salt-adapted cells also displayed a
reduced rate of cell expansion. The correlation between loss of AGPs and less cell wall extensibility suggested that AGPs might aid in cell expansion (Zhu et al., 1993). This was in line with a previous speculation that AGPs function as a cell-wall-loosening factor in auxin-mediated cell extension to facilitate sliding between cell wall polymers (Schopfer, 1990). In this earlier study, deposits of AGPs were identified in the growth-controlling outer epidermal wall of maize coleoptiles. Furthermore, AGPs were able to increase the activity of xyloglucan endotransglycosylases and thus impact the assembly or properties of the cell wall (Takeda and Fry, 2004).

In contrast to results of Zhu et al. (1993), Lamport et al. (2006) reported remarkable upregulation of total AGP as well as soluble/released AGP production by salt-stressed BY-2 cells, although downregulation of PM-bound AGPs was also observed, confirming the connection between PM-bound AGPs and growth rate. A dynamic flux model of AGP movement was proposed in this study: AGPs move from PM to periplasm to cell wall and then to growth medium. Two putative roles of classical AGPs were also discussed. First, periplasmic AGPs may stabilize the PM which experiences high internal hydrostatic pressures. Second, AGPs may function as pectic plasticizers that loosen the pectic network in the cell wall to increase wall extensibility, which may be required by secondary cell wall formation in xylem (Lamport et al., 2006).

Involvement of AGPs in cell expansion was also inferred from β-glucosyl Yariv treatments of Arabidopsis seedlings and suspension-cultured cells. Cell elongation
decreased at the root apex of treated seedlings, leading to reduced root growth and extensive radial expansion of root epidermal cells (Willats and Knox, 1996). Similar growth and morphological effects of Yariv were also observed in a separate study and phenocopied the *Arabidopsis reb1* mutant that contained less and altered AGPs (Ding and Zhu, 1997). Bulging epidermal cells upon Yariv treatment were also described for rape embryos (Tang et al., 2006). When Yariv was applied to carrot and tobacco cultured cells, cell elongation was prevented (Willats and Knox, 1996; Vissenberg et al., 2001). More specifically, reduced L-fucose content of AGPs in a *mur1* mutant had adverse impacts on *Arabidopsis* root cell elongation (van Hengel and Roberts, 2002).

FLAs are candidates involved in cell-cell adhesion. A mutation in *AtFLA4/SOS5* in *Arabidopsis* not only increased the plant’s sensitivity to salt, but also resulted in irregular cell expansion, thinner walls and reduced middle lamella (Shi et al., 2003). More recently, RNAi of *AGP1* in moss *Physcomitrella patens* led to reduced cell length, indicating *AGP1* is indispensable for normal apical cell extension in moss (Lee et al., 2005).

**Cellular signaling**

Six models are proposed for molecular interactions and signaling mediated by AGPs (Figure 1.5) (Showalter, 2001). In the first model, carbohydrate components of AGPs provide an interactive molecular surface in biological processes involving AGPs.
Figure 1.5 Models for AGP involvement in cellular signaling and cell adhesion. (From Showalter, 2001).
(a) Carbohydrate epitopes (small squares) released from AGPs bind to PM receptors to initiate intracellular signaling.
(b) AGPs present ligand molecules (diamonds) to appropriate PM receptors to induce signaling.
(c) Interaction between AGPs on one cell and PM receptors on an adjacent cell.
(d) Signaling by the free GPI anchors following cleavage of GPI-anchored AGPs.
(e) AGPs act as cell adhesion molecules and form PM-cell wall connections essential for normal growth and development. AGPs may interact with other cell wall molecules (black lines) such as pectin.
either as processed polymers or released oligosaccharides, could participate in signaling (Figure 1.5a) (Nothnagel, 1997). More specifically, AGPs are thought to be signaling molecules in somatic embryogenesis (McCabe et al., 1997). Consistent with this idea, sugars were dissociated from AGPs in conifer Pinus caribaea somatic embryogenic cultures, possibly by chitinase activity (Domon et al., 2000). It was also found that processing of AGPs by endochitinases in carrot cultures increased somatic embryogenesis (van Hengel et al., 2001). In the area of plant reproduction, the correlation between the gradient of AGP glycosylation and directional growth of pollen tubes also suggests a signaling role for the carbohydrate moiety of AGPs (Wu et al., 1995). Moreover, sugar modifications of AGPs can significantly affect their biochemical properties (Wu et al., 2000).

Another possible mechanism by which AGPs function in signaling arose from the discovery of GPI anchors in AGPs: AGPs may play biological roles in signal transduction pathways by mechanisms similar to GPI-anchored proteins in animals (Schultz et al., 1998). Supporting this idea, the GPI anchor of LeAGP1 was indispensable for its normal function (Sun et al., 2004a). In animals, a GPI anchor confers the following characteristics to the protein: 1) polarized localization in the PM; 2) association with lipid rafts (microdomains); 3) increased lateral mobility in the lipid bilayer; 4) regulated released from the PM by phospholipases; 5) recycling from the PM (Hooper, 1997; Simons and Ikonen, 1997; Kooyman et al., 1998; Muniz and Riezman, 2000).
Knowledge of GPI-anchored proteins in animals may lead to a better understanding of the function of GPI-anchored AGPs *in planta*. GPI-anchored proteins in animals are associated with signaling; they can bind to extracellular ligands and initiate intracellular signaling pathways. However, GPI-anchored proteins alone, lacking a transmembrane domain, apparently are not capable of reaching the cytoplasm to transduce the signal. Theories suggesting protein-protein interaction between GPI-anchored proteins and TM receptors were put forward and experimentally corroborated. CD14, a GPI-anchored receptor (Wright *et al.*, 1990) forms protein complexes with TM receptors (i.e. Toll-like receptors TLR2 and 4) to relay signals into the cell (Poltorak *et al.*, 1998; Shimazu *et al.*, 1999; Schmitz and Orso, 2002; Triantafilou and Triantafilou, 2005; Finberg and Kurt-Jones, 2006). Surprisingly, CD14 also co-localized with activated TLR3 receptor on lysosomes and enhanced its signaling; in this specific case, extracellular localization of CD14 was not required for its function (Lee *et al.*, 2006). Figure 1.6 summarizes interaction between CD14 and TLR2-4. CD14 brings TLR receptors on the PM in proximity to Src kinases and G proteins that are enriched in lipid rafts to amplify signaling transduction. Additionally, CD14 facilitates ligand (i.e., phosphatidylinositol) uptake into the cell, and this internalization stimulates ligand-mediated signaling cascades (Wang and Munford, 1999; Dunzendorfer *et al.*, 2004; Lee *et al.*, 2006).

AGPs were co-localized with wall-associated kinases (WAKs) at the PM in some immunofluorescence studies (Gens *et al.*, 2000). WAKs have both extracellular and
Figure 1.6 CD14 is a multifunctional adapter protein. (From Finberg and Kurt-Jones, 2006).

CD14 is a GPI-anchored protein that is expressed on the surface. It is associated with src family kinases and heterotrimeric G proteins in lipid rafts. CD14 can bind bacterial lipopolysaccharide and viruses on the cell surface. CD14 interacts with various TLRs to induce cytokines after virus or bacterial infection. The TLR pathway subsequently induces cytokine production through the adaptor proteins MyD88, Mal (also known as TIRAP), and/or Trif and TRAM. CD14 can also bind dsRNA intracellularly and interact with TLR3, which is not expressed on the cell surface.
cytoplasmic domains (Kohorn, 2000; Anderson et al., 2001). Therefore, it is possible that AGPs mediate ligand binding and signal transduction of receptor kinases, including WAKs, through protein-protein interaction (Figure 1.6b).

A third putative mode of action for GPI-anchored AGPs is that they function through the GPI portions following phospholipase cleavage (Figure 1.6c). Protein-free GPI has the ability to generate intracellular messengers (i.e., phosphatidyl-inositol, inositol phosphoglycan and ceramides) upon phospholipase cleavage (Schultz et al., 1998).

A fourth model depicts a hypothetical role for AGPs in intercellular signaling. Here, AGPs may interact with AGPs secreted by or receptor molecules expressed on adjacent cells to promote cell-cell communication (Figure 1.6d). Contactin, a GPI-anchored protein in neurons follows this model. Interaction between contactin with a receptor tyrosin phosphatase on neighbouring cells is essential for cell adhesion and neurite outgrowth (Peles et al., 1995; Peles et al., 1997).

The last model presented here (Figure 1.6e) suggests involvement of AGPs in cell adhesion necessary for normal plant growth and development. In plant cells, adhesion between the PM and cell wall is likely essential, and AGPs may be the connection between the PM, cell wall and ECM. LeAGP1 and AtAGP17 were localized to the PM and Hechtian strands, which are connections between the PM and cell wall (Sun et al., 2004b; Sun et al., 2005). Also, preferred localization and clustering of GPI-anchored
proteins in lipid rafts may stabilize adhesion (Harris and Siu, 2002). Another piece of evidence came from Yariv-induced PCD study in *Arabidopsis* and tobacco BY-2 cultured cells (Gao and Showalter, 1999; Chaves *et al.*, 2002). Yariv can induce abnormal aggregation of AGPs and break up essential AGP connections between and within cells. This is likely to resemble cases in animals, where disrupting PM-ECM connections results in PCD (Showalter, 2001).

Identification of fasciclin domain(s) in FLAs further implicates the involvement of AGPs in cell adhesion (Schultz *et al.*, 2000). Although sharing low sequence similarities, FLA fasciclin domains contain highly conserved H1 and H2 regions (approximately 10 amino acids each) that are characteristic of all fasciclin domains. These domains function as cell-cell adhesion molecules without clearly understood mechanisms (Elkins *et al.*, 1990; Huber and Sumper, 1994; Kawamoto *et al.*, 1998). It was proposed that by forming networks via ionic interactions between fasciclin domains, secreted FLAs in the cell wall control the expansion rate, while GPI-anchored AGPs maintain PM integrity during cell expansion (Vissenberg *et al.*, 2001; Johnson *et al.*, 2003). This speculation was supported by the mutant analyses of sos5, an EMS mutant of *AtFLA4* (Shi *et al.*, 2003). This idea that AGPs stabilize the PM-cell wall interface and regulate cell expansion was re-emphasized by Lamport *et al.* (2006).

Analyses of knockout, knockdown and overexpression mutants of some AGPs have implicated them in hormone signaling pathways. AtAGP30, a nonclassical AGP
containing six cysteines (Cys) in the C-terminus enhances the plant’s response to abscisic acid (ABA) and is required for root regeneration and seed germination (van Hengel and Roberts, 2003). Besides AtAGP30, two Lys-rich AGPs also respond to phytohormones. CsAGP1, a Lys-rich AGP in cucumber, is responsive to GA and implicated in stem elongation based on its endogenous expression pattern and overexpression in tobacco plants (Park et al., 2003). LeAGP1, a tomato Lys-rich AGP, is upregulated by cytokinin, and its overexpression in transgenic tomato results in plants that phenotypically resemble cytokinin-overexpressing plants. Specifically, they are short, highly branched, and produce extremely small seeds. LeAGP1 is thus hypothesized to function in the cytokinin signaling pathway, perhaps, as a co-receptor (Sun et al., 2004a). One incomplete knockout mutant of AtAGP17, designated as ratl (resistance to Agrobacterium), is deficient in binding of Agrobacterium to its roots in water and sucrose solutions (Nam et al., 1999). Further characterization of the mutant indicates that AtAGP17 might regulate Agrobacterium-binding by reducing free salicylic acid levels through signal transduction, which possibly involves the GPI anchor (Gaspar et al., 2004).

**Significance and specific aims of this dissertation research**

Plants are essential for sustainable development of the human society. As noted at the beginning of this chapter, AGPs have important industrial applications being critical
components of valuable plant gums. Despite rapid progress in AGP research, the exact function of AGPs and their underlying mechanisms of action remain poorly understood, especially at the time when my dissertation work started. For example, since the cloning of a tomato Lys-rich AGP LeAGP1 in the lab, its gene and amino acid sequences, expression patterns, subcellular localization, glycosyl composition and linkage, Hyp glycosyl profile and presence of a GPI anchor were characterized (Pogson and Davies, 1995; Li and Showalter, 1996; Gao et al., 1999; Gao and Showalter, 2000; Zhao et al., 2002; Sun et al., 2004b), but its function was not defined. In order to extend the lab’s long-term interest in Lys-rich AGPs and complement previous work with LeAGP1, it was decided to determine the function of three Lys-rich AGPs, namely AtAGP17, 18 and 19, in the genetic model plant Arabidopsis using genetics approaches (Schultz et al., 2002). Revealing the function of these three Lys-rich AGPs in Arabidopsis will provide a basis and guidance for understanding Lys-rich AGPs in other plant species, such as LeAGP1, as well as other classes of AGPs.

Arabidopsis (Figure 1.7) is extensively studied by plant biologists as a genetic model plant given that it has the following advantages (AGI, 2000). First, Arabidopsis behaves like many crop plants. So Arabidopsis research can facilitate the study of commercially important higher plants and enable scientists to readily apply their research findings to improve crop performance and production. Second, the Arabidopsis genome is relatively small and simple to study. It was completely sequenced in 2000, which
Figure 1.7 Anatomy of Arabidopsis.
(Adapted from Genevestigator [https://www.genevestigator.ethz.ch/]).
Arabidopsis at seedling and flowering stages are shown with selected organs and tissues labeled. These organs and tissues will be discussed in subsequent chapters of this dissertation.
significantly facilitates reverse genetics and functional genomics. Third, *Arabidopsis* is inexpensive and easy to grow and produces a large quantity of seeds. This, in combination with its rapid life cycle of approximately six weeks, allows extensive molecular and genetic experiments. Fourth, *Arabidopsis* plants can be transformed efficiently by *Agrobacterium tumefaciens* to carry out genetics studies. Fifth, the availability of many biological resources, such as collections of insertional mutants and microarray data, greatly facilitate *Arabidopsis* research.

Functional elucidation of the Lys-rich AGPs in *Arabidopsis* should provide valuable information on the function of other AGPs; more importantly, it should further our understanding of how plants grow and develop. Discovering the genes and processes underlying *Arabidopsis* growth and development will be of great help in studying crop plants and in eventually producing beneficial transgenic crops.

Plant growth and development is under the concerted control of genes and environmental cues. Development of *Arabidopsis* can be divided to two major phases: vegetative growth and reproductive growth. The transition between vegetative growth and inflorescence development is under the influence of various environmental signals, such as day length, temperature, watering and spacing. However, under standard lab growth conditions, flowering time of *Arabidopsis* can be invariant and therefore can be studied. Indeed, a clear correlation exists between the number of days to flower and the number of leaves produced under certain conditions. Thus, flowering time can not only
be defined in terms of plant age, it can also be defined in terms of leaf number (Koornneef et al., 1991; Howell, 1998; Weigel and Glazebrook, 2002).

Plant growth and development is a highly-ordered process consisting of cell division and cell expansion events. In plants, cell division involves formation of a cell plate, which is not seen in animal cells. Cell expansion is also unique in plants due to the plant cell walls. Proteins in the cell wall, such as expansins and wall-modifying enzymes xyloglucan endotransglycosylases/hydrolases, loosen the polysaccharide wall network to allow for cell expansion (Cosgrove, 2005).

Initiation of a lateral root starts from a series of cell division in the primary root pericycle. When the lateral root primordium has the same cell layers as the primary root, cell expansion helps the lateral root to emerge from the primary root. After that, cell division in the lateral root, like in the primary root, only takes place in the root tip (Howell, 1998). Leaf formation of Arabidopsis, likewise, is a result of both cell division and cell expansion. For example, during the exponential increase of the Arabidopsis first leaf blade area, cell division in the abaxial epidermis is dominant at first. Subsequently, however, the cell division rate drops dramatically, and cell expansion occurs and contributes to the increase in the blade area. Cell division and cell expansion, therefore, contribute to different stages of leaf growth (De Veylder et al., 2001; Autran et al., 2002). Arabidopsis growth and development is an extremely complex and intricate process, which is difficult to describe in a comprehensive manner in only a few pages. In
subsequent chapters of this dissertation, brief descriptions of the relevant developmental and physiological processes associated with the Lys-rich AGPs are presented and discussed as appropriate.

The research on AGP function and other aspects of AGP biology should deepen and broaden the understanding of biological processes in plant growth and development and provide new avenues to investigate their precise mechanism of action in these processes. In the mean time, application of AGPs to produce genetically engineered crops as well as new or improved plant products such as gum additives is expected to be beneficial and profitable.

To better understand the three Lys-rich AGPs in *Arabidopsis* as well as *Arabidopsis* growth and development, my dissertation research included the following three specific aims:

1. Characterize the expression patterns of *AtAGP17, 18* and *19* on the organ, tissue and cellular levels as well as their subcellular localization, which should shed light on their putative biological roles;

2. Employ the RNA interference (RNAi) technique to specifically silence each of the three AGP genes and observe phenotypes of RNAi plants;

3. Examine insertional mutants of *AtAGP17, 18* and *19* for mutant phenotypes and infer biological roles of these three Lys-rich AGPs.
Chapter 2 EXPRESSION AND LOCALIZATION OF

AtAGP17, 18 AND 19
Summary

AtAGP17, 18 and 19 comprise the lysine-rich AGP subfamily in *Arabidopsis* and consist of an N-terminal signal peptide, a classical AGP domain interrupted by a small Lys-rich region and a C-terminal GPI anchor addition sequence. Organ- and tissue-specific expression and subcellular localization of these three AGPs were studied and compared to Lys-rich AGPs in other species. The *AtAGP17* transcript was found in seedlings, rosette leaves, flowers and stems, but not roots. *AtAGP19* mRNA was abundant in stems, moderate in flowers and roots and low in leaves. Similar to *AtAGP19*, *AtAGP18* was expressed in roots, flowers and stems and weakly expressed in seedlings and rosettes. In addition, high promoter activities of *AtAGP18* and 19 were found in the vascular tissues and styles as well as young expanding organs. Microarray and massively parallel signature sequencing (MPSS) data showed largely consistent transcription profiles of these three AGP genes. On the protein level, AtAGP17, 18 and 19 were most abundant in roots and flowers, moderate in stems, seedlings and siliques and low in rosette leaves. Furthermore, AtAGP18 and 19 were localized to the PM and Hechtian strands, adhesion sites between the PM and the cell wall, following plasmolysis of tobacco BY-2 cells expressing EGFP-AtAGP18/19 fusion proteins. Localization of AtAGP18 on the PM was further confirmed by biochemical two-phase fractionation and Western blotting. Finally, expression and inferred function of AGPs is discussed.
Introduction

Seven Lys-rich AGPs have been identified from different plant species: LeAGP1 (Li and Showalter, 1996; Gao et al., 1999) in tomato, NaAGP4 in *Nicotiana alata* (Gilson et al., 2001), AtAGP17, 18 and 19 in *Arabidopsis* (Schultz et al., 2002; Sun et al., 2005), CsAGP1 in cucumber (Park et al., 2003) and PtaAGP6 in pine (Zhang et al., 2003). The information on these AGPs is summarized in Table 2.1. While the consensus intron splicing site for most genes contains the sequence GT…AG, *AtAGP19* has a single intron with a non-consensus intron splicing site: GT…AT. Recently, AtAGP17 has been annotated to have two splice variants, At2g23130.1 and At2g23130.2 (Figure 2.1). Analyses in this dissertation were performed on At2g23130.1 that contains 2 exons and 1 intron. At2g23130.2 has only 1 exon and no intron. Since the second exon in *AtAGP17* encodes the GPI anchor addition sequence, AtAGP17 encoded by At2g23130.2 lacks a GPI anchor.

*LeAGP1* is a major Lys-rich AGP from tomato. It is strongly expressed in flowers and young stems, moderately expressed in roots and young fruits and weakly expressed in leaves, mature fruits and old stems. Its transcription is downregulated by wounding and heat shock and does not change in response to light (Li and Showalter, 1996).

LeAGP1 and PtaAGP6 are characterized on the protein level. PAP antibodies and anti-AGPB antibodies were raised against peptides corresponding to the unique Lys-rich
Table 2.1 Molecular information on the Lys-rich AGPs identified to date.

<table>
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<th>Gene</th>
<th>Accession number</th>
<th>Organism</th>
<th>mRNA size (bp)</th>
<th>Protein size (aa)</th>
<th>Protein ID</th>
</tr>
</thead>
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<td>At2g23130.1</td>
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<td>185</td>
<td>AAP40426</td>
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<td>162</td>
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<tr>
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<td>209</td>
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</tr>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<td>Nicotiana alata</td>
<td>1279</td>
<td>228</td>
<td>AAG24616</td>
</tr>
</tbody>
</table>

In an accession number for an Arabidopsis gene, the number immediately after “At” indicates the chromosome the gene is present on.
Figure 2.1 Two splice variants of AtAGP17 (At2g23130).

(a) Two splice forms of AtAGP17: At2g23130.1 has two exons, while At2g23130.2 has only one.
Nucleotides in yellow background are common to At2g23130.1 and At2g23130.2 exons. Sequence in the red background is unique to At2g23130.2 exon. White background indicates the intron. Blue background indicates the second exon of At2g23130.1.

(b) Alignment of the proteins encoded by two splice variants of AtAGP17.
“*” indicates identical amino acids. “:” indicates strongly conserved residues. “.” indicates weakly conserved residues.
subdomains (non-glycosylated portions of sugar-coated AGPs) of LeAGP1 (PAPAPSKGKVGGKGGKHNA) and PtaAGP6 (HHRKKKKKKH), respectively. These antibodies immunolocalize these two AGPs to differentiating xylem elements and functionally associate them with secondary cell wall thickening and xylem differentiation (Gao and Showalter, 2000; Zhang et al., 2003). LeAGP1 is also abundant in stylar transmitting tissues, with putative roles of guiding and nourishing pollen tube growth (Gao et al., 1999). Western analysis reveals an organ- and tissue-specific expression pattern of LeAGP1 with respect to amount and molecular weight (MW)/glycosylation. Consistent with RNA blotting analysis, LeAGP1 protein accumulation is high in young fruits, flowers, roots and stems and low in leaves (Gao and Showalter, 2000).

-CsAGP1- is a GA-responsive gene, and the increase in transcription in response to GA is restricted to the apical portion of cucumber hypocotyls. CsAGP1 mRNA levels also enhance in presence of exogenous auxin indole-3-acetic acid (IAA). CsAGP1 is expressed throughout cucumber seedlings, including shoot apices, cotyledons, hypocotyls and roots (Park et al., 2003).

NaAGP4 was isolated from styles of Nicotiana alata. As the first ortholog reported for LeAGP1, NaAGP4 and LeAGP1 share high sequence identity. Expression of NaAGP4 is also similar to LeAGP1 and is suppressed by wounding and pathogen infection (Gilson et al., 2001).

Schultz et al. (2002) employed bioinformatics to identify 47 putative AGP genes in
the *Arabidopsis* genome based on their biased amino acid compositions. Among these 47 AGP candidates, there were 13 classical AGPs, 10 AG peptides, 3 Lys-rich AGPs and 21 FLAs, many of which had been reported previously (Schultz *et al*., 2000; Gaspar *et al*., 2001). A novel classical AGP and two new AGP peptides were later added to the list. Moreover, among 248 predicted GPI-anchored proteins in *Arabidopsis*, 100 (40%) contain AG glycomodules, but only 29 (12%) of them are classical AGPs or AG peptides (Borner *et al*., 2003).

Of the three and apparently only three Lys-rich AGPs in *Arabidopsis*, only AtAGP17 has been experimentally demonstrated to be a *bona fide* AGP. Like LeAGP1, AtAGP17 is localized to the PM and Hechtian strands. By characterizing an EGFP-AtAGP17 fusion protein from culture media of transgenic BY-2 cells, AtAGP17 has a biased amino acid composition typical of AGPs, with 84% Pro residues being hydroxylated to Hyp; AtAGP17 contains 86% carbohydrate dry weight, with arabinose and galactose being the two major glycosyl residues; AtAGP17 is precipitated by Yariv reagent; 81% Hyp residues in AtAGP17 are modified by AG polysaccharides, and 12% by oligoarabinoses; and finally, AtAGP17 has glycosyl linkages characteristic of AGPs, with the branched 1,3,6-galactose being the most abundant linkage (Sun *et al*., 2005).

Gaspar *et al*. (2004) reported that *AtAGP17* mRNA was only found in the flowers, while AtAGP18 transcription was high in flowers, moderate in stems and very low in leaves. Neither *AtAGP17* or *18* expression was detected in roots by Northern blotting;
nonetheless, with RT-PCR, the *AtAGP17* transcript was detected in both leaves and roots (Gaspar *et al*., 2004). The *β-glucuronidase (GUS)* reporter gene and *in situ* hybridization show that *AtAGP18* expression spatially and temporally defines the sporophytic to gametophytic transition. *AtAGP18* mRNA is only detected in developing anthers and ovules, in embryos until the globular stage and transiently in vascular tissues. Notably, *AtAGP18* expression initiates in the sporophytic megaspore mother cell, persists in all four products of female meiosis and is abundant in the functional megaspore and the resulting female gametophyte. The *AtAGP18* transcript is also detected in pollen by both methods (Acosta-Garcia and Vielle-Calzada, 2004).

Understanding gene and protein expression serves as the first step towards deciphering the function. In order to provide functional insight to AtAGP17, 18 and 19, comprehensive analyses on genetic and protein expression as well as subcellular localization of AtAGP17, 18 and 19 were performed in the present work.

**Materials and methods**

**Bioinformatics**

The similarities/identities table was generated with MatGAT (Matrix Global Alignment Tool) v2.01 using the BLOSUM62 algorithm
Amino acid sequences of Lys-rich AGPs were aligned with Vector NTI version 6.0 (Invitrogen, Carlsbad, CA), and other alignments were rendered with ClustalW (http://www.ebi.ac.uk/clustalw).

The phylogeny tree was created from 1,000 bootstrap replicates with the PHYML algorithm (http://atgc.lirmm.fr/phyml) (Guindon and Gascuel, 2003; Guindon et al., 2005) using a PHYLIP file of aligned AGP amino acid sequences. The tree was displayed with TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

AGP gene identification numbers (either *Arabidopsis* AGI locus numbers or Genbank accession numbers) are as follows: *AtAGP1*, At5g64310; *AtAGP2*, At2g22470; *AtAGP3*, At4g40090; *AtAGP4*, At5g10430; *AtAGP5*, At1g35230; *AtAGP6*, At5g14380; *AtAGP7*, At5g65390; *AtAGP9*, At2g14890; *AtAGP10*, At4g09030; *AtAGP11*, At3g01700; *AtAGP12*, At3g13520; *AtAGP13*, At4g26320; *AtAGP14*, At5g56540; *AtAGP15*, At5g11740; *AtAGP16*, At2g46330; *AtAGP17*, At2g23130; *AtAGP18*, At4g37450; *AtAGP19*, At1g68725; *AtAGP20*, At3g61640; *AtAGP21*, At1g55330; *AtAGP22*, At5g53250; *AtAGP23*, At3g57690; *AtAGP24*, At5g40730; *AtAGP25*, At5g18690; *AtAGP26*, At2g47930; *AtAGP27*, At3g06360; *AtAGP40*, At3g20865; *PtaAGP6*, AF101785; *CsAGP1*, AB029092; *LeAGP1*, X99147; *NaAGP4*, AF298594.
**Plant material and growth conditions**

Columbia-0 ecotype *Arabidopsis* plants were grown in soil in either a growth room or a growth chamber at 22 °C under 16 h light/8 h dark conditions. Alternatively, *Arabidopsis* seedlings were grown on Murashige and Skoog (MS) plates consisting of 4.3 g/L MS basal salts (Caisson Laboratories, Logan, Utah), 1% sucrose and 0.8% agar (pH 5.8) unless indicated elsewhere. The plates were kept at 24 °C under long day conditions.

*Arabidopsis* suspension cultured cells were grown in Gamborg’s B-5 medium (pH 5.7) containing 3 g/L Gamborg’s B-5 basal salts, 20 g/L sucrose, 1 mg/L nicotinic acid, 10 mg/L thiamine HCl, 1 mg/L pyridoxine HCl, 100 mg/L Myo-Inositol, 500 mg/L 2-[N-Morpholino]ethanesulfonic acid and 1.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich, St. Louis, MO). WT BY-2 (*Nicotiana tabacum* L. cv.) cells were maintained in NT-1 medium (4.3 g/L MS basal salts, 30 g/L sucrose, 180 mg/L KH₂PO₄, 100 mg/L Myo-Inositol, 1 mg/L thiamine HCl, 0.44 mg/L 2,4-D, pH 5.7). Cells were kept on a rotary shaker (120 rpm) in the tissue culture room and subcultured every week.

**RNA extraction**

Total RNA was extracted with the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) from *Arabidopsis* suspension cultured cells and organs. Cultured cells were collected by
filtering at different times after subculturing; aerial parts of 7-day-old *Arabidopsis* seedlings and 4-week-old rosette leaves, roots, flowers and inflorescence stems were harvested.

**Northern blotting**

Northern blotting was carried out according to Sun *et al.* (2005). Briefly, total RNA (15 μg) was electrophoresed in a 1% denaturing agarose-formaldehyde gel and transferred onto Zeta-Probe Genomic Tested Blotting Membrane (Bio-Rad, Hercules, CA) in 10X SSC overnight. Equivalent loading of RNA samples was confirmed by ethidium bromide staining of rRNA. Gene-specific probes corresponding to the coding sequences of *AtAGP17, 18* and *19* and were labeled with α-32P-dCTP using the Prime-a-gene labeling system (Promega, Madison, WI) according to the manufacturer’s protocol.

The membrane was hybridized at 65 °C in 1X hybridization buffer (7% sodium dodecyl sulphate (SDS), 0.25 M Na₂HPO₄, pH 7.2) overnight and washed once in 1/2X hybridization buffer at RT for 5 min, twice in 1/4X hybridization buffer at 65 °C for 15 min and once in 1/7X hybridization buffer at 65 °C for 15 min. Afterwards, the membrane was wrapped in Saran wrap, exposed to Kodak Biomax MS film for 24 to 48 h at -80 °C and developed with Kodak GBX developer and fixer (Fisher Scientific, Hampton, NH). Whenever necessary, the membrane was stripped by boiling in 500 ml of stripping buffer
(0.5% SDS, 0.1X SSC) twice, 20 min each time.

**RT-PCR**

RT-PCR was performed with the OneStep RT-PCR Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. Sometimes, total RNA was treated with DNase (Sigma-Aldrich, St. Louis, MO or QIAGEN, Valencia, CA) before RT-PCR.

One primer set was designed for each of the AGP genes; the reverse primer spanned the intron/exon boundary, based on the rationale that it would only anneal to mRNA but not contaminating DNA. For *AtAGP17*, the primers were 5’-TAA ACC CAC ATC TCC CGC CAT TTC-3’ and 5’-TTC TTT GTG CTC CGC TCT GAT CGT-3’. The primers for *AtAGP18* were 5’-TGG ATC GCA ATT TCC TCC TAA CAG T-3’ and 5’-TCG CTG CTC CGC TCT GAT CGT C-3’. For *AtAGP19*, the primer combination 5’-CTG CTC TCA TCT CTT CCT TTA GTG-3’ and 5’-ATT GAG CCA CAT TAC TGC TCT TCC-3’ were used. Internal control actin primers (5’-GTG CTC GA C TCT GGA GAT GGT GTG-3’ and 5’-CGG CGA TTC CAG GGA ACA TTG TGG-3’) were included in each reaction.

The fragments synthesized by the above four pairs of primers were of different sizes and could be easily differentiated on the gel, allowing for multiplex PCR. To ensure the cycle numbers used for *AtAGP17*, 18 and 19 as well as actin amplification were within
the exponential phase, RT-PCR reactions were set up and removed from the thermocycler at consecutive cycles; product amounts were assessed by electrophoresis.

For each reaction, 1 μg total RNA, 6 μl each pair of \textit{AtAGP17}, \textit{18} and \textit{19} primers and 2 μl actin primers were used. Reverse transcription was performed at 50 °C for 30 min, and the cycling conditions for PCR were as follows: an initial PCR activation at 95 °C for 15 min, 25 cycles of 94°C, 1min; 54 °C, 1min; 72 °C, 1 min and a final extension at 72 °C for 10 min.

\textbf{Cloning}

WT genomic DNA was extracted from \textit{Arabidopsis} leaves either with the Plant DNAZol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions or by the CTAB method (Weigel and Glazebrook, 2002). High fidelity PCR was performed for cloning using the AccuPrime \textit{Pfx} DNA polymerase with a final concentration of Mg$^{2+}$ at 1.5 mM (Invitrogen, Carlsbad, CA). Restriction enzyme sites were added to the ends of gene-specific primers to facilitate cloning. PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). Enzyme digestion products were purified either with QIAquick PCR Purification Kit or QIAEX II Gel Extraction Kit.

Restriction enzymes were purchased from New England BioLabs (Beverly, MA).
Ligation reactions were carried out using Quick Ligation Kit (New England BioLabs, Beverly, MA) with a 1:3 molar ratio of vector to insert. The amount of insert to be used was determined by the following formula: vector amount (µg) * insert size (kb) * 3/vector size (kb).

Chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA) were transformed, and plasmid DNA was extracted with QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). The constructs were verified either by sequencing or diagnostic restriction enzyme digestions or both. Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions and run on an ABI 310 genetic analyzer.

*Nucleic acid quantification*

DNA samples were quantified with gel electrophoresis by comparing intensities of sample bands to a 1 kb DNA ladder (New England BioLabs, Beverly, MA) or λ markers (Promega, Madison, WI) of known quantities. DNA/RNA was also quantified with a spectrophotometer. Samples were diluted, and absorbance was measured at 260 nm and 280 nm. Purity of nucleic acids was determined by $\text{OD}_{260}/\text{OD}_{280}$. Concentrations of DNA (ng/µl) was calculated by $\text{OD}_{260} * 50$, and RNA (ng/µl) by $\text{OD}_{260} * 40$. 
Antibiotic stocks

Kanamycin, streptomycin, timetin and hygromycin (Research Products International, Mount Prospect, IL) stocks were prepared in 50 ml ddH₂O and filter-sterilized. Sterile stock solutions were aliquoted in Eppendorf tubes and stored at -20 °C.

Preparation of Agrobacterium tumefaciens cells for electroporation

Five ml fresh saturated Agrobacterium tumefaciens culture was inoculated in 500 ml LB medium (Research Products International, Mount Prospect, IL). When the cells reached log phase (OD₆₀₀ 0.5 to 0.8), the culture was chilled on ice and centrifuged at 4,000 g for 10 min at 4 °C. The pellet was washed three times with 500 ml, 250 ml and 50 ml of ice-cold sterile ddH₂O, respectively. Finally, cells were resuspended in 5 ml of 10% (v/v) ice-cold sterile glycerol, aliquoted and stored at -80 °C (Weigel and Glazebrook, 2002).

Transformation of Agrobacterium by electroporation

Binary vectors were delivered into Agrobacterium strain LBA4404 by electroporation with the GENE PULSER II system (Bio-Rad, Hercules, CA) using a 1
mm cuvette and the following parameters: capacitance, 25 µF; voltage, 2.0 kV; resistance, 200 Ohm; pulse length, 5 msec (until it beeped). For each electroporation, 1 µl of plasmid was added to 20 µl of competent cells.

Cells were recovered by gentle shaking in 1 ml LB medium for 4 h at 28 °C, collected by centrifugation and plated on LB plates containing kanamycin (25 µg/ml) and streptomycin (25 µg/ml). Plates were incubated at 28 °C for 3 d. Presence of the desired constructs in Agrobacterium was confirmed by colony PCR using Taq PCR Master Mix Kit (QIAGEN, Valencia, CA) before transformation of Arabidopsis or BY-2 cells (Weigel and Glazebrook, 2002).

**Bacterial Stock**

Selected bacteria stocks were mixed with sterile 50% glycerol to reach a final glycerol concentration of at least 15%, frozen in liquid nitrogen and stored at -80 °C. After retrieving Agrobacterium stocks from the freezer, colony PCR was performed again to verify the presence of the correct T-DNA.

**Transformation of Arabidopsis**

WT Arabidopsis plants were transformed by the floral dip method (Clough and Bent,
1998). Briefly, 2 ml *Agrobacterium* overnight culture was inoculated in 500 ml LB medium and allowed to grow at 250 rpm for 16 to 24 h. After reaching an OD between 0.8 and 1.2, *Agrobacterium* was spun down by centrifugation at 5,000 g for 10 min and resuspended in 5% sucrose solution supplemented with 0.02% silwet (LEHLE SEEDS, Round Rock, TX). *Arabidopsis* inflorescence stems were dipped in *Agrobacterium* solution for 15 sec, laid on the side and put away from direct light. After 24 h, the plants were transferred back to the growth room. Sometimes, a second transformation was performed a week after the initial dip.

**Screening of hygromycin resistant seedlings**

Seeds of the dipped plants were collected and dried in paper bags at RT for 4 weeks. Seeds were sterilized by soaking in 70% ethanol for 2 min and then incubating in 30% bleach supplemented with 0.05% Triton X-100 for 10 min. Sterilized seeds were rinsed in sterile ddH₂O at least 5 times and then sown on MS plates containing 1% sucrose and 20 µg/ml hygromycin. After stratification for 3 d at 4 °C, the plates were exposed to light for 4 to 8 h to promote germination and then kept in dark for 5 d. Hygromycin-resistant seedlings had long hypocotyls, while hygromycin-sensitive plants had short hypocotyls. Hygromycin-resistant seedlings were allowed to grow for another 7 d on MS medium without hygromycin before transferring them to soil. Transformed plants were allowed to
self-fertilize, and T2 seeds were collected.

**GUS staining**

For the $P_{AtAGP18}:\text{GUS}$ construct, the $AtAGP18$ promoter (2.5kb upstream of the start codon) was amplified by the primer pair: 5’-GGC CCT TAA TTA AGG TCT CAA CAT GAG AGG TTC CAA C-3’ and 5’-GCC AGG CGC GCC ATT TTG TTA AAA TTT GGA TCA AAC-3’. To build the $P_{AtAGP19}:\text{GUS}$ construct, the $AtAGP19$ promoter sequence was amplified with primers 5’-CCG GTT AAT TAA TGA AAC TGC CTA GTC GGA ACC TGA-3’ and 5’-AAA TGG CGC GCC TGT GTT GTG GAG GAA GCT ACA AGA-3’. The same promoter sequence was used for the complementation of $atagp19$ (Chapter 3), indicating that this promoter successfully drove $AtAGP19$ expression. The PCR products and pMDC164 binary vector (Curtis and Grossniklaus, 2003) were digested by restriction enzymes $\text{PacI}$ (TTAATTAA) and $\text{AscI}$ (GGCGCGCC) and ligated.

GUS staining was performed on T2 transgenic plants. At least 10 independent transgenic lines harboring the $P_{AtAGP18}:\text{GUS}$ or $P_{AtAGP19}:\text{GUS}$ fusions were tested, and representative GUS staining patterns were presented. Samples were harvested at different growth stages and incubated in a GUS staining solution (0.5 M sodium phosphate, 0.2 mM potassium ferricyanide, 0.2 mM potassium ferrocyanide, 0.5% Triton X-100 and 2 mM X-GlcA, pH 7.2) at 37 °C for 12 to 24 h. WT samples were always also incubated in the
same GUS staining solution for same periods of time, but staining of WT samples were never observed. After staining, samples were cleared in absolute ethanol and stored in 70% ethanol. Pictures were taken under the ACE light source (Schott-Fostec, Auburn, NY) with the Nikon COOLPIX 5400 digital camera connected to the Nikon binocular stereo dissecting microscope SMZ1500.

**Histology**

To gain more information on AtAGP19 promoter activity, leaves, hypocotyls and stems of transgenic plants harboring the $P_{AtAGP19}:GUS$ reporter gene construct were stained and sectioned. Immediately following GUS staining, samples were fixed overnight in Safefix II (Fisher Scientific, Hampton, NH), dehydrated in an ethanol series (30, 50, 60, 70, 80, 90, 95, 100%, each for 2 h) and embedded in paraffin. Sections 8 to 25 µm thick were cut with a rotary microtome, depending on the intensity of staining. Sections were dried for 24 h, dewaxed in Citrisolv (Fisher Scientific, Hampton, NH) and rehydrated. Rehydrated sections were either mounted directly in Permount (Fisher Scientific, Hampton, NH) or counter-stained with 1% aqueous Safranin O for 15 min or phloroglucinol/HCl for a few seconds. Safranin O stained sections were dehydrated and mounted. Phloroglucinol/HCl stained slides were viewed immediately under a microscope.
Expression data retrieval

Microarray data of endogenous gene expression in WT *Arabidopsis* (Columbia-0 ecotype) were obtained from Genevestigator (Zimmermann et al., 2004) at https://www.genevestigator.ethz.ch/ and plotted. Microarray data of gene expression under stress conditions were retrieved from the Arabidopsis Membrane Protein Library (http://www.cbs.umn.edu/arabidopsis/) and TAIR Microarray Expression Search (http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression) and analyzed. Alternative sources of expression data include the Stanford MicroArray Database (http://genome-www5.stanford.edu/).

Co-expression of *AtAGP17*, 18 and 19 with other genes was examined at *Arabidopsis thaliana* trans-factor and cis-element prediction database (ATTED) at http://www.atted.bio.titech.ac.jp/ and the *A.thaliana* Co-Response Database (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath.html).

MPSS data were obtained from the Arabidopsis MPSS Plus Database (http://mpss.udel.edu/at/) (Meyers et al., 2004a). Results of the classic and signature MPSS methods were processed separately. Transcripts per million (TPM) values (sums of abundance for classes 1, 2, 5 and 7 signatures) of the 17 bp and 20 bp signatures were averaged and plotted.
Construction of EGFP-AtAGP18/19 fusions

To construct the EGFP-AtAGP18/19 fusion proteins, the LeAGP1 signal sequence followed by the EGFP sequence was amplified from the pBI121- SS<sup>com</sup>-EGFP-AtAGP17 vector (Sun et al., 2005) with a 5’ primer containing a XhoI site (CTCGAG) (5’-CCG GCT CGA GGG TCT ATA TTT TCT TTA GCT ACC ATG-3’) and a 3’ primer containing a ClaI site (ATCGAT) (5’-AAA TTA ATC GAT GTA CAG CTC GTC CAT GCC GAG AGT GA-3’).

The AtAGP18/19 coding regions without the signal sequence were amplified with primers: 5’-CCT GCG ATC GAT CAA TCT CCT ATC TCT TCT CCG ACC-3’ and 5’-CGC TCT AGA TTA GAA TGC CAT AAC GAG AAC GGC CCA-3’ for AtAGP18 and 5’-GAC TGC ATC GAT GTA AAT GCA CAA GGA CCT GCT GCT-3’ and 5’-GGC CCT CTA GA TAG GCT GTC ATA GCA AGT AGA AAG AGG A-3’ for AtAGP19. A ClaI site (ATCGAT) and an XbaI site (TCTAGA) were added to the 5’ and 3’ ends of the PCR products, respectively. The PCR products were successively cloned into the vector pKANNIBAL (Wesley et al., 2001), and the fragment containing the 35S CaMV promoter, EGFP and AtAGP18/19 was then inserted into the binary vector pART27 (Gleave, 1992).
Transformation of BY-2 cells

Tobacco BY-2 cells were transformed following published methods (Persson et al., 2001; Love et al., 2002; Zhao et al., 2002). Briefly, 3- or 4-day-old BY-2 cells were allowed to sediment, and the liquid medium was removed. Four ml of cells were supplemented with 1 ml fresh NT-1 medium and pipetted up and down 15 times to cause small lesions in cells to allow for high transformation efficiency. After incubation with 100 µl of an overnight Agrobacterium culture in the dark at 28 °C for 3 d, cells were washed three times in fresh NT-1 medium, spread on plates containing NT-1 medium supplemented with 8 g/L agar, 300 µg/ml timetin and 100 µg/ml kanamycin. After 1 month, microcalli were transferred to 1 ml Schenk and Hildebrandt (SH) liquid medium (3.2 g/L SH basal salts [Caisson Laboratories, Logan, Utah], 34 g/L sucrose, 1X SH vitamin powder [Sigma-Aldrich, St. Louis, MO], 0.1 mg/L kinetin, 2.1 mg/L p-chlorophenoxy acetic acid, 1 g/L Myo-Inositol, 0.44 mg/L 2,4-D, pH 5.8) supplemented with 300 µg/ml timetin and 100 µg/ml kanamycin in 24 Well Costar Cell Culture Cluster plates (Corning Incorporated, Corning, NY) and kept in the dark at 24 °C on a rotary shaker. Transgenic suspension-cultured cells were subjected to microscopic analysis and maintained by subculturing every 2 to 3 weeks with SH medium containing only 100 µg/ml kanamycin.
Localization of the EGFP-AtAGP18/19 fusion proteins

Transgenic BY-2 cells expressing the GFP-AtAGP18 and GFP-AtAGP19 fusion proteins were observed at a wavelength of 488 nm using a Zeiss confocal laser scanning fluorescence microscope LSM510 with a GFP filter set (Zeiss, Germany). Cells were plasmolyzed with 4% NaCl for 15 min. Images were processed with the Zeiss LSM image browser.

Isolation of PM

PM vesicles were isolated and purified following published procedures (Larsson et al., 1987; Komalavilas et al., 1991; Sun et al., 2004b). Briefly, transgenic BY-2 cultured cells expressing EGFP-AtAGP18 were harvested 10 to 14 days after subculture by vacuum filtering. The cells were homogenized with a PTA 20S polytron (Brinkmann, Westburg, NY) using several bursts in a homogenizing buffer (50 mM Tris-HCl, 10 mM KCl, 1 mM EDTA, 0.1 mM MgCl₂, 8% sucrose, 1 mM phenylmethylsulphonylfluoride, pH 7.5). The homogenate was filtered through cheese cloth to remove cell debris, and all subsequent steps were performed at 4 °C. Chloroplasts and mitochondria were pelleted at 10,000 g for 15 min, and the supernatant was ultracentrifuged at 100,000 g for 45 min to pellet microsomes, which were resuspended in a buffer (0.33 M sucrose, 3 mM KCl, 5
mM potassium phosphate, pH 7.8). Two-phase partitioning was carried out in the cold room with a clinical centrifuge exactly as described by Larsson et al. (1987). The PM and PM-depleted phases were diluted with ddH2O, pelleted at 100,000 g to collect PM and intracellular membranes and re-dissolved in ddH2O. Protein concentrations were determined with the DC RD Protein Assay Kit (Bio-Rad, Hercules, CA). The samples were aliquoted and stored at -80 °C. Equal amounts of protein (10-20 μg) were used for Western blotting.

Isolation of AGPs from PM

Purified PM was incubated with 1% Triton X-100 overnight at 4 °C and centrifuged at 100,000 g for 1 h. Absolute ethanol was added to the supernatant to reach a final concentration of 80% and incubated overnight again at 4 °C to precipitate AGPs. AGPs were pelleted by centrifugation, washed twice with ethanol and air-dried (Sun et al., 2004b).

Production of LeAGP1, AtAGP17, 18 and 19 antibodies

Previous reports demonstrated the feasibility of raising specific antibodies against the unique Lys-rich subdomains of Lys-rich AGPs (Gao et al., 1999; Gao and Showalter,
2000; Zhang et al., 2003). The same approach was used to produce antibodies against AtAGP17, 18 and 19 as well as LeAGP1. Before peptide injection, rabbit preimmune sera were screened, and two rabbits with the lowest immune responses to Arabidopsis total leaf proteins were chosen for production of each antibody (data not shown). As shown below, peptides (20 aa) were synthesized (Genemed Synthesis, South San Francisco, CA) corresponding to the Lys-rich regions of LeAGP1 and AtAGP17, 18 and 19:

LeAGP1 peptide: PAPAPSKGKVKGKKGKKKHNA (Gao et al., 1999)

AtAGP17 peptide: PAPALTKHKKTCHKTAPA

AtAGP18 peptide: PAPAPSKHKKTCKSKKHKQA

AtAGP19 peptide: PAPAPAPTCHKRKHKKHKRHH

The synthetic peptides were conjugated to keyhole limpet hemocyanin (KLH) to promote immune responses in rabbits. Peptide antibodies were purified by passing antisera through affinity columns filled with synthetic peptides, and they were referred to as purified (anti-peptide) antibodies or simply antibodies in this dissertation. The flow-through portions of two rabbits from affinity column purification were combined and designated as antisera. Enzyme-linked immunosorbant assay results of these antibodies were excellent (data not shown). Purified antibodies in PBS buffer, antisera flow-through and preimmune sera were stored in aliquots at -80 °C; they were also stable at 4 °C for at least one month.
**Protein extraction**

Total proteins were extracted from BY-2 cells or *Arabidopsis* seedlings, leaves, roots, stems, flowers and siliques. Briefly, plant tissues were ground in liquid nitrogen with a mortar and pestle. The powder was added to 2 volumes of 1X SDS-PAGE sample buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100mM dithiothreitol or 5% β-mercaptoethanol) or 1 volume of 2X SDS-PAGE sample buffer and heated at 100 °C for 5 min (Sambrook and Russell, 2001; Weigel and Glazebrook, 2002). The samples were centrifuged at maximum speed for 10 min, and the supernatant was quantified and used for Western blotting.

**Protein electrophoresis and transfer**

Electrophoresis and transfer of proteins were carried out using the Mini-PROTEAN 3 Electrophoresis Cell and Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). Equal amounts of protein samples were run on 5-10% SDS-PAGE gels with Precision Plus Protein All Blue Standards (Bio-Rad, Hercules, CA). For colorimetric development, purified EGFP-AtAGP17 (Sun *et al.*, 2005), purified EGFP-LeAGP1 (Sun *et al.*, 2004b) or 100 µg *Arabidopsis* total protein was loaded per well; for
chemiluminescence development, 30-60 µg *Arabidopsis* total protein was loaded in each lane. After protein electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) for at least 15 min. Proteins were electro-blotted to a PVDF membrane (Bio-Rad, Hercules, CA) in transfer buffer for 1 h at 100 V with stirring. PVDF membrane was wet in 100% methanol before use. After transfer, the membrane was washed three times in ddH₂O before next step.

*Coomassie blue staining*

The gel was stained in Coomassie staining solution (0.25% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid) for 1 h to overnight. It was destained in several changes of destaining solution (50% methanol, 10% acetic acid).

The membrane was stained in 0.1% Coomassie blue R-250 in 40% methanol for 1 min. It was then destained in several changes 50% methanol until the blot turned light blue, and the bands were visible.

*Western blotting*

The membrane was treated with blocking buffer (5% milk, 0.05% Tween 20, 1X PBS/TBS) for 1 h at RT and then incubated with primary antibodies diluted in blocking
buffer. Usually, GFP Living Colors A.v. Peptide Antibody (Clontech, Mountain View, CA) were diluted between 1:100 to 1:400; purified AtAGP17, 18 and 19 and LeAGP1 antibodies were diluted between 1:500 to 1:1,000; preimmune and antisera were diluted between 1:100 to 1:500. Incubation time in GFP antibodies was 1 h at RT; otherwise, the incubation time was at least 3 h at RT or overnight at 4 °C.

Membranes were washed 3 times in washing buffer (0.05% Tween 20, PBS/TBS) for 5 min each time and then incubated with goat anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO and Bio-Rad, Hercules, CA) diluted in blocking buffer according to the manufacturer’s instructions. Before color development, the membrane was washed three times with washing buffer and another time with PBS/TBS only.

To achieve higher detection sensitivity, goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies were used in combination with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). The membrane was washed at least 4 times to minimize background before secondary antibody incubation and before exposing the membrane to film (Denville Scientific, Metuchen, NJ). Due to extremely high sensitivity of this chemiluminescence method, washing is critical, and three washes resulted in a noisy background. Preimmune sera virtually did not produce signals. All blocking and incubation steps were performed with gentle shaking.
Membrane stripping

Blots were stripped in TBS with 7 μl/ml β-mercaptoethanol and 2% SDS for 1 h at RT with agitation. The blots were then washed with TBS for 3 times, re-blocked and incubated with secondary antibodies to check stripping efficiency.

Dot blotting

Protein samples (1 μl) were spotted directly on nitrocellulose membrane or pre-wet PVDF membrane. The membranes were allowed to dry completely after sample loading. Blocking and immunoblotting were same as described for Western blotting.

Immunolocalization

Paraffin sections of Arabidopsis stem (apical portions) were dewaxed, rehydrated, attached to Poly L-lysine coated microscopic slides (Electron Microscopy Sciences, Hatfield, PA), blocked in 10% goat serum in PBS/BSA and incubated with primary antibodies (1:100 for purified antibodies and 1:20 for antisera and preimmune sera) overnight at 4 °C in a humid chamber. After washing with PBS/BSA, the sections were incubated with FITC-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO).
After rinsing, slides were mounted in 50% glycerol in PBS and observed under a Nikon fluorescence microscope.

**Yariv staining**

For Yariv staining of gels, the gel was submerged in 10 ml Yariv solution (200 μM Yariv, 1% NaCl) overnight at RT and rinsed in PBS. For Yariv staining of membranes, the membrane was treated with blocking buffer (3% milk, 1X PBS) for 1 h at RT, washed in PBS for 5 min and then immersed in Yariv solution (200 μM Yariv, 1% NaCl) for 15 min. The membrane was then rinsed in PBS to wash away nonspecific binding.

**Results**

**Lysine-rich AGPs**

All Lys-rich AGPs identified to date have an N-terminal signal sequence, a central AGP domain containing a small Lys-rich region, and a C-terminal GPI anchor addition sequence (Figure 2.2a). Amino acid sequences of the Lys-rich AGPs were aligned (Figure 2.2b), and the similarities/identities were analyzed (Table 2.2). LeAGP1 and NaAGP4 have high amino acid sequence similarity (82.5%) and identity (79.6%). Other AGPs
Figure 2.2 Lys-rich AGPs.
(a) Protein backbone organization of Lys-rich AGPs.
(b) Amino acid sequence alignment of seven Lys-rich AGPs. Identical amino acids are in a black background, while similar amino acids are in a gray background. Signal peptide sequences are shown by a dashed underline. Lys-rich regions are underlined. A triangle indicates the predicted cleavage sites for GPI anchor addition.
Table 2.2 Amino acid similarities/identities among the seven Lys-rich AGPs.

<table>
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<th>AtAGP18</th>
<th>AtAGP19</th>
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<td>62.3</td>
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Similarities and identities are indicated in the left and right hand sides of the diagonal respectively.
have amino acid sequence similarities ranging from 37.7% to 64.2% and sequence identities ranging from 27.9% to 53.7%. In particular, while AtAGP17 and 18 are 64.1% similar and 53.7% identical to each other, AtAGP19 has only 37.7% and 27.9% amino acid sequence similarity and identity to AtAGP17 and 44.9% and 33.6% similarity and identity to AtAGP18.

In order to provide some insight on the relationship among Lys-rich AGPs and other AGPs, a phylogeny tree was created (Figure 2.3). As expected, all known Lys-rich AGPs clustered together; AtAGP17 and AtAGP18 were closely related, as are LeAGP1 and NaAGP4. In contrast, AtAGP19 was most closely related to PtaAGP6 and AtAGP9, a classical Arabidopsis AGP lacking a lysine-rich domain. An alignment between AtAGP19 and AtAGP9 was generated (Figure 2.4). As predicted, the Lys-rich region of AtAGP19 did not align with AtAGP9.

**Northern blotting and RT-PCR**

Northern blot analyses using gene specific probes revealed that expression of AtAGP17, 18 and 19 in Arabidopsis was organ-specific (Figure 2.5a). The AtAGP17 transcript was found in seedlings, rosette leaves, flowers and stems, but not roots. AtAGP19 transcript levels were high in stems, moderate in roots and flowers, low in seedlings and barely detectable in mature rosette leaves. Similarly, AtAGP18 was
Figure 2.3 Phylogeny tree of AGPs.
All classical AGPs and AG peptides in *Arabidopsis* as well as Lys-rich AGPs from other plants were analyzed. The Lys-rich AGPs from *Arabidopsis* are underlined. Capitalized letters after the protein names indicate the classes of AGPs: C, classical AGPs; K, Lys-rich AGPs; P, AG peptides. Bootstrap values from 1000 trials are indicated at branch points. The scale indicates the branch length.
Figure 2.4 Amino acid sequence alignment of AtAGP19 and AtAGP9.
This alignment was performed because AtAGP19 and AtAGP9 showed close evolutionary relationship in the phylogeny tree (Figure 2.3).
“*” indicates identical amino acids. “:” indicates strongly conserved residues. “.” indicates weakly conserved residues.
Figure 2.5 Northern blot analyses of AtAGP17, 18 and 19.

(a) Northern blotting of AtAGP17, 18, and 19 in Arabidopsis plants and cell cultures. Left panel, organ-specific expression patterns of AtAGP17, 18, and 19 in Arabidopsis. Aerial parts of 10-day-old seedlings (SL) and 4-week-old roots (RT), rosette leaves (RL), flowers (FL) and stems (ST) were examined. Right panel, transcript abundance of AtAGP17, 18, and 19 in Arabidopsis cell cultures. Predicted sizes of AtAGP17, 18, and 19 mRNA are indicated. rRNAs were stained with ethidium bromide to show equal loading.

(b) Northern blotting of AtAGP17 in Arabidopsis cultured cells, done independent of (a).
expressed in roots, flowers and stems and weakly expressed in seedlings and rosettes. Transcription of \textit{AtAGP17}, 18 and 19 in \textit{Arabidopsis} cell culture was also examined with Northern blotting. \textit{AtAGP19} transcript was not detected after three attempts. \textit{AtAGP17} and 18 showed high expression in exponentially growing cells, and the expression decreased with age and disappeared in cells in the stationary stage (Figure 2.5a,b). RT-PCR generally supported Northern blotting results and showed that the three AGP transcripts were present in various organs. Furthermore, expression of \textit{AtAGP17} in roots was not detectable by RT-PCR, either (Figure 2.6).

\textit{Promoter activity of \textit{AtAGP18}}

To investigate the expression of \textit{AtAGP18} at the tissue level, expression of the \textit{GUS} gene under the control of the \textit{AtAGP18} promoter was examined in transgenic \textit{Arabidopsis} plants (Figure 2.7). In light-grown seedlings, cotyledons, hypocotyls and roots were stained. The lower portion of the hypocotyl displayed heavier GUS staining than the upper portion. Roots were generally stained except root tips, including the vasculature, root hairs and other root cells. In 3-day-old dark-grown seedlings, GUS staining was throughout the seedlings; in 5-day-old dark-grown seedlings, the upper portion of hypocotyl was stained. Strong \textit{AtAGP18} promoter activity was found in young rosette leaves. As the leaf matured, staining diminished from the midvein region first, while
Figure 2.6 RT-PCR of *AtAGP17, 18* and *19*.
(a) *AtAGP19* amplification in RT-PCR reactions stopped at consecutive cycles. This was an example of effort to ensure the cycle number used for multiplex RT-PCR was within the exponential amplification phase.
(b) Multiplex RT-PCR of *AtAGP17, 18* and *19*. Actin mRNA provided an internal control.
Figure 2.7 Promoter activity of AtAGP18 in Arabidopsis.
Promoter activity of AtAGP18 was revealed by GUS staining of transgenic Arabidopsis plants harboring the P<sub>AtAGP18</sub>-GUS construct.
(a) and (b) Light-grown 3- and 7-day-old seedling, respectively.
(c) Root of light-grown 7-day-old seedling.
(d) and (e) Dark-grown 3- and 5-day-old seedling, respectively.
(f) Shoot of 10-day-old seedling grown in light.
(g) Rosette. Note staining was heavy in young leaves but absent in old leaves.
(h) and (i) Maturing rosette leaves. Staining started to disappear from the midvein.
(j) Magnified view of a hydathode in (i). Trichome base cells and stomata were also stained.
(k) Inflorescence.
(l) Flowers.
(m) Developing silique at a later stage than the one in (k).
Scale bars = 1 mm in (a), (b), (d) to (i) and (k), 100 μm in (c) and (j) and 500 μm in (l) and (m).
staining of the hydathodes (epidermal structures specialized for water secretion) persisted. All staining disappeared in old and senescent leaves. Stomata, trichomes and trichome base cells exhibited GUS staining. Intense AtAGP18 promoter activity was also observed in young stems, flowers (especially styles) and elongating siliques. In addition to styles, GUS staining was found in vascular tissues of sepals, petals and stamens but not pollen. Papillae cells on the stigma sometimes appeared blue (data not shown), but it was unclear whether it was caused by direct staining or by diffusion of the stain from adjacent tissues. In older siliques, AtAGP18 expression was restricted to seeds, but not seed pods. From the above, AtAGP18 expression was specific to tissues and developmental stages; vascular tissues throughout the plant and styles as well as young organs (i.e., leaves, stems and siliques) were preferentially stained by GUS.

**Promoter activity of AtAGP19**

Promoter activity of AtAGP19 was also monitored by expression of the GUS gene (Figure 2.8). AtAGP19 GUS staining was consistently found in the vasculature of leaves, roots, young stems and flowers (pedicel, sepals, petals and filaments). Both light- and dark-grown 3-day-old seedlings expressed GUS in the cotyledons, hypocotyls and roots, but not in root hairs or root tips. In 7-day-old seedlings, the root staining pattern resembled that of the 3-day-old seedlings; although the staining in hypocotyls was
Figure 2.8 Promoter activity of AtAGP19 in Arabidopsis.
Promoter activity of AtAGP19 was revealed by GUS staining of transgenic Arabidopsis plants harboring the P_{AtAGP19}:GUS construct.
(a) and (b) Light-grown 3- and 7-day-old seedling, respectively.
(c) Dark-grown 3-day-old seedling.
(d) Rosette. Note staining was heavy in young leaves but restricted to the vasculature in more mature leaves. Arrow indicates a hydathode.
(e) and (f) Root and root tip.
(g) Inflorescence including a cauline leaf.
(h) Magnified view of the stem in (g).
(i) Open flower.
(j) Carpel in an unopened flower. Note the style, ovary wall and transmitting tract were stained.
(k) Silique.
Bars = 1 mm for (a) to (d) and (g) and 0.1 mm for (e), (f) and (h) to (k).
stronger and broader, with the apical portion of the hypocotyl being more heavily stained than the basal portion. In rosette leaves, the vascular tissues in the blade and petiole were stained, with pronounced staining at the hydathodes. Staining in new leaves was strong throughout, but decreased and became restricted to the vasculature as the leaves matured. Old or senescent leaves showed little or no staining. Staining in cauline leaves was similar to rosette leaves. GUS activity was also found in leaf and stem trichomes. Young stems were stained throughout their lengths, while older stems showed greatest staining in the apical portion (data not shown). Anthers lacked GUS staining, while the style, ovary walls and transmitting tract were stained. Siliques also demonstrated GUS staining. These results indicated that *AtAGP19* expression was also developmentally controlled, with seedlings and young organs being preferentially stained, and organ- and tissue-specific, with the vasculature, style and developing siliques displaying the greatest amount of GUS staining.

*AtAGP19* promoter activity was also studied on the tissue level. Consistent with whole mount GUS staining in leaves, all leaf cells were stained in newly developed leaves (Figure 2.9a). As the leaf matures, *AtAGP19* expression became restricted to the abaxial half of the leaf (Figure 2.9b) and later only to the vascular bundles (Figure 2.9c). Both xylem and phloem displayed GUS staining. Eventually, *AtAGP19* expression was not detectable in old/senescent leaves. In hypocotyls undergoing secondary thickening, *AtAGP19* GUS activity was found in xylem, vascular cambium, phloem and periderm,
Figure 2.9 Expression of \textit{AtAGP19} in \textit{Arabidopsis} leaves.

Leaves of transgenic plants harboring the \textit{P}_{\text{AtAGP19}}:GUS reporter gene construct were stained and sectioned.

(a) A transverse section of a young leaf showing strong GUS staining in leaf epidermal and mesophyll cells.

(b) In developing leaves, GUS staining gradually diminished from the adaxial portion but retained longer in the abaxial portion (spongy mesophyll cells and abaxial epidermis). PA, palisade mesophyll. SP, spongy mesophyll. V, vascular bundle.

(c) GUS activity of the \textit{AtAGP19} promoter was confined to vascular tissues in developed leaves.

(d) A transverse section of the petiole showing that GUS activity was associated with both xylem and phloem. X, xylem. P, phloem.

(b) and (d) were counter-stained by Safranin O after GUS staining and sectioning. Bars = 100 µm.
substantiating the previous report on the presence of AGPs in the periderm (Dolan and Roberts, 1995). The collapsing outer layers, including endodermis, cortex and epidermis, did not show GUS activity (Figure 1.10a,b). In secondary xylem, xylem parenchyma cells and developing xylem vessel elements were stained, but not differentiated vessels containing lignified secondary cell walls Figure 1.10c-e). Staining in the periderm decreased in hypocotyls at later developmental stages (Figure 1.10e).

In young inflorescence stems where high *AtAGP19* expression was observed, virtually all cells were stained (Figure 1.10f,g). In vascular bundles, phloem, procambium, xylem parenchyma cells and developing xylem vessels displayed GUS activity (Figure 1.10h-k). As development progressed, staining faded away from the pith region first (Figure 1.10j). GUS staining was also associated with fiber cells in the stem interfascicular region (Figure 1.10j,k) and hypocotyl secondary xylem region (Figure 1.10e) even after they were lignified, suggesting additional roles in living fiber cells.

**Microarray expression data**

**Endogenous expression patterns**

In addition to Northern blotting, RT-PCR and GUS analyses, *AtAGP17, 18* and *19* expression was also examined by accessing publicly available microarray data at
Figure 2.10 Promoter activity of *AtAGP19* in *Arabidopsis* hypocotyls and stems. Hypocotyls and stems of transgenic plants harboring the *P_{AtAGP19}*::GUS reporter gene construct were stained and sectioned.
(a) and (b) Transverse sections of a hypocotyl undergoing secondary thickening showing GUS staining in all cells.
(c) A magnified view of secondary xylem in (b). Differentiating vessels and xylem parenchyma cells displayed GUS staining.
(d) and (e) Transverse sections of hypocotyls at later stages of secondary thickening.
(f) and (g) Transverse sections of a young stem showing GUS staining in all cells.
(h) and (i) Enlarged views of a vascular bundle. Mature xylem elements were not stained.
(j) and (k) Transverse sections of stems at later developmental stages.
(a) and (f) were GUS stained only; (b), (c), (g), (h) and (j) were counter-stained by Safranin O; (d), (e), (i) and (k) were stained by phloroglucinol/HCl.
Bars = 100 µm in (a), (b), (f) and (g) and 50 µm in (c) to (e) and (h) to (k).
Genevestigator (Zimmermann et al., 2004). The microarray results indicated that, in general, \textit{AtAGP18} was the most highly expressed of the three, followed by \textit{AtAGP17}, and then by \textit{AtAGP19}, which was the least abundant (Figure 2.11). \textit{AtAGP17, 18} and \textit{19} expression was detected in cell suspension culture and various plant organs and tissues. The signal intensities were averaged from a pool of microarray experiments performed by different investigating labs.

In microarray experiments, \textit{AtAGP17} transcript was most abundant in rosette leaves, followed by seedlings, flowers and stems and lowest in roots (Figure 2.12a). This was essentially consistent with the Northern blotting profile of \textit{AtAGP17}. Microarray data confirmed the widespread expression of \textit{AtAGP18} and \textit{AtAGP19} and were largely in line with the Northern blotting and GUS staining results (Figure 2.12b,c). However, there were some discrepancies with regard to relative expression intensities. For example, microarray experiments with Affymetrix GeneChips 25k (ATH1 chips covering all \textit{Arabidopsis} genes) detected higher levels of \textit{AtAGP18} and \textit{19} mRNA in rosettes than in flowers. Complementary DNA (cDNA) chips used in tissue comparison experiments, however, detected 5 to 6 times more \textit{AtAGP18} transcript in flowers relative to leaves (data not shown), which was similar to the Northern blotting results (Figure 2.5a).
Figure 2.11 Relative expression intensities of *AtAGP17, 18* and *19* in *Arabidopsis*.

Expression intensities of *AtAGP17, 18* and *19* in *Arabidopsis* (Columbia-0 ecotype) were examined by microarray analyses and compiled by Genevestigator (https://www.genevestigator.ethz.ch/). Signal indicates expression intensity.
Figure 2.12 Microarray analyses of *AtAGP17*, *18* and *19* in *Arabidopsis*. The same data in Figure 2.11 are separately presented for a better view of the tissue-specific expression patterns of *AtAGP17* (a), *AtAGP18* (b) and *AtAGP19* (c).
Stress responses

*AtAGP17, 18 and 19* expression levels in *Arabidopsis* under various abiotic and biotic stress conditions were also examined. Limited data were available on *AtAGP19*, as it was not detected in many microarray experiments. Therefore, the focus of this section was on *AtAGP17* and 18. Microarray data of some abiotic treatments were summarized at the Arabidopsis Membrane Protein Library ([http://www.cbs.umn.edu/arabidopsis/](http://www.cbs.umn.edu/arabidopsis/)). *AtAGP17* and 18 transcript levels decreased in the presence of cold, osmotic and UV stress (data not shown), but not by wounding (Figure 2.13).

Biotic stress experiments were found via a TAIR Microarray Expression Search ([http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression](http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression)), and expression data were manually analyzed. Details of the experiments can be viewed at the website. For hormone treatments, WT *Arabidopsis* seedlings were treated with 10 μM ABA, 1 μM auxin (IAA), 10 nM brassinosteroid (brassinolide), 1 μM cytokinin (zeatin), 10 μM ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid), 1 μM GA (GA3) and 10 μM jasmic acid (methyl jasmonate), and expression was examined over time with two replicates. *AtAGP17* and 18 signals were downregulated by ABA after 3 h, and *AtAGP18* expression was upregulated 2 to 3 fold by brassinolide after 3 h. All other treatments did not show any significant changes. Suppression of *AtAGP18* transcription
Figure 2.13 Responses of *AtAGP17* and *18* to wounding. Microarray data were obtained from TAIR Microarray Expression Search ([http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression](http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression)) and analyzed. The leaves of 16-days-old plants were wounded; shoots and roots were harvested at different time points to test for wounding responses.

(a) Expression of *AtAGP17* did not change significantly in response to wounding. Only *AtAGP17* expression in shoots was shown, because its expression in roots was too low.

(b) Expression of *AtAGP18* did not change significantly in response to wounding.

Error bars represent SE (*n* = 2).
by ABA was corroborated by Northern blotting (Sun, 2004). \textit{AtAGP19} mRNA levels did not change significantly (data not shown).

Pathogen-mediated regulation of AGP expression was also examined. \textit{AtAGP17} and \textit{18} transcription was significantly suppressed by the bacterial pathogen \textit{Pseudomonas syringae} pv. \textit{tomato} DC3000, but not by a nonpathogenic mutant version \textit{Pseudomonas syringae} pv. \textit{tomato} DC3000 \textit{hrcC} that had a mutation in the \textit{hrcC} gene (Figure 2.14). Figure 2.15 shows responses of \textit{AtAGP17} and \textit{18} to some pathogen-derived elicitors. \textit{AtAGP17} mRNA levels decreased more than half compared to controls after both 1 h and 4 h treatments. Substantial inhibition of \textit{AtAGP17} and \textit{18} expression was also observed upon non-host \textit{Arabidopsis} pathogen \textit{Phytophthora infestans} invasion, but not with pathogenic fungi \textit{Erysiphe orontii} (data not shown). These expression changes under stresses should be confirmed by Northern blotting or RT-PCR, and significance should be pursued.

\textit{Co-expression of AGP genes with other genes}

No co-expression data were available for \textit{AtAGP19}. \textit{AtAGP17} co-expression genes were retrieved from ATTED, and no data for \textit{AtAGP17} were available at the \textit{A.thaliana} Co-Response Database. \textit{AtAGP18} co-expression data were available on both websites.
Figure 2.14 Responses of *AtAGP17* and *18* to pathogenic bacteria. Microarray data were obtained from TAIR Microarray Expression Search (http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression) and analyzed. DC3000, bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. DC3000 hrcC-, a nonpathogenic version of *Pseudomonas syringae* pv. *tomato* DC3000 hrcC, which had a mutation in the hrcC gene. *AtAGP17* (a) and *AtAGP18* (b) expression was suppressed by pathogenic bacteria, but not the nonpathogenic counterparts. Error bars represent SE (*n* = 3).
Microarray data were obtained from TAIR Microarray Expression Search (http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression) and analyzed. Rosette leaves of 5-week-old plants under 8 h light/16 h dark conditions were infiltrated with controls (GST, water and CaCl$_2$+MgCl$_2$) or elicitors (Hairpin Z, GST-NPP1, flagellin and lipopolysaccharide) for 1 or 4 hours and then harvested.

NPP1, necrosis-inducing *Phytophthora* protein 1 (NPP1) purified from *P. parasitica*. Flagellin, *P. syringae*-derived peptide elicitor. Hairpin Z, a proteinaceous elicitor plant hypersensitive responses. Lipopolysaccharide, elicitor present in the pathogen cell wall.

(a) Expression of *AtAGP17* was down-regulated by specific pathogen-derived elicitors of plant defense responses.

(b) Expression of *AtAGP18* did not change significantly in response to the pathogen-derived elicitors.

Error bars represent SE ($n = 3$).
Interestingly, \textit{AtAGP17} and \textit{18} were also co-expressed with each other as well as with other AGPs, such as AtFLA2, AtAGP7 and AtAGP21.

Moreover, \textit{AtAGP17} and \textit{18} were co-regulated with other genes involved in cell wall biosynthesis and signaling, such as genes encoding glycosyltransferases, protein kinases and GTP binding proteins.

\textit{MPSS expression data}

Another approach to analyze gene expression is MPSS, which is more sensitive and accurate in quantifying gene expression at low levels compared to microarray analysis (Meyers \textit{et al.}, 2004a). Classic and signature MPSS are two variations of MPSS. Starting from same cDNA libraries, classic MPSS involves cloning the entire fragment from the 3’ most DpnII site to the poly(A) tail for sequencing, while in the signature MPSS methodology, only the 21 to 22 nt fragment including the \textit{Dpn}II site is cloned for sequencing. The signature MPSS method produces iso-length fragments and eliminates processing biases against different sizes of fragments resulting from the classic MPSS methodology (Meyers \textit{et al.}, 2004b).

Consistent with microarray data, MPSS also showed that \textit{AtAGP18} and \textit{AtAGP19} mRNA levels were generally the highest and lowest among three AGP genes, respectively.
(Figure 2.16). TPM values of $AtAGP17$ signatures, obtained by classic and signature MPSS, were similar; $AtAGP17$ expression was extremely low in roots, high in leaves and moderate in inflorescence and siliques. $AtAGP18$ was the only Lys-rich AGP whose expression was detected by MPSS analyses in callus. Its transcript was most abundant in seedlings, high in flowers, siliques and roots and also present in leaves. Relative levels of $AtAGP18$ and 19 mRNA in roots, leaves, flowers and siliques were different as detected by classic and signature MPSS methods. For example, while signature MPSS showed a higher TPM value of $AtAGP18$ in immature inflorescence relative to leaves, classic MPSS detected similar expression levels of $AtAGP18$ in the two organs. $AtAGP19$ was most highly expressed in elongating siliques and immature inflorescence. $AtAGP19$ transcripts were also found in germinating seedlings, and the abundance was low in roots and leaves.

*Subcellular localization of AtAGP18 and 19*

EGFP-AtAGP18/19 fusion proteins were expressed in transgenic tobacco BY-2 cells under the control of the $35S CaMV$ promoter, and an identical localization pattern was seen (Figures 2.17 and 2.18). EGFP was inserted between the signal sequence and the rest of coding sequences (Figure 2.18a). While WT BY-2 cells showed no background
Figure 2.16 MPSS expression patterns of *AtAGP17*, *18* and *19* in *Arabidopsis*.
(a) Expression of *AtAGP17*, *18* and *19* detected by the signature MPSS method.
(b) Expression of *AtAGP17*, *18* and *19* detected by the classic MPSS method.
TPM, transcripts per million. Roots and leaves were 21 days old, seedlings were 3 days old, and immature inflorescence (including floral meristems and immature floral buds) and developing siliques were harvested from 5-week-old plants. Plants were of Columbia-0 ecotype.
Figure 2.17 AtAGP18 was localized to the PM and Hechtian strands. Left panel, GFP fluorescence was observed on the cell surfaces before plasmolysis. Right panel, the EGFP-AtAGP18 fusion protein was localized to the PM and Hechtian strands after plasmolysis with 4% NaCl. CW, cell wall. H, Hechtian strands. PM, plasma membrane. Scale bars = 20 µm.
Figure 2.18 AtAGP19 was localized to the PM and Hechtian strands.
(a) Organization of the EGFP-AtAGP19 fusion construct.
(b) GFP fluorescence was observed on the surfaces of transgenic BY-2 cells expressing the EGFP-AtAGP19 fusion protein before plasmolysis.
(c) The EGFP-AtAGP19 fusion protein was localized to the PM and Hechtian strands after plasmolysis with 4% NaCl.
fluorescence, green fluorescence was observed on the surfaces (the PM and the cell wall) of transgenic tobacco BY-2 cells. When the cells were plasmolyzed with 4% NaCl, green fluorescence was localized to both the PM and Hechtian strands, which are adhesion sites between the PM and cell wall. The above results indicated that AtAGP18 and 19 were localized to the PM, presumably via a GPI lipid anchor, and in Hechtian strands.

To confirm the PM localization of AtAGP18, biochemical two-phase fractionation and Western blotting were employed. A purified PM phase and a PM-depleted phase were isolated from BY-2 cells overexpressing the EGFP-AtAGP18 fusion protein. Equal amounts of proteins from the two fractions were used for Western blotting, and polyclonal GFP antibodies revealed that the majority of EGFP-AtAGP18 is contained in the purified PM fraction, but not in the fraction depleted of PM. This finding indicated that AtAGP18 was indeed present on the PM (Figure 2.19).

**Specificity of antibodies and antisera**

Antibodies were generated against the Lys-rich regions of AtAGP17, 18 and 19 as well as LeAGP1. An alignment of the four synthetic peptides is shown in Figure 2.20. The purified antibodies were first tested for specificity. When synthetic peptides were used in dot blotting, little or no cross-reactivity was observed (Figure 2.21). LeAGP1
Figure 2.19 AtAGP18 was localized to the purified PM fraction.

(a) Western blot analysis of 20 µg purified EGFP-AtAGP17 (lane 1) and EGFP-LeAGP1 (lane 2) with GFP antibodies.

(b) EGFP-AtAGP18 was localized to the purified PM fraction of transgenic BY-2 cells overexpressing EGFP-AtAGP18.

Top panel, Western blotting with GFP antibodies. Bottom panel, Yariv staining of a duplicate gel. Lane 1, lysate of WT BY-2 cells. Lane 2 to 5, biochemical protein preparations from transgenic BY-2 cells overexpressing the EGFP-AtAGP18 fusion protein. Lane 2, microsomes. Lane 3, purified PM fraction. Lane 4, PM-depleted fraction. Lane 5, AGPs precipitated from PM. Ten µg proteins were loaded in lanes 1 to 4, 5 µg proteins were loaded in lane 5. Sizes of protein markers are labeled on the left.
Figure 2.20 Alignment of peptides used for antibody production.

“*” indicates identical amino acids. “:” indicates strongly conserved residues. “.” indicates weakly conserved residues.

LeAGP1  --PAPAFSKGVKGGKKKHKHA  20
AtAGP18  --PAPAFLHHKTTKSKHQA  20
AtAGP17  --PAPALTTHHTKHKHTAP  20
AtAGP19  PAPAPAPTHHHR--HKHHHRHH--  20
      ****  : * : . : *
LeAGP1 antibodies | AtAGP17 antibodies

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Figure 2.21 Antibody specificity test with western dot blots of synthetic peptides. Four identical blots were made by loading 1 µl of LeAGP1, AtAGP17, 18 and 19 synthetic peptides at indicated concentrations onto a nitrocellulose membrane. The blots were air dried, blocked and then incubated in the specified primary and secondary alkaline phosphatase-conjugated antibodies to test specificity and reactivity of the LeAGP1, AtAGP17, 18 and 19 antibodies.
antibodies cross-reacted with the highest concentrations of AtAGP18 peptide, and vice versa. In contrast, the AtAGP17 and AtAGP19 antibodies did not recognize any other peptides. Besides being specific, these antibodies exhibited strong reactivity against the peptides.

Additionally, specificity of AtAGP17, 18 and 19 antibodies and antiserum flow-through were examined using purified EGFP-AtAGP17 protein (Figure 2.22). Preimmune sera of AtAGP17 did not recognize the protein, while antisera and antibodies did. Antisera and preimmune sera for AtAGP18 and 19, but not their purified peptide antibodies, reacted with EGFP-AtAGP17. These results indicated that the purified antibodies were specific, while antisera could cross-react.

Reactivity of antibodies and antiserum

Purified GFP-LeAGP1 and GFP-AtAGP17 fusion proteins were also used to test the reactivity of the antibodies. The LeAGP1 antibodies generated in the present work were referred to as purified anti-LeAGP1 peptide antibodies in order to differentiate them from PAP antibodies produced previously. Since the same synthetic peptide sequence was used for PAP and LeAGP1 antibody production, it was expected that the newly generated antibodies would have similar activities compared to PAP antibodies. Surprisingly,
Figure 2.22 Specificity test of antibodies and antisera using EGFP-AtAGP17. Ten μg purified EGFP-AtAGP17 was loaded each lane and incubated with antisera (1:200 dilution), preimmune sera (1:200 dilution) and affinity-purified antibodies (1:500 dilution) of AtAGP17, 18 and 19. The peptides that antisera/antibodies were raised against are indicated on the left. Arrows indicate labeled EGFP-AtAGP17.
anti-LeAGP1 peptide antibodies could barely recognize GFP-LeAGP1, even when incubating 75 μg of EGFP-LeAGP1 antigen overnight with the antibodies (Figure 2.23).

In contrast, AtAGP17 antibodies were more reactive, and the antisera could recognize purified EGFP-AtAGP17 above 250 ng (Figure 2.24). In addition, against 1 μg purified EGFP-AtAGP17, a series of dilutions was tested for AtAGP17 antibodies and antisera. The highest dilution was 1:1,000 for purified AtAGP17 antibodies and 1:500 for the antisera (data not shown) to generate a detectable colorimetric signal. Greater dilutions resulted in weak signals; lower dilutions were used in some experiments to increase signal intensities.

A lysate of transgenic BY-2 cells overexpressing EGFP-AtAGP18 was used to test activity of the purified AtAGP18 antibodies. A faint protein smear corresponding to the EGFP-AtAGP18 fusion protein and two non-specific bands were observed (Figure 2.25). These data indicated that the purified AtAGP18 antibodies had low reactivity against the target protein. In contrast, GFP antibodies readily generated signals with as little as 5 μg cell Lysate (data not shown), while AtAGP18 antibodies barely did so with 100 μg lysate.
Figure 2.23 LeAGP1 antibodies were not reactive against EGFP-LeAGP1. Duplicate membranes were incubated with PAP antibodies diluted 1:1,000 (left panel) or LeAGP1 antibodies diluted 1:500 (right panel). Different quantities of the purified EGFP-LeAGP1 fusion protein were loaded. Lanes 1, 20 μg. Lanes 2, 40 μg. Lanes 3, 60 μg. Lanes 4, 75 μg. Secondary antibodies were goat anti-rabbit alkaline phosphatase-conjugated antibodies.
Figure 2.24 Reactivity of AtAGP17 antibodies and antisera against EGFP-AtAGP17.

Left panel, different quantities of the purified EGFP-AtAGP17 fusion protein incubated with the AtAGP17 antibodies (1:500 dilution). Lane 1, 20 μg. Lane 2, 40 μg. Lane 3, 60 μg. Lane 4, 75 μg. Right panel, different quantities of the purified EGFP-AtAGP17 fusion protein incubated with the AtAGP17 antisera (1:300 dilution). Lane 5, 5 μg. Lane 6, 2.5 μg. Lane 7, 1 μg. Lane 8, 500 ng. Lane 9, 250 ng. Lane 10, 75 ng. Lane 11, 50 ng. Lane 12, 10 ng. Lane 13, 1 ng. Lane 14, 100 pg. Secondary antibodies were goat anti-rabbit alkaline phosphatase-conjugated antibodies.
Figure 2.25 Reactivity of AtAGP18 antibodies. AtAGP18 antibodies were diluted 1:500, and secondary antibodies were alkaline phosphatase-conjugated. Arrow indicates smear corresponding to AtAGP18. Stars indicate non-specific bands. Lane 1, 25 μg lysate of WT BY-2 cells. Lane 2 to 6, lysate of transgenic BY-2 cells expressing EGFP-AtAGP18 fusion protein. Lane 2, 20 μg. Lane 3, 40 μg. Lane 4, 60 μg. Lane 5, 80 μg. Lane 6, 100 μg.
**Western blotting with AtAGP17, 18 and 19 antibodies and antisera**

Total proteins were extracted from *Arabidopsis* whole seedlings and various organs from mature plants and used in Western blotting. Organ-specific expression of AtAGP18 and 19 was obtained with their respective antibodies using chemiluminescence detection (Figure 2.26). The smears of different size ranges on the Western blots indicated that these AGPs had different MW/glycosylation in different organs. AtAGP18 and 19 expression patterns were very similar to each other; AtAGP18 and 19 levels were highest in roots and flowers, followed by seedlings, stems and siliques. Low abundance of AtAGP18 and 19 was found in rosette leaves. Similar expression patterns of AtAGP17 and AtAGP19 were also obtained with their antisera (Figure 2.27). Incubating an identical membrane with preimmune sera produced virtually no signal. AtAGP17, 18 and 19 all had similar expression patterns.

**Immunolocalization of AtAGP19**

AtAGP19 antisera, preimmune sera and purified antibodies were used to localize AtAGP19 in WT *Arabidopsis* stem sections. AtAGP19 antisera labeled all the cells in the stem section; preimmune sera did so too, but signal intensity was weaker. AtAGP19
Figure 2.26 Western blot analyses of AtAGP18 and 19.

Total protein (30 μg) from *Arabidopsis* 12-day-old seedlings (SL), 35-day-old rosette leaves (RL), roots (RT), stems (ST), flowers (FL) and siliques (SiL) was loaded each lane. Identical membranes were either stained by Coomassie blue or incubated with AtAGP18 or AtAGP19 purified antibodies overnight (1:500 dilution). Secondary antibodies were horseradish peroxidase-conjugated.

(a) Coomassie blue staining of total proteins on PVDF membrane.
(b) and (c) Organ-specific expression pattern of AtAGP18 and 19, respectively.
Figure 2.27 Western blotting with AtAGP17 and 19 antisera. Total protein (60 μg) from *Arabidopsis* young leaves (YL), old leaves (OL), cauline leaves (CL), stems (ST), flowers (FL), green siliques (SiL) and roots (RT) and loaded each lane. An identical protein gel was stained by Coomassie blue to ensure the quality of protein samples (data not shown). Duplicate membranes were incubated with AtAGP17 (top) or AtAGP19 (bottom) antisera (1:200 dilution). The same membrane did not give any signal when incubated with preimmune sera (1:200 dilution, data not shown). Consistent results were obtained in two independent experiments. Star indicates nonspecific bands.
antibodies, on the other hand, produced a weak signal that was not strong enough to be captured by the camera (data not shown). This experiment requires further optimization of conditions and should be repeated with higher concentrations of antibodies and lower concentrations of antisera. Due to time restraints, immunolocalization with AtAGP17 and 18 antisera and purified antibodies were not commenced.

Discussion

AtAGP17, 18 and 19 are Lys-rich classical AGPs

AtAGP17, 18 and 19 are Lys-rich classical AGPs in Arabidopsis and are predicted to be anchored to the PM by a GPI anchor (Schultz et al., 2002; Borner et al., 2003). These three genes are related to four other AGP genes characterized to varying extents in other plant species; all are members of the Lys-rich classical AGP gene subfamily, and all are predicted to specify GPI anchors.

AtAGP19 is a unique member of the Lys-rich AGP family in Arabidopsis. Its gene has an unusual intron splicing site; the protein has relatively low amino acid sequence similarities/identities to its two homologs and is most closely related to PtaAGP6 and AtAGP9, but not AtAGP17 and 18, in the phylogeny tree; moreover, in addition to AG
polysaccharide addition sites at non-clustered Hyp residues, AtAGP19 is predicted to contain a greater percentage of oligoarabinoside addition sites at clustered Hyp residues compared to its two homologs (Sun et al., 2005).

LeAGP1 and AtAGP17 are localized to the PM by examining transgenic expression of EGFP-LeAGP1 and EGFP-AtAGP17 in cultured BY-2 cells (Zhao et al., 2002; Sun et al., 2004b; Sun et al., 2005). Biochemical analysis further confirms a GPI anchor in LeAGP1. When the GPI anchor addition sequence is missing in the EGFP-LeAGP1 fusion protein, green fluorescence is uniformly distributed at the cell wall-PM interface, and the protein is absent in PM preparations. Here, EGFP-AtAGP18/19 fusion proteins produce an identical PM localization pattern as with these two other Lys-rich AGPs, corroborating the GPI anchor prediction in AtAGP18 and AtAGP19 and making them candidates for participating in cell signaling. Reinforcing the GFP pattern, immunoblotting with GFP antibodies localizes EGFP-AtAGP18 to the purified PM fraction.

Comparison among expression analyses

In the present work, transcription of AtAGP17, 18 and 19 was examined by Northern blotting, RT-PCR, promoter-GUS expression, microarray analyses and MPSS data. The
expression data are summarized in Table 2.3. These approaches produce largely consistent results although small discrepancies are observed with respect to the relative transcript abundance in different organs. Two reasons may account for such differences. First, Arabidopsis plants used for Northern, microarray and MPSS experiments are grown under different conditions and may not be harvested at identical stages. In particular, microarray signals are averaged from a pool of microarray experiments done by different labs (Johnson et al., 2003). Second, each analysis has its limitations; for example, detection sensitivity of microarray experiments are limited by RNA quantities (Meyers et al., 2004a), and AtAGP17 and 19 transcript abundance is relatively low. With MPSS, slightly different classic and signature MPSS could produce different TPM values and relative signal intensities, too.

The RNA gel blot hybridization patterns presented here are similar to patterns obtained in independent experiments (Gaspar et al., 2004; Sun et al., 2005). Low accumulation of AtAGP18 mRNA in leaves is also observed with RT-PCR (Pereira et al., 2006). On the other hand, in situ hybridization only detectes AtAGP18 mRNA in developing anthers and ovules as well as transiently in clusters of companion cells closely associated with stem vascular elements (Acosta-Garcia and Vielle-Calzada, 2004). Moreover, AtAGP18 promoter activity is not observed in pollen in the present study, contradictory to results reported by Acosta-Garcia and Vielle-Calzada (2004); this may be
Table 2.3 Summary of AtAGP17, 18 and 19 expression.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>SL</td>
</tr>
<tr>
<td>AtAGP17</td>
<td>-</td>
</tr>
<tr>
<td>AtAGP18</td>
<td>++++</td>
</tr>
<tr>
<td>AtAGP19</td>
<td>+++</td>
</tr>
</tbody>
</table>

Expression intensities of the gene/protein are summarized from Northern blotting, GUS analysis and Western blotting data and are indicated by “+” or “−”. The stronger the expression, the more “+”. The expression intensities of one gene/protein in various organs are not comparable with other genes/proteins. RT, roots. SL, aerial parts of seedlings. OL, mature rosette leaves. FL, flowers. ST, stems. SiL, siliques.
attributed to different promoter sequences used by the two groups.

In general, \textit{AtAGP18} and \textit{19} GUS staining patterns resemble each other. For example, \textit{AtAGP18} and \textit{19} promoter activities are detected consistently and abundantly in vascular tissues and styles, but not in root tips or pollen. Moreover, they are both developmentally regulated, with young expanding organs being more highly and thoroughly stained. Despite the similarities, differences are also observed. First, \textit{AtAGP18} promoter is stronger than that of \textit{AtAGP19}; GUS staining proceeds faster in transgenic \textit{Arabidopsis} plants harboring the \textit{P_{AtAGP18}:GUS} construct (data not shown). Second, interestingly, \textit{AtAGP18} and \textit{AtAGP19} are preferentially expressed in the basal and apical regions of the hypocotyl in light-grown seedlings, respectively. Third, \textit{AtAGP18} is expressed in other root cells, including root hairs, in addition to root vasculature, while \textit{AtAGP19} is only expressed in root vascular tissues.

\textit{Expression of Lys-rich AGPs}

Expression of Lys-rich AGPs is developmentally-controlled and organ- and tissue-specific. The expression patterns of \textit{AtAGP18} and \textit{AtAGP19} are similar to that of \textit{LeAGP1} and \textit{NaAGP4}. \textit{LeAGP1} is strongly expressed in flowers and young stems, moderately expressed in roots and young fruits and weakly expressed in leaves and old
stems (Li and Showalter, 1996). LeAGP1 and PtaAGP6 are both localized to differentiating xylem elements; LeAGP1 is also abundant in stylar transmitting tissues (Gao et al., 1999; Gao and Showalter, 2000; Zhang et al., 2003). Similarly, strong AtAGP18 and 19 promoter activities are observed in styles and vasculature. In particular, AtAGP19 transcription is associated with developing xylem elements. Like CsAGP1 (Park et al., 2003), AtAGP18 and 19 transcripts are found throughout seedlings.

Similarities and differences are found with respect to stress responses of Lys-rich AGPs. In response to wounding, AtAGP17 and 18 do not change their mRNA levels, while LeAGP1 and NaAGP4 transcripts rapidly decrease (Li and Showalter, 1996; Gilson et al., 2001). On the other hand, transcription of AtAGP17 and 18 as well as NaAGP4 (Gilson et al., 2001) is downregulated by pathogens. Resembling LeAGP1 (Sun et al., 2004a), AtAGP17 and 18 expression is suppressed by ABA; unlike LeAGP1 and CsAGP1 (Park et al., 2003; Sun et al., 2004a), they do not respond to cytokinin, GA or IAA, suggesting differences in the function of these AGPs, which remain to be identified.

**Antibodies**

The AtAGP17, 18, 19 and LeAGP1 antibodies, generated using synthetic peptides, demonstrate excellent specificity and reactivity with respect to synthetic peptides.
However, they behave differently with purified proteins or plant total protein extracts. Purified anti-LeAGP1 peptide antibodies have little reactivity against the target protein; AtAGP18 antibodies have much lower activity than EGFP antibodies. It is possible that the carbohydrates on the AGPs sterically hinder antibody-antigen interactions or native antigen configuration is necessary for more efficient antibody binding. Deglycosylated protein samples and native protein gels can be tested for higher detection sensitivity.

Using total protein preparations, antisera and preimmune sera render adequate differences in signal intensity for specific antigen detection; preimmune sera either do not react at all or reacted only to a minimal degree. Purified antibodies are sufficiently specific, while antisera and preimmune sera may produce nonspecific signals since antisera of AtAGP18 and 19 also recognize purified EGFP-AtAGP17.

Protein expression patterns obtained by antibodies or antisera are very similar for all three AGPs. They are very abundant in roots and flowers, moderate in seedlings, stems and siliques and low in rosette leaves. This is expected for AtAGP18 and 19; their mRNA levels are low in leaves, but high in roots, stems and flowers, as shown by Northern blotting. Furthermore, GUS staining reveals high expression of \textit{AtAGP18} and \textit{19} in seedlings, styles and young organs (leaves, stems and siliques). Surprisingly, little AtAGP17 is detected in leaves, while a large amount of AtAGP17 is found in roots; this is opposite to the Northern blotting data. Although the amount of mRNA does not always
agree with the amount of protein and the Western blotting results presented reflect the endogenous protein expression of AtAGP17, it is still likely that the pattern of AtAGP17 detected here with the antibodies may, at least partially, derive from cross-reactivity of the antisera. However, this inconsistency between AtAGP17 protein and mRNA levels is interesting considering that in an *AtAGP17* mutant, mutant phenotypes are observed in the roots, but not other parts of the plant (Nam *et al.*, 1999; Gaspar *et al.*, 2004). Therefore, more work is worthwhile confirming the protein expression of AtAGP17 in *Arabidopsis* roots.

The organ-specific distribution patterns of AtAGP17, 18 and 19 are reminiscent of LeAGP1. These AGPs are abundant in roots, flowers, stems and fruits and are less abundant in leaves. These four AGPs apparently also have different MW/glycosylation in different organs, which may be critical for organ-specific function (Gao and Showalter, 2000). In the future, antibodies should be further characterized with respect to detection conditions and cross-reactivity, and the negative control (proteins from null knockout mutants of AtAGP17, 18 and 19) should be included to test antibody specificity. It is worthwhile immunolocalizing these three AGPs in *Arabidopsis*. 
Expression and function

The endogenous expression and co-expression patterns of AGPs as well as their expression in response to stress have provided guidance in elucidating their biological roles. Based on the expression of AGPs in young and expanding tissues, it was proposed that AGPs, in coordination with expansins and XTHs, contribute to cell wall expansion (Gilson et al., 2001). Enrichment of AGPs, including LeAGP1, AtAGP18, 19 and TTS proteins, in the style suggests that they could guide, stimulate and nourish pollen tube growth (Cheung et al., 1995; Wu et al., 1995; Gao et al., 1999).

AtAGP18 and 19 promoter activities were consistently found in the vasculature throughout the plant. In particular, AtAGP19 promoter activity was present in cambium, xylem parenchyma cells and differentiating xylem vessels in hypocotyls and stems and absent in differentiated xylem elements with lignified cell walls. This is not surprising because expression patterns of many other AGPs, at the protein or mRNA levels, were specifically associated with pattern formation and vascular development.

Antibodies are powerful tools in probing AGP expression. Although some AGPs were localized to phloem (Dolan and Roberts, 1995; Gao and Showalter, 2000), most studies using the JIM, PAP and anti-AGPB antibodies localized AGPs in different stages of xylem differentiation, and the localization was specific in a spatially and temporally
controlled manner. These studies implicated AGPs in vascular development (Knox et al., 1989; Stacey et al., 1990; Knox et al., 1991; Dolan et al., 1995; Dolan and Roberts, 1995; Schindler et al., 1995; Stacey et al., 1995a; Stacey et al., 1995b; Casero et al., 1998).

Available gene sequences allow for transcription analyses of individual AGPs. Many AGP genes also showed xylem-specific or -preferential expression patterns. Two such examples were in situ hybridization of two homologous FLA genes in Arabidopsis and Zinnia. The AtFLA11 transcript was restricted to the stem and silique sclerenchyma cells (Ito et al., 2005), while ZeFLA11 was specifically expressed in differentiating xylem elements with reticulate type wall thickenings (Dahiya et al., 2006). Two other studies highlighted AtFLA11 as a gene co-regulated with secondary cell wall cellulose synthase genes and also implicated it in secondary wall formation (Brown et al., 2005; Persson et al., 2005).

All the above correlations between AGPs and xylem differentiation suggested AGPs as cell position markers; however, xylogen, a chimeric AGP, is unequivocally established as a mediator in xylem differentiation. In Zinnia stems, xylogen is expressed in procambium and immature xylem cells. In particular, it is directionally localized to the apical side of differentiating treachery elements, possibly bringing neighboring cells into differentiation and vascular network. A double xylogen mutant in Arabidopsis (atxyp1 atxyp2) has discontinuous leaf venation patterns (Motose et al., 2004). Tight association
of *AtAGP19* expression with vascular tissues suggests that AtAGP19 may also play a role in vascular development.

In summary, expression and localization studies of AtAGP17, 18 and 19 further our understanding of these three Lys-rich AGPs and provide a basis for functional identification of them.
Chapter 3 T-DNA MUTANTS OF *AtAGP17, 18 AND 19*
Summary

*Arabidopsis* T-DNA insertion mutants were obtained and examined to elucidate the function of the three Lys-rich AGPs, *AtAGP17, 18* and *19*. *atagp17* and *atagp19* were both null knockout mutants, while the *AtAGP18* mutant had only slightly truncated *AtAGP18* mRNA. *atagp17* and *18* did not have any mutant phenotypes under normal growth conditions. The *atagp19* mutant, however, displayed multiple readily identified phenotypes under normal growth conditions compared to WT: 1) smaller, rounder and flatter rosette leaves, 2) lighter green leaves containing less chlorophyll, 3) delayed growth and flowering, 4) fewer lateral roots, 5) shorter hypocotyls, 6) shorter and thinner inflorescence stems, 7) reduced secondary growth and 8) compromised fertility. Further analyses of mutant phenotypes indicated defects in cell division, cell expansion and cell packing. Complementation of *atagp19* with the WT *AtAGP19* gene restored the WT phenotypes and confirmed that AtAGP19 functions in various aspects of plant growth and development, including cell division and expansion, leaf formation, lateral root initiation, reproduction and vascular development. Functional implications of Lys-rich AGPs are discussed.
Introduction

Insertional mutagenesis

Great progress has been made in *Arabidopsis* functional genomics. This is largely attributed to the sequencing of the *Arabidopsis* genome and implementation of large-scale insertion mutagenesis. There are two major types of insertions, transfer DNA (T-DNA) and transposable elements.

*T-DNA mutagenesis*

T-DNA was first identified in *Agrobacterium tumefaciens*, which inserts T-DNA into a dicot nuclear genome and induces crown gall formation in infected plants. Modification of the tumor-inducing (Ti) plasmid into a binary system has enabled T-DNA mutagenesis to become one of the most important and commonly used genetic engineering techniques in plant biotechnology (Hoekema *et al.*, 1983). Actually, RNAi is also based on T-DNA delivery into the plant genome.

Integration of T-DNA into the genome is based on the formation of a double-stranded break in the chromosomal DNA and insertion of T-DNA. If inserted into
a chromosomal gene, T-DNA disrupts gene expression and function. Sometimes, T-DNA insertions can cause large chromosomal rearrangements, including deletions, inversions and chromosomal translocation (Nacry et al., 1998; Laufs et al., 1999; Kaya et al., 2000).

T-DNA mutagenesis has been used as a forward genetics approach: researchers first identified T-DNA mutant with desired phenotypes and then traced back to the mutated genes that were responsible for the phenotypes. Recently, T-DNA mutagenesis has also become a convenient and fast reverse genetics approach (Krysan et al., 1999), thanks to several large populations of T-DNA mutants that are generated and contributed as a public resource, from which other researchers can obtain mutant lines of interest and study their favorite genes. Therefore, time and energy are saved for scientists in generating their own mutants. This has already benefited AGP function research, providing clues to the biological roles of at least two AGPs. Analysis of a null mutant of AtAGP30, a nonclassical AGP containing six cysteines in the C-terminus, suggests that it plays a role in ABA responses, root regeneration and seed germination (van Hengel and Roberts, 2003). One incomplete knockout mutant of AtAGP17 (CS12955), previously designated as rat1 (resistance to Agrobacterium), has a T-DNA insertion in the promoter region of AtAGP17, which results in reduced binding of Agrobacterium to rat1 roots in water and sucrose solutions. Further characterization of the mutant indicates that AtAGP17 may regulate Agrobacterium-binding either by providing a binding site on the
root surface or by reducing free salicylic acid levels through signal transduction, which possibly involves the GPI anchor (Gaspar et al., 2004).

*Transposon mutagenesis*

The second commonly used mutagen is the *Activator/Dissociation* (*Ac/Ds*) transposable element system. The *Ac* element encodes a transposase and catalyzes transposition of the *Ds* element into the genome, which also results in disruption of gene expression. The *Ds* element can be moved again by the *Ac* element from one locus to another in the genome. Like T-DNA lines, there are also a large number of transposon-bearing plants available for public research (Martienssen, 1998).

*Available insertion mutants for AtAGP17, 18 and 19*

The available insertion mutants cover a majority of genes in the *Arabidopsis* genome, with many genes bearing multiple insertion alleles. A summary of the mutant lines for *AtAGP17, 18 and 19* is provided in Table 3.1; it should be updated periodically to add in new lines as they become available. Information about the mutant resources is summarized in Table 3.2. The *Arabidopsis* Biological Resource Center (ABRC) and
Table 3.1 Available insertion mutant lines of *AtAGP17, 18* and *19*<sup>a</sup>

<table>
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<sup>a</sup>More detailed information on these mutant collections is presented in Table 3.2.

<sup>b</sup>Although claimed to have an insertion in *AtAGP17*, neither PCR nor sequencing had identified any alteration in the gene.

<sup>c</sup>Not verified.
Table 3.2 Information of insertion mutant collections

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<td>ABRC/NASC</td>
<td>Kanamycin</td>
<td>Yes</td>
<td>Not Columbia</td>
</tr>
<tr>
<td>RIKEN RIKEN Genomic Sciences Center Plant Functional Genomics Research Group</td>
<td>Transposon</td>
<td>RIKEN Center BioResource</td>
<td>Hygromycin</td>
<td>Yes</td>
<td>Nössen</td>
</tr>
<tr>
<td>SAIL&lt;sup&gt;b&lt;/sup&gt; Syngenta Biotechnology, Inc.</td>
<td>T-DNA</td>
<td>ABRC/NASC</td>
<td>BASTA</td>
<td>No</td>
<td>Columbia</td>
</tr>
<tr>
<td>SALK Salk Institute Genomic Analysis Laboratory</td>
<td>T-DNA</td>
<td>ABRC/NASC</td>
<td>Kanamycin</td>
<td>No</td>
<td>Columbia</td>
</tr>
<tr>
<td>Wisconsin Knockout Facility at the University of Wisconsin</td>
<td>Transposon</td>
<td>ABRC</td>
<td>BASTA</td>
<td>No</td>
<td>Columbia</td>
</tr>
<tr>
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<td>T-DNA</td>
<td>ABRC</td>
<td></td>
<td>No</td>
<td>Wassilevskija</td>
</tr>
</tbody>
</table>

<sup>a</sup>This collection was originally named Genoplante.
<sup>b</sup>This collection was previously named TMRI/GARLIC.

All the above collections have been integrated to the SALK database and are searchable with the SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress).

Nottingham Arabidopsis Stock Centre (NASC) are two largest *Arabidopsis* stock centers. In the present study, SALK T-DNA mutant lines of *AtAGP17, 18* and *19*, plus one transposon mutant of *AtAGP18*, were examined.

**Materials and methods**

**Verification of insertions and isolation of homozygous lines**

SALK T-DNA lines were identified by the SIGnal "T-DNA Express" Arabidopsis Gene Mapping Tool ([http://signal.salk.edu/cgi-bin/tdnaexpress](http://signal.salk.edu/cgi-bin/tdnaexpress)) and obtained from ABRC. The seeds that arrived were the T3 generation. Kanamycin was not used as a selectable marker for SALK lines, since it might be silenced through the generations.

PCR was used to verify the insertion and isolate homozygous lines for phenotypic analyses with the Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, St. Louis, MO). For all SALK T-DNA insertion mutant lines, the LBa1 primer (5’-TGG TTC ACG TAG TGG GCC ATC G-3’) specific to the T-DNA left border region was used. Two gene specific primers for each AGP mutant line were designed with the SIGnal T-DNA verification primer design tool ([http://signal.salk.edu/isects.html](http://signal.salk.edu/isects.html)). A rationale of primer design for PCR screening is provided in Figure 3.1.
Figure 3.1 Primer design for insertion mutants.
LBA1, T-DNA specific primer. LP, left gene specific primer. RP, right gene specific primer.
For the *AtAGP17* mutant line SALK_016294, 5’-CCA GAC TGG GCC TAG CTC AAA-3’ and 5’-TGT GCT CCG CTC TGC AGT AAA-3’ were used. For another *AtAGP17* mutant line SALK_101062 (*atagp17*), 5’-TTT GTT TTC GTA TGC GTG TCC-3’ and 5’-CAG CTC CCA CCA TTT GTA TCA-3’ were the gene specific primers.

The original primer set designed by the website did not work. For the *AtAGP18* mutant line SALK_117268, the forward primer was 5’-GCT TTG CTT ATT GTT ACA TAG ACG-3’ and the reverse primer was 5’-AGT TAC ATT GAT CTG CAT TGT CGT-3’. For the SALK_038728 line (*atagp19*), two gene specific primer sets worked well and had the same reverse primer (5’-GTG CTG GTG GTG GTG ATA CAG-3’) but different forward primers; one was 5’-AAA GAC AAG AAC TCA GAT CCA CCT-3’ and the other was 5’-CAG TTC ATC CAT TCC ATC CTA AG-3’. The original primer set designed by the website 5’-TCC CAA ACT CCT CAA CAA GAT GC-3’ and 5’-AGG AGG TGG AGA AGC TGG TGC-3’ did not work. Sequencing reactions were performed as described in Chapter 2 on *atagp19* to specify the exact insertion site.

Kanamycin was used to screen the CSHL GT6565 gene trap line at the concentration of 50 μg/ml. For PCR verification of kanamycin resistant plants, two *Ds* specific primers were used together with two genomic flanking sequence specific primers. 5’-CGA TTA CCG TAT TTA TCC CGT TC-3’ (right *Ds* specific primer) and 5’-GCA ATT TCC TCC TAA CAG TTA CA-3’ (left gene specific primer) were a pair; 5’-TAC GAT AAC GGT
CGG TAC GG-3’ (left *Ds* specific primer) was used in combination with 5’-CAT CAC TGA CAG ATA TGA ATT CTT-3’ (right gene specific primer).

*RNA extraction, Northern blotting and RT-PCR*

Methods for RNA extraction, Northern blotting and RT-PCR (with same primers) were described in Chapter 2. Inflorescence stems were the preferred source of total RNA, especially for transformed plants, for two reasons. First, stems provided enough material for RNA isolation from individual plants. Second, new stems grew following the harvest of stem material and allowed for seed set. 25-28 cycles were used in RT-PCR.

*Complementation*

Since only *atagp19* showed visible mutant phenotypes, it was complemented to solidify the causal relationship between silencing of *AtAGP19* and the multiple phenotypes. The WT *AtAGP19* gene sequence, including its endogenous promoter and coding sequence (from the 1836 nucleotide upstream of the *AtAGP19* start codon to the stop codon), was amplified with the primers 5’-CCG GTT AAT TGA AAC TGC CTA GTC GGA ACC TGA-3’ and 5’-CCC GCG AGC TCT TAG GCT GTC ATA GCA
AGT AGA AAG-3’ and cloned into the binary vector pMDC110 at \textit{PacI} (TTAATTAA) and \textit{SacI} (GAGCTC) sites (Curtis and Grossniklaus, 2003), followed by the nopaline synthase (NOS) terminator. The constructed vector was verified by diagnostic restriction enzyme digestions. Transformation of \textit{Agrobacterium} and Arabidopsis was performed as described in Chapter 2.

\textit{Genetic analyses of complemented mutants}

PCR and RT-PCR were conducted to confirm that the WT \textit{AtAGP19} gene (in the complementation construct), the \textit{AtAGP19} mRNA as well as the T-DNA (mutant alleles) were present in complemented mutants. Three PCR reactions using three different primer sets were carried out for each independent complemented mutant line. The first combination was to confirm the presence of T-DNA in the complemented plants, and the primers used were the same as those used for \textit{atagp19} mutant verification. The primers specific to the \textit{AtAGP19} sequence introduced by the complementation construct were: 5’-GTA GCT TCC TCC ACA ACA CAA TGG AAT C-3’ (specific to \textit{AtAGP19}) and 5’-CGA CTA GTC CGA TCT AGT AAC ATA GAT GAC AC-3’ (specific to the NOS terminator following \textit{AtAGP19}). The primers specific to the hygromycin resistance gene were: 5’-GGT TTC CAC TAT CGG CGA GTA CTT CTA C-3’ and 5’-AGA TCG TTA
Plate-based growth stage analyses

Plate-based growth stage phenotypic analyses were carried out as described previously (Boyes et al., 2001) except that the MS growth medium used in this study contained 1% sucrose and 0.4% Phytagel (Sigma-Aldrich, St. Louis, MO). Observations were terminated when all seedlings developed four true leaves larger than 1mm. Germination rates, true leaf numbers, primary root length and lateral root numbers were recorded at the end of the 13-day period.

Agrobacterium binding assays

Agrobacterium binding assays were performed according to the published method (Nam et al., 1999) using Agrobacterium strain LBA4404. Briefly, 4-day-old seedlings grown on vertical MS plates were incubated in water or 0.4% sucrose with 20 μl saturated Agrobacterium culture at least overnight. Seedlings were briefly rinsed in water and placed in water for microscopic observation.


**Seedling cytokinin response assays**

Cytokinin 6-benzylaminopurine (BAP) stock (1 mg/mL) was prepared by dissolving BAP in ddH₂O. *Arabidopsis* seedlings were grown on vertical plates, and seedling responses to exogenous cytokinin were tested according to the published method (To *et al.*, 2004) with minor modifications. Root lengths at days 4 and 7 were marked on the plates. At day 7, the plates were photographed, and root growth between day 4 and 7 was measured using the ImagePro software (Media Cybernetics, San Diego, CA). Total lateral roots per seedling were counted under a dissecting microscope. For chlorophyll assays, seedlings were allowed to grow until 14 days old.

**Shoot initiation assay**

MS plates contained 1% sucrose, 0.4% phytagel and 30 combinations of BAP and naphthaleneacetic acid (NAA) concentrations. BAP concentrations ranged from 0 to 3000 ng/mL, and NAA concentrations ranged from 0 to 1000 ng/mL.

*Arabidopsis* WT and mutant seedlings were grown on vertical plates in the dark for 3 d and then in dim light for 3 d to produce elongated and firm hypocotyls. Hypocotyls were excised from the seedlings and transferred to MS plates containing different
concentrations of hormones. Three to five hypocotyl explants were used for each genotype on each plate. The plates were maintained horizontally in the tissue culture room under long day conditions for 30 d and then subjected to continuous light for another 8 d. One representative callus at each concentration was selected and arranged to create a composite photograph for each genotype (To et al., 2004).

**Visualization of lateral root primordia**

Seedlings grown on 1/2X MS plates were cleared by autoclaving in 90% lactic acid. A differential interference contrast (DIC) microscope was used to count the number of lateral root primordia at different stages along the primary root.

**Hypocotyl measurement**

Seven-day-old seedlings grown on horizontal plates were cleared in lactic acid, and digital pictures of hypocotyls were taken. Hypocotyls and hypocotyl epidermal cells were measured with Image J software (http://rsb.info.nih.gov/ij/index.html).
Quantification of chlorophyll and anthocyanin levels

Shoot systems of 14-day old WT and mutant plants growing on MS plates were harvested and immediately ground to a homogenized slurry with the mortar and pestle (without liquid nitrogen). Chlorophyll was extracted with 80% acetone. Sample absorbance was spectrophotometrically measured at 645nm and 663nm. Total chlorophyll and chlorophyll a and b contents were calculated according to the following equations:

Total chlorophyll (µg/ml) = (20.2 \times A_{645} + 8.02 \times A_{663}) \times ml extraction volume / g fresh weight, Chlorophyll a (µg/ml) = (12.7 \times A_{663} - 2.69 \times A_{645}) \times ml extraction volume / g fresh weight, Chlorophyll b (µg/ml) = (22.9 \times A_{645} - 4.68 \times A_{663}) \times ml extraction volume / g fresh weight (Arnon, 1949).

Anthocyanin levels were assayed according to published methods (Laby et al., 2000; Macinelli and Schwartz, 1984). In brief, shoot systems of soil-grown 3-week old WT and atagp19 plants were weighed and soaked in 99:1 methanol:HCl (v/v) at 4 °C for 12 to 24 h. Sample absorbance was measured at 530nm and 657nm. Relative anthocyanin levels were determined using the following formula to eliminate absorbance of chlorophyll degradation products at 530 nm: (A_{530} - 0.25 \times A_{657}) \times ml extraction volume / g fresh weight. The above experiments were repeated at least two times with at least three parallel samples each time.
**Chloroplast visualization**

First leaves were fixed in 3.5% glutaraldehyde in the dark for 1 h and then heated in 100 mM EDTA (pH 9.0) at 60 °C for 2.5 h. The softened leaf was transferred to a drop of EDTA on a microscopic slide, and intact mesophyll cells were released by tapping the coverslip. A DIC microscope was used to look at the chloroplasts in the mesophyll cells (Pyke and Leech, 1991). Since the number of chloroplasts was proportional to the size of the mesophyll cell, only mesophyll cells of similar sizes from WT and atagp19 were compared.

**Leaf epidermal and mesophyll cell analysis**

Fully elongated first or third leaves (from 21-day-old WT and atagp19 plants grown in soil) were placed in methanol overnight and then cleared and stored in lactic acid. Abaxial epidermal cells of first leaves and mesophyll cells of third leaves were drawn on white paper using a DIC microscope equipped with a drawing tube.

Measurements of abaxial epidermal cells were carried out as described previously (De Veylder et al., 2001; Autran et al., 2002). Briefly, for each leaf, at least 30 abaxial
epidermal cells were drawn from regions located 25% and 75% of the distance between
the tip and base of the blade, halfway between the midrib and leaf margin. The drawing
tube images were scanned in the grayscale mode and processed by Image J
(http://rsb.info.nih.gov/ij/index.html). The cells should have defined and closed edges.
After scanning, the images were processed by “Binary” orders to further define the whole
drawing area as well as individual cells. The following parameters were determined in the
drawing: total area of all cells, total number of cells and number of guard cells. The scale
was set, and the total area of the drawing was measured. Average epidermal cell area was
obtained by dividing the total area by the number of epidermal cells in the drawing. The
total number of cells in the drawing was counted by painting counted cells black with
Image J. Leaf blade area was measured by Image J on digital pictures. Average cell area
of each leaf was calculated by averaging cell areas of the 25% and 75% regions. Total
number of cells per leaf was estimated by dividing leaf blade area by the average cell area.
Stomatal index was the fraction of stomata in the total population of epidermal cells. Nine
first leaves from 9 independent plants were analyzed.

Mesophyll cells were drawn and scanned as described above for epidermal cells.
They were also processed by Image J: the cells were painted gray, and intercellular space
was kept white.
**Scanning electron microscopy (SEM)**

Plant samples were fixed in freshly made 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and 2% formaldehyde in 0.1 M phosphate buffer (pH 6.8) overnight at 4 ºC. After rinsing three times (5 min each time) in phosphate buffer, the samples were post-fixed in 2% buffered osmium tetroxide for 1 to 2 hours. The samples were dehydrated in 30, 50, 70, 80, 90, 95 and 100% ethanol (each for 15 min) and then stored in 100% ethanol. Critical point drying was carried out with either 100% ethanol or amyl acetate (Electron Microscopy Sciences, Hatfield, PA), and dried samples were mounted onto metal stubs with conductive adhesive. A very thin layer of metals (approximately 15 nm gold and palladium) was deposited on the sample surface using a sputter coater. 100% ethanol was stored with H$_2$O-absorb molecular sieves (Fisher Scientific, Hampton, NH).

**Histology**

A vibratome (Vibratome Company, St. Louis, MO) and a microtome were utilized to compare WT and *atagp19* plants on the tissue and cellular levels. Fresh, young hypocotyls and roots and glutaraldehyde-fixed petioles were embedded in 5% low melt
agarose (Fisher Scientific, Hampton, NH), and transverse sections 60 to 100 µm in thickness were obtained with the vibratome. The operating parameters for the vibratome were: speed, 2 (for hypocotyls and roots) or 8 (for petioles); vibration, 8-9; angle, 12-15 degrees.

A rotary microtome was used to section leaves, stems, hypocotyls and roots from mature WT and atagp19 plants. Plants that finished flowering were considered mature. Mature WT plants were approximately 6 weeks old, and mature atagp19 plants were 8 weeks old. Plant samples were harvested, fixed overnight in Safefix II (Fisher Scientific, Hampton, NH), dehydrated and embedded in paraffin. Sections 7-11 µm in thickness were cut, dried, dewaxed in Citrisolv (Fisher Scientific, Hampton, NH) and rehydrated in an ethanol series (100, 95, 70, 30%, each for 3 min). The sections were stained in either 0.05% toluidine blue or phloroglucinol/HCl for a few seconds. If necessary, stained sections were dehydrated and mounted in Permount (Fisher Scientific, Hampton, NH) before viewing under the microscope.

**Measurements and statistics**

Flowering time was determined by two criteria: first, when floral buds were visible without a magnifier; second, when the first flower opened. Reproduction was examined
in mature WT and atagp19 plants immediately after they finished flowering. Flowers and fully expanded siliques from the same positions on WT and atagp19 plants were compared, and the siliques close to either end of inflorescence stems were avoided.

Stem height was measured with a ruler; stem width, root length and leaf length and width were measured with Image-Pro software (Media Cybernetics, San Diego, CA). Lateral roots and abaxial trichomes were counted under a dissecting microscope. Measurements of sections were performed with SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI). Statistical analyses and data plotting were performed with Sigma Plot 8.0 (SPSS, Chicago, IL) and Microsoft Excel.

Vein Patterning

Cotyledons and leaves of 3-week-old WT and atagp19 plants were incubated in 100% methanol overnight to remove chlorophyll. Cotyledons were cleared in 90% lactic acid at RT overnight, while leaves were cleared in lactic acid at 70 °C for 3 h. Pictures were taken with a dark-field microscope.

An alternative protocol to clear leaves was to incubate leaves in 95% ethanol at 70 °C for 30 min and then in 1:1:1:1 (lactic acid: phenol: glycerol: H2O) at 90 °C for 30 min. This protocol proved to be better than the one described above.
Drought treatment

Water was withheld from 3-week-old plants for 2 weeks. The survival rate was recorded 6 d after rewatering.

Low humidity stress

WT and atagp19 seedlings grown horizontally on MS plates were challenged by low humidity by removing the lids from the plates. Seedlings were observed every 30 min for several hours to compare wilting of the cotyledons and leaves of WT and atagp19 plants.

Germination assays

Circular pieces of filter paper 9cm in diameter were placed in square petri dishes (Fisher Scientific, Hampton, NH) saturated with either ddH2O or indicated concentrations of osmoticums or ABA. Approximately 100-200 seeds were germinated in each plate. The plates were stratified at 4 °C in dark for 3 days and then moved to the tissue culture room with long day conditions. At least three independent experiments were performed
for control and each stress treatment.

For salt treatment, both ionic and non-ionic stress was tested: NaCl (100 mM and 175 mM) represented ionic stresses, and mannitol (200 mM and 350 mM) represented non-ionic stresses. Fresh salt solutions were made in ddH₂O each time. Radical emergence was used to determine germination. Germinated seeds were scored every day for 5 d (the day the plates were transferred to the tissue culture room was day 1).

For ABA responses, ABA stock solution (5 mM) in ethanol was stored at -20 °C. Four concentrations of ABA solutions (0.25 µM, 0.5 µM, 1 µM and 1.5 µM) were prepared fresh in ddH₂O each time. Seed germination was determined by cotyledon greening and scored at day 3 after plates being transferred to the tissue culture room.

**Auxin responses**

Approximately 100 WT and *atagp19* seeds were sown on MS plates containing 1/2X MS medium, 1% sucrose and 0.5% Agargel (Sigma-Aldrich, St. Louis, MO). The plates were stratified for 3 d in darkness and then placed vertically under long day conditions for an additional 3 d. The seedlings were transferred to MS plates with only the above ingredients or supplemented with IAA or 2,4-D. The seedlings were grown vertically for 3 more days, harvested and cleared in lactic acid. A DIC microscope was
used to score lateral root primoridea.

**Generation of double mutants**

Double mutants *atagp17 atagp19* and *atagp18 atagp19* were produced by crossing the SALK_101062 (*atagp17*) and SALK_117268 (*atagp18*) lines with the SALK_038728 (*atagp19*) line respectively, according to the method described by Weigel and Glazebrook (2002). Reciprocal crosses were performed, meaning each genotype was used as both the male and female parent. Homozygous double mutants were generated and confirmed by PCR and RT-PCR, and phenotypes were observed under normal growth conditions.

**Results**

*SALK_016294 failed to show mutated AtAGP17*

SALK_016294, was the only available T-DNA line of *AtAGP17* when this project started. However, PCR of all plants received from ABRC produced only bands of the WT size (data not shown), indicating the absence of a T-DNA insertion. Sequencing was carried out on PCR products from at least five individual plants, and the sequences were
identical to WT (data not shown). Southern blotting was not performed. Since no heterozygous or homozygous mutants were identified, this line was not studied further. Not long after this, another SALK T-DNA mutant line for *AtAGP17*, SALK_101062, became available.

*SALK_101062 was a knockout mutant line of AtAGP17*

This SALK line for *AtAGP17* became available after SALK_016294. Homozygous mutants were isolated. The insertion was in the first exon, leading to complete knockout of the *AtAGP17* expression. mRNA levels of *AtAGP18* and *19* did not change significantly in *atagp17* heterozygous and homozygous mutants (Figure 3.2).

The incomplete knockout mutant of *AtAGP17* (CS12955 or *rat1*) was shown to be deficient in binding of *Agrobacterium* to its roots in water and sucrose. It was expected that it would show similar, if not stronger, defects in *Agrobacterium* binding, since SALK_101062 was a null knockout mutant line of *AtAGP17*, The *Agrobacterium* binding assay was carried out with WT and *atagp17* roots in both water and a sucrose solution. In initial experiments, defects in *Agrobacterium* binding to *atagp17* roots were observed (Figure 3.3), similar to the previous report (Nam *et al.*, 1999); however, in a second experiment, no obvious differences were observed (data not shown).
Figure 3.2 SALK_101062 was a null knockout mutant of *AtAGP17*. HM, homozygous. HZ, heterozygous.

(a) The position of the T-DNA insertion in *AtAGP17*. The T-DNA insertion is not drawn to scale.

(b) RT-PCR of stem RNA showed the *AtAGP17* mRNA was absent in *atagp17*, but present in WT and *atagp17* heterozygous plants.

(c) Northern blotting of stem RNA corroborated with RT-PCR results.
Figure 3.3 *atagp17* roots were deficient in *Agrobacterium* binding. Arrow indicates *Agrobacterium* bound to roots.

(a) Binding of *Agrobacterium* to WT roots in water.
(b) *Agrobacterium* was not able to bind *atagp17* roots in water.
(c) Binding of *Agrobacterium* to WT roots in sucrose.
(d) *Agrobacterium* was not able to bind *atagp17* roots in sucrose, either.
Cotyledon and hypocotyl binding of *Agrobacterium* was also tested, and *atagp17* and WT behaved similarly. Shoots exhibited lower binding capacities than roots. Cotyledons and hypocotyls showed minimally binding in water; while in sucrose, binding of *Agrobacterium* to cotyledons and hypocotyls was enhanced, although still to a lesser extent compared to roots (data not shown).

Other phenotypic analyses, including plate-based growth stage phenotypic analysis and cytokinin responses analysis, were performed on the mutant seedlings. *AtAGP17* did not show striking differences from WT during the plate-based growth stage phenotypic analysis (Figure 3.4 and Table 3.3).

Since LeAGP1 is implicated in cytokinin signaling (Sun et al., 2004a), responses of *atagp17* plants to different concentrations of cytokinin were examined with respect to primary root elongation, lateral root number and chlorophyll content (Figure 3.5). Consistent with previous reports, concentrations of cytokinin higher than 0.005 µM inhibited root elongation and lateral root formation in WT plants; chlorophyll levels also decreased in response to high concentrations of cytokinin. However, no appreciable differences were observed in *atagp17* mutants which behaved similarly to WT.
Figure 3.4 Early development of WT and *atagp17*.
Rosette leaf number per plant, lateral root number per plant and primary root length of WT and *atagp19* seedlings were recorded at the end of the plate-based growth stage analysis (see Table 3.3 for statistical analysis of WT and *atagp17* growth stage development). Error bars indicate SE ($n > 50$).
<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Description</th>
<th>WT Days</th>
<th>atagp17 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>Stratification</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>0.50</td>
<td>Radical visible</td>
<td>4.05 ± 0.21</td>
<td>4.04 ± 0.19</td>
</tr>
<tr>
<td>0.70</td>
<td>Hypocotyl and cotyledons visible</td>
<td>5.22 ± 0.42</td>
<td>5.09 ± 0.29</td>
</tr>
<tr>
<td>1.00</td>
<td>Cotyledons fully expanded</td>
<td>7.65 ± 0.63</td>
<td>7.86 ± 0.40</td>
</tr>
<tr>
<td>1.02</td>
<td>2 true leaves &gt;1mm</td>
<td>9.19 ± 0.39</td>
<td>9.08 ± 0.28</td>
</tr>
<tr>
<td>1.04</td>
<td>4 true leaves &gt;1mm</td>
<td>11.06 ± 0.45</td>
<td>10.94 ± 0.35</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n > 100).
Figure 3.5 Responses of atagp17 to treatment with exogenous cytokinin BAP. Seedlings were grown vertically on MS plates supplemented with indicated concentrations of BAP.
(a) Elongation of primary roots between days 4 and 7. Error bars represent SE (n > 20).
(b) Lateral root numbers of 7-d-old seedlings. Error bars represent SE (n > 20).
(c) Chlorophyll measurement in 14-d-old seedlings. Error bars represent SE (n = 4).
GT6565 was a gene trap line of \textit{AtAGP18}

GT6565, a gene trap line of \textit{AtAGP18} (Martienssen, 1998), was attained via the \textit{Ac/Ds} transposable system. The \textit{Ds} element carries a \textit{GUS} gene and lacks a promoter. Therefore, only when the \textit{Ds} element is present within a chromosomal gene can the \textit{GUS} gene be expressed, in a way mimicking that of the chromosomal gene. Theoretically, \textit{GUS} expression in GT6565 can be assayed for the normal expression pattern of \textit{AtAGP18}. In addition, since the gene is disrupted by the \textit{Ds} element, mutant phenotypes may be observed.

By PCR, it was confirmed that T3 seeds sent by CSHL were homozygous for the transposon insertion (data not shown). However, neither GUS activity nor abnormal phenotypes of GT6565 plants were detected; the positive control \textit{P\textsubscript{DRS}:GUS} seedlings exhibited GUS staining with the same GUS staining solution (data not shown). RT-PCR analysis indicated similar levels of the \textit{AtAGP18} transcript in GT6565 plants compared to the WT (data not shown). These observations might be because the insertion was close to the end of \textit{AtAGP18} second exon. It was still likely that the transcript size was altered in the inserted plants, but this possibility was not examined experimentally.
AtAGP18 mRNA in SALK_117268 was truncated

SALK_117266 and SALK_117268 were both putative mutant lines of *AtAGP18*. SALK_117266 was ordered but not studied. This was because no primers could be designed with the SIGnAL T-DNA verification primer design tool. SALK_117268 was verified by PCR to have a T-DNA insertion close to the end of *AtAGP18* (Figure 3.6a). The homozygous mutants were obtained, and *AtAGP18* message was examined in the mutant with RT-PCR and Northern blotting. Although the transcript level was not altered (Figure 3.6b), a slightly smaller size of the transcript was observed (Figure 3.6c). Therefore, the T-DNA insertion in the second exon of *AtAGP18* resulted in truncated *AtAGP18* mRNA expressed at WT levels and did not seem to affect expression of *AtAGP17* and 19.

An *Agrobacterium* binding assay was carried out with *atagp18* roots and shoots in both water and sucrose solution. No obvious differences were found between WT and *atagp18* seedlings (data not shown). SALK_117268 plants were also tested for the responses to different concentrations of cytokinin BAP with respect to primary root elongation, number of lateral roots and chlorophyll content (Figure 3.7). Similar to WT, SALK_117268 plants exhibited reduced root elongation, lateral root formation and chlorophyll levels in the presence of cytokinin concentrations higher than 0.005 µM.
Figure 3.6 RT-PCR and Northern blotting results of SALK_117268.
(a) The position of the T-DNA insertion in *AtAGP18*. The T-DNA insertion is not drawn to scale.
(b) RT-PCR revealed similar levels of *AtAGP18* mRNA in WT and mutant seedlings.
(c) Northern blotting of WT seedling (1) and stem (2) as well as SALK_117268 seedling (3, 4) and stem (5) RNA showed a slightly truncated *AtAGP18* transcript in the mutant. Stripping and reprobing the same membrane with the *AtAGP19* probe indicated the size shift was specific to *AtAGP18* mRNA and *AtAGP19* transcript levels were similar in *atagp18* and WT.
Figure 3.7 Responses of SALK_117268 plants to exogenous cytokinin BAP.
(a) Primary root elongation between days 4 and 7. Error bars represent SE (n > 20).
(b) Lateral root numbers of 7-d-old seedlings. Error bars represent SE (n > 20).
(c) Chlorophyll content of 14-d-old seedlings. Error bars represent SE (n = 4).
Occasionally the mutants showed overbranching phenotypes (data not shown), mimicking \textit{AtAGP18} RNAi plants (see Chapter 4). Nonetheless, these mutant phenotypes were not consistently observed, and no further effort was made to study the phenotypes of this specific mutant line. Since no mutant phenotypes could be conclusively associated with SALK\textsubscript{117268} plants, it was likely that, despite the truncated \textit{AtAGP18} transcript, the mutant still expressed functional AtAGP18 protein due to the position of the T-DNA insertion. However, AtAGP18 protein was not studied in the mutant.

Besides SALK T-DNA and CSHL lines, more \textit{AtAGP18} mutant alleles are available from other mutant collections, including GABI-Kat (Table 3.1). These mutants should be ordered and examined for a null mutant line of \textit{AtAGP18}.

\textit{atagp19} was a null mutant of \textit{AtAGP19}

SALK\textsubscript{038728} (Figure 3.8a,b) was the only insertion mutant line available for \textit{AtAGP19}. No \textit{AtAGP19} mRNA was detected in \textit{atagp19} by RT-PCR or Northern blotting (Figure 3.8c and data not shown), indicating that it was a null knockout mutant. \textit{atagp19} was the most intensely studied mutant in this research project, because it showed both widespread and dramatic phenotypes with respect to vegetative and reproductive growth of \textit{Arabidopsis} life cycle, in contrast to \textit{AtAGP17} and \textit{AtAGP18} mutants that had virtually...
Figure 3.8 atagp19 is a null knockout mutant.

(a) The position of the T-DNA insertion in AtAGP19. The T-DNA insertion is not drawn to scale. The arrows indicate the positions of primers used for RT-PCR.
(b) PCR screening of WT, atagp19 homozygous (HM) and atagp19 heterozygous (HZ) plants.
(c) RT-PCR of total stem RNA showed that AtAGP19 mRNA was absent in the atagp19 mutant but restored in the complemented plants. Actin mRNA was used as the internal control. The results were confirmed by Northern blotting (data not shown).
no mutant phenotypes. Complementation of the *atagp19* mutant with the WT *AtAGP19* gene under the control of its own promoter restored *AtAGP19* mRNA (Figure 3.8c) as well as the WT phenotypes, confirming the mutant phenotypes were caused by the knockout of *AtAGP19*. The phenotypes of *atagp19* were analyzed in detail and are reported below.

*Molecular phenotypes*

Since there are three Lys-rich AGPs in *Arabidopsis*, it was important to know whether the two homologs of *AtAGP19*, namely *AtAGP17* and *18*, were compensating for *AtAGP19* in the *atagp19* null mutant. As shown in Figure 3.9, both *AtAGP17* and *18* transcription was down-regulated in the stem. In complemented mutants, *AtAGP17* and *18* expression returned to normal levels. In *atagp19* seedlings, while *AtAGP18* mRNA was elevated, the *AtAGP17* mRNA level showed little change. In contrast, in mutant rosettes, *AtAGP18* expression did not change, while the *AtAGP17* mRNA level increased.
Figure 3.9 Northern blotting of AtAGP17 and 18 in atagp19.
Lane 1, WT seedling. Lane 2, atagp19 seedling. Lane 3, WT rosette. Lane 4, atagp19 rosette. Lane 5, WT stem. Lane 6, atagp19 stem. Lane 7 to 10, stem RNA of complemented atagp19 mutant lines 3, 6, 10 and 14. rRNAs were stained with ethidium bromide to show equal RNA loading.
atagp19 grew slower and had fewer lateral roots

Early growth and development of atagp19 was studied using the plate-based growth stage phenotypic analysis, which also provided information on root development (Boyes et al., 2001). The results are summarized in Table 3.4. atagp19 and WT germinated at the same time at a 100% germination rate, indicating that atagp19 was not defective in germination. However, atagp19 seedlings showed delayed growth after hypocotyl and cotyledon emergence. At the end of the 13-d analysis period, atagp19 mutants had developed fewer rosette leaves and lateral roots and had shorter primary roots. As the plants aged, the mutant consistently had fewer lateral roots (Figure 3.10).

Decreased numbers of lateral roots in atagp19 could be due to impaired lateral root initiation or elongation. In order to specify the aspect of lateral root development that atagp19 was defective in, seedlings were grown vertically on half strength MS medium, which promoted lateral root development and primary root elongation, for 6 d. Counting of lateral root primordia at different stages on cleared roots under a DIC microscope indicated that it was lateral root initiation, and not elongation, that was impaired in atagp19 (data not shown). Despite these root abnormalities, atagp19 had normal root cell architecture (Figure 3.11a) and did not have defects in Agrobacterium-binding ability as reported for atagp17 (data not shown).
Table 3.4 Plate-based growth stage analysis of WT and *atagp19* seedlings

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Description</th>
<th>WT Days</th>
<th><em>atagp19</em> Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Stratification</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>0.5</td>
<td>Radicle visible</td>
<td>4.2 ± 0.2</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>0.7</td>
<td>Hypocotyl and cotyledons visible</td>
<td>5.5 ± 0.2</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>1.0</td>
<td>Cotyledons full expanded</td>
<td>6.8 ± 0.2</td>
<td>8.0 ± 0.3*</td>
</tr>
<tr>
<td>1.02</td>
<td>2 true leaves &gt;1mm</td>
<td>8.8 ± 0.2</td>
<td>9.6 ± 0.2*</td>
</tr>
<tr>
<td>1.04</td>
<td>4 true leaves &gt;1mm</td>
<td>11.3 ± 0.2</td>
<td>12.6 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± 95% confidence interval (*n* > 40).

*Data from *atagp19* were significantly different from WT (*α* = 0.05).
Figure 3.10 \textit{ata gp}19 had fewer lateral roots throughout the life cycle. 
(a) Rosette leaf numbers, lateral root numbers and primary root length of WT and \textit{ata gp}19 seedlings at the end of the plate-based growth stage analysis (see Table 3.4 for statistical analysis of WT and \textit{ata gp}19 growth stage development). Error bars indicate SE ($n > 50$).
(b) 18-d-old \textit{ata gp}19 seedlings produced fewer lateral roots than WT of the same age.
(c) and (d) Mature WT plants had bigger and more roots than \textit{ata gp}19.
Scale bars = 1 cm.
Figure 3.11 Roots and hypocotyls of WT and *atagp19*.
(a) Transverse sections of 6-d-old WT (left) and *atagp19* (right) roots showing that WT and *atagp19* roots had similar root cell architecture.
(b) WT seedlings (top) had longer hypocotyls than *atagp19* (bottom). Seven-d-old seedlings grown in soil under a low light intensity were compared.
(c) Transverse sections of hypocotyls from 7-d-old WT (left) and *atagp19* (right) grown on MS plates showing that WT and *atagp19* hypocotyls had similar cellular organization. (a) and (c) were stained with toluidine blue. Scale bars = 25 µm in (a), 1cm in (b) and 50 µm in (c).
Hypocotyl cell length in atagp19 was reduced

Atagp19 hypocotyls were 75% of the length of WT when grown under long day conditions (Figure 3.11b), which corresponded to a reduction in hypocotyl cell length, but not cell number. However, hypocotyl length was not compromised when atagp19 seedlings were grown in the dark (Table 3.5). These results indicated that AtAGP19 function was regulated by light. Moreover, although cell length was reduced in the light-grown mutant, hypocotyl diameter, cell width and cell layers were all similar to WT (Figure 3.11c and Table 3.5).

Atagp19 had reduced pigmentation

Atagp19 mutants, including leaves, stems and sepals, were lighter green in color than WT plants throughout the life cycle. Pigment content analyses demonstrated that atagp19 rosette leaves contained less chlorophyll and anthocyanin compared to WT (Figure 3.12a). Microscopic analysis showed that there was no significant difference in chloroplast numbers in mesophyll cells of similar sizes between the mutant and WT (Figure 3.12b). It was difficult to tell if there were morphological differences between WT and atagp19 chloroplasts on the DIC level, but sometimes, there were many tiny green granules in
Table 3.5 Hypocotyl parameters of WT and atagp19 seedlings grown on MS media

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Parameters</th>
<th>WT</th>
<th>atagp19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypocotyl length (mm)</td>
<td>2.8 ± 0.1</td>
<td>2.1 ± 0.1*</td>
</tr>
<tr>
<td>Light</td>
<td>Hypocotyl cell number</td>
<td>18.1 ± 0.8</td>
<td>19.8 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td>Average epidermal cell length (µm)</td>
<td>155.9 ± 6.4</td>
<td>110.3 ± 8.4*</td>
</tr>
<tr>
<td>Light</td>
<td>Hypocotyl diameter (µm)</td>
<td>275.7 ± 14.2</td>
<td>267.1 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>Average epidermal cell width (µm)</td>
<td>11.0 ± 0.7</td>
<td>11.1 ± 0.6</td>
</tr>
<tr>
<td>10µM GA</td>
<td>Hypocotyl length (mm)</td>
<td>4.5 ± 0.3</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>Dark</td>
<td>Hypocotyl length (mm)</td>
<td>16.2 ± 0.5</td>
<td>16.5 ± 1.3</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± 95% confidence interval.

*Data from atagp19 were significantly different from WT (α = 0.05).
Figure 3.12 *atagp19* had reduced pigmentation.
(a) *atagp19* had lower chlorophyll and anthocyanin levels than WT. Error bars indicate SE ($n = 5$).
(b) Chloroplast numbers did not significantly alter in *atagp19* mesophyll cells. Left, WT. Right, *atagp19*. 
atagp19 mesophyll cells, which were not seen in WT. Whether this was indeed a phenotype or just an artifact needs to be determined.

**atagp19 had altered leaf morphology**

During vegetative growth, atagp19 had fewer rosette leaves compared to WT plants at the same age. Moreover, atagp19 rosette leaves were smaller and more round and had shorter petioles than WT (Figure 3.13).

In order to explore causes of the smaller leaf size, the abaxial epidermis of the first two true leaves of WT and atagp19 were studied. Both the size and number of the epidermal cells were found to be decreased in the mutant. However, stomata cells appeared normal in terms of morphology and fraction of total epidermal cells (Figure 3.14).

Leaves of atagp19 are more round compared to their WT counterparts, resulting in lower leaf length/leaf width ratios (Table 3.6). Therefore, it is likely that atagp19 leaf development was arrested in the juvenile phase. To study this possibility, the presence of abaxial trichomes on atagp19 and WT rosette leaves was scored to determine the number of juvenile leaves, given that juvenile leaves do not have abaxial trichomes (Bollman et al., 2003). Mutants were thus found to have more juvenile leaves than WT (Table 3.7).
Figure 3.13 Morphology differences between WT and atagp19 rosettes.
(a) 3-week-old rosettes of WT (left), atagp19 (middle) and complemented plants (right).
(b) Rosette leaves of WT (top), atagp19 (middle) and complemented plants (bottom).
(c) Trichomes of atagp19 have normal morphology.
Bars = 1 cm in (a) and (b) and 100 μm in (c).
Figure 3.14 Epidermis of *atagp19* first leaves contained smaller and fewer cells. 
(a) 3-week-old first leaves of *atagp19* were only half the size of WT, consistent with smaller and fewer cells in *atagp19* abaxial epidermis. However, *atagp19* and WT had similar stomata to total epidermal cell ratios. Error bars indicate SE ($n = 9$). 
(b) Drawing tube images of the abaxial epidermis of WT (top) and *atagp19* (bottom) plnats. Bars = 50 µm.
Table 3.6 Morphometry of the longest rosette leaf in mature WT and *atagp19* plants

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th><em>atagp19</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf length (cm)</td>
<td>5.19 ± 0.22</td>
<td>2.77 ± 0.15</td>
</tr>
<tr>
<td>Petiole length (cm)</td>
<td>1.51 ± 0.11</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>Leaf width (cm)</td>
<td>1.56 ± 0.07</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>Length/width ratio</td>
<td>3.33 ± 0.12</td>
<td>2.30 ± 0.08</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± 95% confidence interval (n > 10). All data from *atagp19* were significantly different from WT (α=0.05).
Table 3.7 Flowering time and leaf numbers of WT and *atagp19* plants

<table>
<thead>
<tr>
<th></th>
<th>Days</th>
<th>Juvenile leaf number</th>
<th>Rosette leaf number</th>
<th>Cauline leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>31.6 ± 0.5</td>
<td>4.5 ± 0.2</td>
<td>15.2 ± 0.4</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td><em>atagp19</em></td>
<td>37.6 ± 1.3</td>
<td>6.2 ± 0.3</td>
<td>13.7 ± 0.5</td>
<td>2.6 ± 0.2</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± 95% confidence interval (*n* > 40). All data from *atagp19* were significantly different from WT (*α*=0.05).
The trichomes appeared normal (Figure 3.13c).

As plants matured, WT rosette leaves curled down, and *atagp19* mutant leaves remained flat (Figure 3.15a) or even curled upwards (data not shown). To determine the cause of flat mutant leaves, a DIC microscope was used to visualize mesophyll cells. Palisade mesophyll cells were smaller in the mutant than in WT (Figure 3.15b,c). Whereas spongy mesophyll cells in the *atagp19* mutant were more regular in shape and more densely packed than in WT (Figure 3.15d,e), this abnormal cellular packing may result in flat leaves in *atagp19*. In contrast, spongy mesophyll cells in WT were irregular in shape and characterized by large intercellular spaces. *atagp19* leaves were thinner, which was probably due to the compact spongy mesophyll layers with little intercellular space (Figure 3.16a,b).

*atagp19* had shorter petioles

The petioles of *atagp19* were shorter, contributing to higher blade length/petiole length ratios (Figure 3.17a and Table 3.6). However, cells in WT and *atagp19* petioles were of similar length (Figure 3.17b,c) and cellular organizations (Figure 3.17c), suggesting that a reduction in cell division, instead of altered cell expansion, caused shortened petioles in *atagp19*. 
Figure 3.15 Mature *atagp19* rosette leaves are flat.
(a) 49-d-old rosettes of WT (left) and *atagp19* plants (right). The inflorescence stems were removed from the rosettes before the picture was taken.
(b) and (c) Paradermal views of palisade mesophyll cells in WT and *atagp19* third leaves, respectively.
(d) and (e) Paradermal views of spongy mesophyll cells in WT and *atagp19* third leaves, respectively.
Gray represents cells, and white represents intercellular space. Similar phenotypes were observed with WT and *atagp19* first and fifth leaves, too. At least 10 leaves in total were examined.
Bars = 1 cm in (a) and 10 µm in (b) to (e).
Figure 3.16 Leaf phenotypes of *atagp19*.
(a) and (b) Transverse sections of WT and *atagp19* leaves. AB, abaxial epidermis. AD, adaxial epidermis. PA, palisade mesophyll. SP, spongy mesophyll.
(c) and (d) Transverse sections of WT and *atagp19* leaves showing the midvein. P, phloem. X, xylem.
Bars = 100 µm.
Figure 3.17 Petiole phenotypes of *atagp19*.
(a) Fully expanded rosette leaves of *atagp19* had higher blade/petiole ratios than the WT counterparts. Error bars indicate SE (*n* = 10).
(b) and (c) Longitudinal sections of WT and *atagp19* elongated petioles showing cells of similar length in WT and *atagp19* petioles.
(d) Transverse sections of WT (left) and *atagp19* (right) petioles of the third leaves showing that WT and *atagp19* petioles had similar cellular organization.
(b) to (d) were stained with toluidine blue.
Bars = 100 µm.
atagp19 had normal venation

Because atagp19 had abnormal leaf shape and size, and the promoter activity of AtAGP19 was consistently associated with leaf vasculature (Chapter 2), vein patterns were examined in atagp19. The vascular tissues are arranged collaterally in WT and atagp19 leaves, with xylem located towards the adaxial epidermis (Figure 3.16c,d). atagp19 also displayed normal venation in cotyledons and foliar leaves with respect to both vein complexity and continuity (Figure 3.18 and Table 3.8).

Delayed flowering in atagp19

Emergence of floral buds and opening of the first flower were used as criteria to determine flowering time. Floral buds were visible in WT plants approximately 25 d after sowing, and 29 d in the case of atagp19 mutants. The first flower opened in 31-d-old WT and 37-d-old atagp19 plants. Many late flowering Arabidopsis mutants have more rosette and cauline leaves compared to WT plants (Koornneef et al., 1991), but atagp19 did not (Table 3.7). Since rosette leaf number serves as a cue for flowering and Arabidopsis plants, under standard growth conditions, flower after reaching a certain number of rosette leaves, delayed flowering of atagp19 was probably caused by its slower
Figure 3.18 *atagp19* cotyledons and leaves had normal venation patterns. (a) and (b) Cleared cotyledons of WT and *atagp19* plants, respectively. (c) and (d) Cleared first leaves of WT and *atagp19* plants, respectively. Bars = 1mm.
Table 3.8 Quantitative analysis of cotyledon venation complexity

<table>
<thead>
<tr>
<th></th>
<th>No. examined</th>
<th>Number of areoles</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>WT</td>
<td>202</td>
<td>55 (27.2%)</td>
<td>81 (40.1%)</td>
<td>66 (32.7%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><em>atagp19</em></td>
<td>117</td>
<td>34 (29.1%)</td>
<td>46 (39.3%)</td>
<td>37 (31.6%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

The proportion of cotyledons containing each number of areoles (spaces delimited by veins) over the total number of cotyledons examined was indicated in parentheses.
vegetative growth and in particular its slower leaf initiation rate.

*Secondary growth was highly reduced in atagp19*

The hypocotyl and root of *Arabidopsis* undergo secondary growth to increase vascular cylinder diameter and organ thickness (Dolan and Roberts, 1995; Chaffey *et al.*, 2002). Secondary xylem development in hypocotyls occurs in two phases. In phase I, only vessel elements differentiate, and the rest of the cells from vascular cambium division remain as thin-walled xylem parenchyma cells; and in phase II, vessels and fibers form (Chaffey *et al.*, 2002). The lignified xylem cell walls (of vessels and fibers) stained bright blue with toluidine blue, appeared red with phloroglucinol/HCl and glowed under polarized light, and cells with thin walls (i.e. xylem parenchyma and phloem) were purple after toluidine blue staining and were not stained by phloroglucinol/HCl.

While young hypocotyls and roots of *atagp19* had similar architecture and diameter (Figure 3.11a,c) to their WT counterparts, mature *atagp19* hypocotyls and roots were much thinner than those of WT (Figure 3.10c,d and Table 3.9). This was mainly due to diminished secondary xylem area resulting from reduced secondary growth in *atagp19* (Figure 3.19). Moreover, although phase I regions of mature WT and *atagp19* hypocotyls were similar in size, the radius of the phase II region in *atagp19* was only 30% of that of
Table 3.9 Histological analysis of mature WT and *atagp19* roots and hypocotyls

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th><em>atagp19</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Root radius (µm)</td>
<td>517.2 ± 55.9</td>
<td>278.2 ± 8.8*</td>
</tr>
<tr>
<td>Root secondary xylem radius (µm)*</td>
<td>334.1 ± 27.4</td>
<td>180.3 ± 5.5*</td>
</tr>
<tr>
<td>Hypocotyl xylem phase I radius (µm)*</td>
<td>147.1 ± 13.2</td>
<td>134.0 ±16.1</td>
</tr>
<tr>
<td>Hypocotyl xylem phase II radius (µm)*</td>
<td>244.7 ± 22.9</td>
<td>69.6 ± 4.1*</td>
</tr>
</tbody>
</table>

*aThe measurement is illustrated in Figure 5.

Values are expressed in mean ± 95% confidence interval (*n* = 6).

*Data from *atagp19* were significantly different from WT (*α* = 0.05).
Figure 3.19 Secondary growth in *atagp19* hypocotyls and roots was reduced. 
(a) and (b) Transverse sections of mature WT and *atagp19* hypocotyls, respectively. X, secondary xylem. P, secondary phloem. 
(c) and (d) Views of (a) and (b) under polarized light, respectively. I, phase I. II, phase II. Radii of hypocotyl secondary xylem phase I and II regions were measured. 
(e) and (f) Transverse sections of mature WT and *atagp19* roots, respectively. R, root secondary xylem radius. See Table 3.9 for statistical analysis of root secondary xylem as well as hypocotyl xylem phase I and II radii. 
(a) and (b) were stained with toluidine blue; (e) and (f) were stained with phloroglucinol. Bars = 100 µm.
WT (Table 3.9). Therefore, only phase II xylem growth was affected in *atagp19*. No distinctive alterations were noted in secondary phloem or periderm.

*atagp19 showed distinctive stem phenotypes*

The mutant inflorescence stems were shorter, thinner (Figure 3.20a,b and Table 3.10), and tended to recline (data not shown). Epidermal and cortex layers appeared normal except that cortex cells in *atagp19* were collapsed (Figure 3.20c,d).

The vascular bundles in *atagp19* stems have a normal collateral pattern (xylem towards the inside and phloem towards the outside), and they were more flattened than those in WT (Figure 3.20e to h). Indeed, the radii of the fascicular xylem and interfascicular fiber were reduced in half compared to the WT (Table 3.10), consistent with the notion that differentiation processes of xylem and fiber share a common molecular mechanisms (Zhong *et al.*, 2001). Little difference was seen with respect to secondary wall thickness in xylem elements or interfascicular fibers.

Thinner mutant stems were largely due to a lower number of pith cells, with normal cell size, across the *atagp19* stem diameter (Figure 3.20a,b and Table 3.10), indicating division of pith cells was reduced in *atagp19*. Similarly, shorter *atagp19* stems were attributed to reduced cell division since pith cell length was not compromised (Figure
Figure 3.20 Stem phenotypes of *atagp19*.
(a) and (b) Transverse sections of WT and *atagp19* stems, respectively.
(c) and (d) The cortical cells were collapsed in *atagp19* stems (d), but not in WT (c).
(e) and (f) Xylem areas in *atagp19* stems (f) were smaller than those in WT (e). Radii of fascicular xylem and interfascicular region were measured (Table 3.10).
(g) and (h) WT and *atagp19* stem sections viewed with polarized light, respectively.
(i) and (j) Pith cells from the base of mature WT and *atagp19* stems had similar length.
(a) to (d), (i) and (j) were stained with toluidine blue; (e) and (f) were stained with phloroglucinol/HCl. CO, cortex. EP, epidermis. FX, fascicular xylem. IF, interfascicular region. PI, pith. Bars = 100 µm.
Table 3.10 Histological analysis of mature WT and atagp19 stems

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>atagp19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem length (cm)</td>
<td>33.8 ± 1.1</td>
<td>20.1 ± 1.2</td>
</tr>
<tr>
<td>Stem radius (µm)</td>
<td>580.3 ± 97.8</td>
<td>329.9 ± 36.1</td>
</tr>
<tr>
<td>Side bolts from shoot apex (&gt; 1 cm)</td>
<td>1.3 ± 0.3</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Auxillary branches</td>
<td>3.1 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Pith cell number across the center</td>
<td>15.5 ± 0.9</td>
<td>8.9 ± 0.8</td>
</tr>
<tr>
<td>Number of vascular bundles</td>
<td>8.0 ± 0.0</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>Fascicular xylem radius (µm)</td>
<td>170.2 ± 20.6</td>
<td>87.0 ± 11.3</td>
</tr>
<tr>
<td>Interfascicular radius (middle part) (µm)</td>
<td>132.6 ± 9.2</td>
<td>79.3 ± 7.2</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± 95% confidence interval (n > 10).
All data from atagp19 were significantly different from WT (α=0.05).
Therefore, division of stem pith cells was affected in *atagp19*.

It has been reported that WT *Arabidopsis* stems typically contained 6 to 8 discrete vascular bundles separated by interfascicular regions (Turner and Somerville, 1997; Jun *et al.*, 2002). We found that all 15 WT stems examined consisted of 8 vascular bundles; while among 16 *atagp19* stems, 3 (~20%) had only 5 vascular bundles, and the rest had 6 to 8 bundles (Table 3.10).

*Compromised fertility in atagp19*

*atagp19* had shorter, more slender inflorescence stems with fewer auxillary branches and side bolts (Figure 3.21a and Table 3.11). *atagp19* produced fewer flowers than WT (Figures 3.21b,c and 3.22a,b). *atagp19* also had fewer and shorter siliques, fewer seeds per silique and a higher percentage of sterile siliques (Table 3.11), resulting in less seed production. More than half of the *atagp19* flowers were fertile; and while they were smaller than WT flowers, they opened normally and had normal arrangements and numbers of floral organs (Figures 3.21d and 3.22c,d). Some *atagp19* sterile flowers were open while others remained closed (Figure 3.21e). One reason for sterility was the failure of the stamens to elongate beyond the pistil at floral stage 14 (Figures 3.21f,g and 3.22e), while in WT, the stamens were longer than the pistil and brushed against the stigma to
Figure 3.21 Inflorescence and silique phenotypes of atagp19.  
(a) Inflorescence stems of WT (left), atagp19 (middle) and complemented plants (right). 
(b) and (c) Inflorescence of WT and atagp19.
(d) A WT flower (left) was larger than a fertile *atagp19* flower (right), but the floral organization was similar.

(e) Top view of a closed *atagp19* flower. Sometimes the carpel extended beyond the sepals, as in this case.

(f) A dissected closed *atagp19* flower showing abnormal petal and stamen development.

(g) A sterile *atagp19* flower had the carpel longer than stamens at floral stage 14, preventing pollination.

(h) Siliques of WT (top) and *atagp19* (bottom) plants. The siliques shown were of representative lengths. *atagp19* contained an abnormally high proportion of short/sterile siliques (Table 3.11).

(i) *atagp19* (middle and bottom) had higher aborted ovule rates than WT (top). Middle silique corresponded to longest *atagp19* silique, and bottom silique belonged to middle length range in (g).

Bars = 1 cm in (a) and (h), 1 mm in (b) to (d) and (i), and 0.5 mm in (e) to (g).
Table 3.11 Reproduction comparison between WT and atagp19 plants

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>atagp19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silique number</td>
<td>121.0 ± 21.4</td>
<td>19.4 ± 3.2</td>
</tr>
<tr>
<td>Silique length (mm)</td>
<td>13.5 ± 0.4</td>
<td>10.8 ± 0.4</td>
</tr>
<tr>
<td>Seeds per silique</td>
<td>54.0 ± 2.4</td>
<td>28.2 ± 4.6</td>
</tr>
<tr>
<td>Sterile siliques (%)</td>
<td>4.0 ± 0.5</td>
<td>31.5 ± 5.1</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± 95% confidence interval (n > 30).
All data from atagp19 were significantly different from WT (α=0.05).
Figure 3.22 SEM of WT and *atagp19* flowers.
(a) and (b) Inflorescence of WT and *atagp19*, respectively.
(c) A WT flower.
(d) A normal *atagp19* flower.
(e) A sterile *atagp19* flower that had short stamens when it opened.
(f) Stamens of *atagp19* dehiscence normally.
Scale bars= 2 mm in (a) and (b), 1 mm in (c) to (e) and 200 μm in (f).
allow for pollination and fertilization (Smyth et al., 1990). Stamens of open *atagp19* flowers had normal morphology and dehiscence time (Figure 3.22f). Siliques of *atagp19* could be divided into three length ranges, with the longest ones being slightly shorter than WT siliques (Figure 3.21h). The shortest *atagp19* siliques were completely sterile, while the medium-length siliques were semi-sterile, bearing less than 5 seeds per silique. Even when pollination occurred normally, there was a high percentage of aborted ovules without signs of early seed development (Figure 3.21i); therefore, shorter stamens do not seem to be the only cause of sterility. Moreover, reciprocal crosses suggested that female reproduction was possibly defective in *atagp19*, because few seeds were recovered when *atagp19* plants were used as female parents, and normal fertility was observed with *atagp19* plants as the male parents (data not shown).

SEM of WT and *atagp19* floral organs only identified two morphological differences between them (Figure 3.23). First, *atagp19* sepal epidermal cells looked more collapsed. Second, *atagp19* petal epidermal cells were pointier, while they were rather flat in WT. More work, however, is needed to confirm these were real phenotypic differences.
Figure 3.23 Close-up SEM of WT and *atagp19* flowers.
(a) and (b) Unopened floral buds of WT and *atagp19*, respectively.
(c) and (d) Distal portions of WT and *atagp19* sepals from (a) and (b), respectively.
(e) and (f) Distal portions of WT and *atagp19* petals, respectively.
(g) and (h) Pedicles of WT and *atagp19* immediately below flowers.
Scale bars = 1 mm in (a) and (b), 100 μm in (c) and (d) and 50 μm in (e) to (h).
*atagp19 was more resistant to drought stress*

Although *atagp19* showed multiple phenotypes under normal conditions, the mutants were also challenged with stresses to provide more insight into the function of AtAGP19. The first stress challenge was drought. 3-week-old WT and *atagp19* plants grown side by side in the growth chamber were not watered for 2 weeks. All WT rosettes wilted, while all mutant plants appeared normal. Six days after rewatering, only 30% WT were alive (Figure 3.24). Although *atagp19* mutants may truly be drought-resistant, one possible reason to explain this is that *atagp19* plants were smaller when they were of the same age of WT, therefore they might need less water to grow or stay alive.

Consistently, *atagp19* seemed to be more resistant to low humidity stress. When plants grown on petri dishes were exposed to air (low humidity stress) for 2 h, WT leaves wilted, but *atagp19* did not (data not shown).

*Salt treatment*

Two salt treatments were applied to WT and *atagp19* plants. One was to water 3-week-old plants with 300 mM NaCl solution every three days for 3 times and then to observe survival rate after 10 days. No conclusion could be drawn from the observations.
Figure 3.24 *atagp19* plants were more drought tolerant. Water was withheld from 3-week-old WT and *atagp19* plants for 2 weeks. (a) WT (left) and *atagp19* (right) plants at the end of drought treatment. (b) WT (left) and *atagp19* (right) plants at day 6 after rewatering. During drought treatment, WT growth was completely arrested.
The plants were placed side by side in the same tray, but there was great variation even among pots growing plants of the same genotype (from 0% to 100% survival), and eventually all WT and atagp19 plants died. The experiment was not repeated.

Another salt treatment was to germinate WT and mutant seeds in ionic (NaCl) and non-ionic (mannitol) osmotic solutions (Figure 3.25). atagp19 generally showed lower germination rates in response to NaCl treatment and seemed to be more sensitive to ionic stress. Although more atagp19 seeds germinated in presence of non-ionic stress (mannitol) initially, WT seeds eventually caught up. Large fluctuations were observed among samples of the same treatment prepared at different times, as previously reported (van Hengel and Roberts, 2003). It was suggested that WT and mutant seeds used for germination assays should be collected at the same time from plants grown under the same conditions, and the seeds should be stored for the same period of time under the same conditions. In addition, parallel experiments should be performed on the seeds collected from different plants at different times.

*atagp19 was more resistant to ABA*

WT and *atagp19* responses to ABA were examined by germinating seeds in four concentrations of ABA (0.25 µM, 0.5 µM, 1 µM and 1.5 µM). Although there were
Figure 3.25 Germination of WT and *atagp19* seeds in various osmotic solutions.
Germination was examined in the presence of 100 mM NaCl (a), 175 mM NaCl (b), 200 mM mannitol (c) and 350 mM mannitol (d). Error bars represent SE ($n = 3$) for (a) and (b) and SE ($n = 5$) for (c) and (d). On water control plates, 100% germination was reached after the first 24 h. Seeds with protruding radicals were determined as germinated.
variations among samples in the same treatment, \textit{atagp19} appeared to be more resistant than WT to ABA at high concentrations (> 1 µM) of ABA (Figure 3.26).

\textit{Seedling cytokinin response assays}

Due to reported connection between \textit{LeAGP1} and cytokinin signaling (Sun \textit{et al.}, 2004a), responses of \textit{atagp19} to cytokinin were tested. \textit{atagp19} had fewer lateral roots, shorter primary roots and less chlorophyll relative to WT under control conditions. After addition of exogenous BAP to MS media, similar to WT, \textit{atagp19} plants also showed a reduction in lateral root number, primary root elongation and chlorophyll levels (Figure 3.27). Therefore, \textit{atagp19} plants under normal conditions mimicked WT plants in presence of exogenous cytokinin.

\textit{Shoot initiation assay}

Since cytokinin promotes cell division and initiates shoots in concert with auxin in cultured plant tissues, shoot initiation abilities of excised hypocotyls from WT and AGP mutant seedlings were examined in response to various concentrations of cytokinin BAP and auxin NAA (Figure 3.28). This was intended as an additional means (other than cytokinin response assays) to determine if \textit{AtAGP17}, 18 and 19 are involved in cytokinin
Figure 3.26 Germination of WT and \textit{atagp19} seeds in the presence of ABA. Approximately 100 to 200 seeds were sowed on each plate. Seedlings with green cotyledons were counted as germinated after 3 days. This experiment was repeated three times. Error bars represent SE ($n = 3$).
Figure 3.27 Responses of atagp19 to exogenous cytokinin BAP.
(a) Primary root elongation between days 4 and 7. Error bars represent SE (n > 20).
(b) Lateral root numbers of 7-d-old seedlings. Error bars represent SE (n > 20).
(c) Chlorophyll content of 14-d-old seedlings. Error bars represent SE (n = 4).
Figure 3.28 Shoot initiation assays of WT and atagp17, 18 and 19 mutants. atagp17, 18 and 19 mutants formed elaborate shoot structures on low cytokinin concentrations. Five hypocotyls of each genotype were examined at each concentration. One hypocotyl representative of the response at each concentration was selected and arranged to create a composite photograph for each genotype. Scale bars = 1cm.
signaling and responses. At low cytokinin:auxin ratios, all genotypes initiated roots. Between 30 to 300 ng/mL BAP and 30 to 300 ng/mL NAA, large leafy and flowering structures were found in the AGP mutants, while WT hypocotyl explants formed only green calli but no recognizable shoots. This behavior of WT was expected because it was reported that the Columbia ecotype did not efficiently form shoots from undifferentiated tissues in culture (To et al., 2004). Surprisingly, in addition to roots, WT explants formed green calli at some low cytokinin:auxin ratios, such as at 30 ng/mL BAP and 100 to 300 ng/mL NAA; it was expected that green calli formed only under high cytokinin:auxin ratios. This discrepancy may be due to the effectiveness of different cytokinins or different shoot induction conditions. At the most extreme hormone concentrations, few calli or only undifferentiated calli formed, which was similar for both WT and the mutants. The only observed difference between the WT and AGP mutants was that the AGP mutants tended to form recognizable shoot structures while WT formed only green calli at certain concentrations. This difference may be due to altered sensitivity and responsiveness of the AGP mutants to cytokinin or auxin. Together with the seedling cytokinin response analyses, no significant differences were observed in the mutants with respect to their responses to cytokinin, suggesting that AtAGP17, 18 and 19 may not involved in cytokinin signaling pathways.
Complementation of atagp19 and restoration of WT phenotypes

PCR was conducted to confirm that the T-DNA (mutant alleles), WT AtAGP19 gene introduced by the complementation construct and hygromycin resistance gene were present in independent complemented mutant lines (Figure 3.29 and data not shown). Complementation of the mutant with the WT AtAGP19 gene under the control of its own promoter restored AtAGP19 mRNA expression (Figure 3.8c) as well as WT phenotypes. In particular, complemented plants displayed normal leaf size, shape, color and growth rate (Figure 3.13). In addition, stem length and thickness as well as seed production was also restored in the complemented mutants (Figure 3.21a). These data established the causal relationship between the mutant phenotypes and the knockout of AtAGP19.

Double mutants

Homozygous double mutants atagp17 atagp19 and atagp18 atagp19 were generated by crossing the SALK_101062 (atagp17) and SALK_117268 (atagp18) lines with SALK_038728 (atagp19), respectively. These double mutants were confirmed by both PCR and RT-PCR (data not shown). However, no distinctive additional phenotypes were apparent in the double mutants (Figure 3.30).
Figure 3.29 Genetic analyses of complemented *atagp19* plants.
(a) PCR with primers specific to hygromycin resistance gene within the complementation construct showed that the gene was only present in the complemented *atagp19* plants, but not untransformed WT or *atagp19* plants.
(b) PCR with primers specific to *AtAGP19* within the complementation construct showed that the gene was only present in the complemented *atagp19*, but not untransformed WT or *atagp19* plants. One primer was *AtAGP19* specific, and the other primer was specific to the NOS terminator following *AtAGP19*. 
Figure 3.30 *atagp17 atagp19* and *atagp18 atagp19* double mutants. 
*aatagp17 atagp19* (a) and *atagp18 atagp19* (b) double mutants looked like *atagp19* (c). Rosettes were taken from 5-week-old plants.
Discussion

*AtAGP19 functions in plant growth and development*

The *atagp19* mutant displays multiple and dramatic mutant phenotypes which are statistically different from WT. These phenotypes affect both during both vegetative and reproductive growth and include altered leaf morphology, lighter green coloration, slower growth and flowering, fewer lateral roots, shorter hypocotyls and inflorescence stems, reduced secondary growth and compromised fertility. Phenotypes of *atagp19* correspond to the position and timing of *AtAGP19* expression and indicate that AtAGP19 is essential for normal plant growth and development, including cell division and expansion, leaf formation, lateral root initiation, stem growth, vascular development and seed production.

It is not yet clear how the loss of AtAGP19 results in lower pigment levels in *atagp19*. With respect to reproduction, it is likely that a high percentage of aborted ovules seen in *atagp19* siliques are a result of several factors, such as abnormal stamen development, compromised female gametogenesis and poor pollen tube guidance in the style. A reduction in female reproductivity was apparent in reciprocal crosses when *atagp19* was used as the female parent, few seeds were produced.

AtAGP19 function is regulated by light with respect to seedling growth. In light, *atagp19* hypocotyls are approximately 75% as long as WT, but when grown in the dark,
there is no length difference. *AtAGP19* expression, as determined by GUS staining, is found in both light- and dark-grown *Arabidopsis* seedlings. Similarly, *LeAGP1* transcription is not affected by light (Li and Showalter, 1996). Thus, this light-regulated function of AtAGP19 occurs at a posttranscriptional level and probably involves interactions with other components which are more directly regulated by light to control hypocotyl length. A good example relating to this speculation is seen with the HY5 and COP1 proteins that perform antagonistic roles in the light-regulated control of *Arabidopsis* seedling growth. COP1 does not affect mRNA accumulation of *HY5* but instead targets HY5 for proteasomal degradation in the dark through direct protein-protein interaction. COP1 activity, in turn, is directly repressed by two cryptochromes (blue/UV-A light receptors) that are activated by light, also through a direct physical interaction between proteins (Osterlund *et al.*, 2000; Wang *et al.*, 2001).

The precise mechanism(s) for the observed function of AtAGP19 in plant growth and development remains to be elucidated. There are several indications, however, that AtAGP19 acts through cellular signaling pathways. First, *atagp19* has multiple abnormal phenotypes, and the disruption of one or more signaling pathways in this mutant would provide a plausible explanation to account for these pleiotropic effects. Second, many of the phenotypes observed are related to alterations of phytohormone pathways. Third, AtAGP19 has a predicted GPI anchor, and GPI-anchored proteins have putative roles in cellular signaling and communication (Schultz *et al.*, 1998; Youl *et al.*, 1998).
**Functional relationships of AtAGP17, 18 and 19**

Despite some changes in *AtAGP17* and *18* mRNA accumulation in the *atagp19* mutant and the similar expression patterns of *AtAGP18* and *19*, there appears to be little functional compensation. This idea is further supported by the observation that no additional mutant phenotypes are seen in *atagp17 atagp19* and *atagp18 atagp19* double mutants, although it was not clear whether truncated *AtAGP18* mRNA could lead to functional *AtAGP18* protein. In *AtAGP17* or *AtAGP18* T-DNA insertion mutants and *AtAGP18* RNAi mutants, no gene compensation by the other Lys-rich AGPs occurs in the organs examined (Acosta-Garcia and Vielle-Calzada, 2004). Overall, the picture that emerges from the expression data (Chapter 2) and from characterization of mutants of the three Lys-rich AGP is that all three Lys-rich AGPs have unique and independent function in *Arabidopsis*. Future experiments to test this idea might involve determining whether the other two Lys-rich AGP genes can complement a specific Lys-rich AGP mutant (e.g., *atagp19*) when expressed under the control of the promoter of the mutated AGP gene (e.g., *P_{AtAGP19}; AtAGP17* and *P_{AtAGP19}; AtAGP18*).
**AtAGP19 and cell division**

Plant growth involves cell division and cell elongation. *atagp19* has smaller rosette leaves, which consist of fewer abaxial epidermal cells, indicating a decrease in cell division. *atagp19* also has shorter petioles and inflorescence stems. In these cases, reduction in length results from suppressed cell division, but not inhibition of cell elongation, since cell length in the *atagp19* petiole and stem are normal. Moreover, slower growth and delayed flowering of *atagp19* suggest a lower cell division rate during vegetative growth.

Consistent with results presented here, previous studies have implicated AGPs in promoting cell proliferation (Serpe and Nothnagel, 1994; Langan and Nothnagel, 1997). AtAGP19-dependent cell division regulation is specific to certain tissues and organs. Abnormal cell division is associated with leaf blade epidermal cells, petioles and inflorescence stems.

**AtAGP19 and cell expansion**

AtAGP19 also plays a role in cell expansion; this is seen from shorter hypocotyl cells, smaller rosette epidermal cells and more regularly shaped spongy mesophyll cells in *atagp19*. The dwarf phenotype of *atagp19* is a result of impaired cell division and cell
expansion.

Earlier work indicated that AGPs, either individually or collectively, function in cell expansion (Schopfer, 1990; Zhu et al., 1993; Willats and Knox, 1996; Ding and Zhu, 1997; Vissenberg et al., 2001; van Hengel and Roberts, 2002; Park et al., 2003; Shi et al., 2003; Lee et al., 2005; Lamport et al., 2006), and the findings reported here support this functional role. Although the exact underlying mechanisms of action are not known, AGPs are thought to loosen cell wall polymers so as to increase wall extensibility and stabilize the PM.

**AtAGP19 and leaf formation**

In WT plants, the first four true rosette leaves are juvenile leaves; they are round and have low blade length/petiole length ratios and no abaxial trichomes (Bollman et al., 2003). Since atagp19 leaves are more round (having higher blade width/length ratios) compared to their WT counterparts, it is likely that atagp19 leaf development is arrested in the juvenile phase of vegetative growth. This idea is also supported by the delayed appearance of abaxial trichomes on mutant rosette leaves. Hence, it is expected that atagp19 leaves also have low blade length/petiole length ratios; however, atagp19 rosette leaves have higher blade length/petiole length ratios than their WT counterparts. This unexpected phenomenon can probably be attributed to the abnormally short length of the
petioles in the mutant.

**AtAGP19 and stem growth**

Two Lys-rich AGPs, CsAGP1 from cucumber and LeAGP1 from tomato, are involved in stem elongation, although they seem to play opposite roles: overexpression of *CsAGP1* in tobacco results in taller plants (Park *et al.*, 2003), while tomato plants overexpressing *LeAGP1* are significantly shorter (Sun *et al.*, 2004a). Nonetheless, in both cases, differences in stem height become apparently only at later growth stages. It is proposed that CsAGP1 functions downstream of plant hormone (i.e., auxin and GA) signaling pathways to maintain cell wall extensibility; this hypothesis is supported by its upregulation by auxin. On the other hand, *LeAGP1* expression is downregulated by auxin, and it may be involved in cytokinin signaling.

Similar to CsAGP1 and LeAGP1, AtAGP19 also functions in stem elongation since *atagp19* stems are shorter. Unlike CsAGP1 or LeAGP1, AtAGP19 is also involved in stem radial growth because *atagp19* stems are thinner. Moreover, AtAGP19 and CsAGP1 seem to function in different aspects of stem development, since CsAGP1 may regulate stem cell expansion, while *atagp19* participates in cell division. Although the precise mechanisms by which Lys-rich AGPs control stem growth are elusive, their functional importance is clearly demonstrated here.
AtAGP19 and vascular development

AtAGP19 promoter activity is consistently associated with the vasculature throughout the plant, including cambium, xylem parenchyma cells and differentiating xylem vessels in hypocotyls and stems. Differentiated xylem elements with thick, lignified cell walls did not show GUS staining (Chapter 2). Although no defects were evident in leaf vein patterning or xylem/phloem arrangement/morphology, atagp19 showed decreased secondary growth in mature roots and hypocotyls, indicating that AtAGP19 promotes secondary xylem development. Indeed, AGPs have long been proposed to function in vascular development, and the evidence was summarized in Chapters 1 and 2.

Xylogen, a chimeric AGP, directly mediates local intercellular communication necessary for TE differentiation of Zinnia mesophyll suspension cells given that addition of xylogen greatly increases the TE differentiation rate at low cell densities (Motose et al., 2001). A double xylogen mutant in Arabidopsis (atxyp1 atxyp2) has discontinuous leaf venation patterns with normal vasculature in the hypocotyl and stem (Motose et al., 2004). In contrast, atagp19 shows normal vein patterning in leaves but has a decreased xylem area with fewer xylem elements in the root, hypocotyl and stem. This difference in mutant phenotypes suggests that AtAGP19 and xylogen function in different aspects of
vascular development. Consistent with the notion that differentiation processes of xylem and fiber share a common molecular mechanisms (Zhong et al., 2001), both xylem and interfascicular fiber regions are thinner in atagp19. Considering that only phase II of hypocotyl secondary thickening is reduced, while phase I developed normally, the reduced xylem development in atagp19 is likely to be caused by slower cell division activities in the vascular cambium. Based on all the above data, AtAGP19 plays a positive role in xylem development.

Expression and function of AtAGP19

AtAGP19 expression is tissue-specific and developmentally controlled. Notably, vascular tissues and young organs display the highest AtAGP19 promoter activity (Chapter 2). The phenotypes of atagp19 correspond to the position and timing of AtAGP19 expression, providing valuable insight to AtAGP19 function. For example, in developing leaves, AtAGP19 is highly expressed in leaf epidermal and spongy mesophyll cells; atagp19 leaves are composed of fewer/smaller abaxial epidermal cells and abnormally expanded and densely packed spongy cells. Reduced secondary growth and xylem area in mature atagp19 plants is also not surprising considering AtAGP19 transcription is tightly associated with vascular tissues, and in particular, with differentiating, but not developed, xylem elements. In young stems, AtAGP19 promoter
activity is present in virtually all cells, including epidermal, cortical, interfascicular, vascular, procambium and pith cells, which is consistent with the multiple phenotypes identified in \textit{atagp19} stems. In addition to the reduced radii of fascicular xylem and interfascicular fiber regions in \textit{atagp19} stems, cortical cells are collapsed, and pith cell numbers greatly decrease. A role for \textit{AtAGP19} in cell division is further supported by the expression of \textit{AtAGP19} in hypocotyl vascular cambium and stem procambium.

Furthermore, \textit{AtAGP19} may somehow be connected to auxin for the following four reasons. Auxin, a key player in vascular differentiation, is synthesized in leaf hydathodes (Aloni, 2001), where pronounced \textit{AtAGP19} expression is observed via GUS staining. Second, \textit{AtAGP19} promoter activity is also closely associated with vascular tissues, and \textit{atagp19} has under-developed xylem. Third, auxin controls lateral root initiation and development (Casimiro \textit{et al.}, 2001), and there are indeed fewer lateral roots in \textit{atagp19}. Last but not least, auxin is also essential for normal cell division and expansion, and \textit{atagp19} has abnormal cell division and expansion. AGPs have been proposed to interact with auxin to regulate cell wall loosening during expansion (Schopfer, 1990). Preliminary data showed that \textit{atagp19}, like WT, also increases lateral root formation in presence of exogenous auxin (data not shown). This observation suggests that if auxin is indeed involved, auxin transport/distribution is altered in \textit{atagp19}.
AGP mutants provide insight to AGP function

AGP mutants are helping to identify the function of AGPs and provide a framework to explore the underlying mechanisms of action responsible for AGP function. The overexpression study of LeAGP1 connected this AGP to cytokinin signaling (Sun et al., 2004a). In contrast, atagp19 responds to cytokinin in a way similar to WT; for example, chlorophyll content, primary root length and lateral root numbers of both WT and mutant seedlings decrease in response to cytokinin BAP concentrations higher than 0.005 µM. These observations imply that function of AtAGP19 may be different from LeAGP1. Analysis of a null mutant of AtAGP30, a nonclassical AGP gene, indicates that it may play a role in ABA responses and root regeneration (van Hengel and Roberts, 2003). The implication that AGPs are associated with phytohormone pathways is in line with our hypothesis that AtAGP19 is involved in cell signaling pathways. Mutant analyses also shed light on the other two Lys-rich AGPs in Arabidopsis: an insertion in the promoter region of AtAGP17 results in reduced binding to Agrobacterium (Nam et al., 1999; Gaspar et al., 2004), while AtAGP18 RNAi mutants display high ovule abortion rates (Acosta-Garcia and Vielle-Calzada, 2004). Similar to the AtAGP18 RNAi mutants, atagp19 siliques contain many aborted ovules; the reason for this remains unknown. On the other hand, roots of atagp19 have normal Agrobacterium-binding capacities.

Results presented in this dissertation indicate that AtAGP19 is essential for cell
division and expansion, leaf formation, lateral root initiation, stem development, secondary growth and reproduction in *Arabidopsis*. Defects in cell expansion in *atagp19* are reminiscent of three other AGP mutants. Moss *AGP1* RNAi plants represent one such mutant and display reduced apical cell extension (Lee *et al.*, 2005). A mutant of *AtFLA4* in *Arabidopsis* shows abnormal cell expansion (Shi *et al.*, 2003). Tobacco plants overexpressing *CsAGP1* illustrate a third mutant; these plants are taller due to greater stem elongation (Park *et al.*, 2003). Together with this study, there is now compelling evidence that AGPs are required for normal cell expansion.

In addition, a double xylogen mutant in *Arabidopsis* (*atxyp1 atxyp2*) shows discontinuous leaf venation patterns (Motose *et al.*, 2004). Although this particular phenotype is not observed in *atagp19*, *atagp19* has smaller vascular cylinders in the mature roots and hypocotyls compared to WT. The smaller vascular region in the mutant is due to fewer cells and not to changes in cell size, shape and wall thickness of the xylem elements, which appear normal.

Considering all AGP mutants examined to date, *atagp19* shows the broadest and most readily apparent phenotypes without the need to resort to special screens. The underlying mechanisms explaining how AtAGP19, as well as the other AGPs from characterized mutants, regulate various cellular and physiological processes remain to be elucidated, but efforts are underway to address this important and challenging issue.
Chapter 4 RNAi OF *AtAGP17, 18 AND 19*
RNAi was adopted as an alternative approach to elucidate the function of \textit{AtAGP17}, \textit{18} and \textit{19} in addition to insertion mutagenesis. It was expected to provide functional insights on AGPs (especially \textit{AtAGP19} which lacked insertion mutants when this study was initiated) as well as to corroborate phenotypes and implications of insertion mutants. RNAi constructs of \textit{AtAGP17}, \textit{18} and \textit{19} were made, one for each gene, and delivered into \textit{Arabidopsis} plants. PCR confirmed stable integration of the constructs into the genome. RT-PCR showed that \textit{AtAGP17} and \textit{18} mRNA was absent in the T1 generation of their respective RNAi plants. However, in the T2 generation, their transcript levels reverted to normal levels. \textit{AtAGP19}, in contrast, was not silenced even in the T1 transformants. Overbranching phenotypes were shown by RNAi transformants of \textit{AtAGP17}, \textit{18} and \textit{19}. The correlation between phenotypes observed in putative RNAi plants and RNA silencing was discussed.

**Summary**

RNAi was adopted as an alternative approach to elucidate the function of \textit{AtAGP17}, \textit{18} and \textit{19} in addition to insertion mutagenesis. It was expected to provide functional insights on AGPs (especially \textit{AtAGP19} which lacked insertion mutants when this study was initiated) as well as to corroborate phenotypes and implications of insertion mutants. RNAi constructs of \textit{AtAGP17}, \textit{18} and \textit{19} were made, one for each gene, and delivered into \textit{Arabidopsis} plants. PCR confirmed stable integration of the constructs into the genome. RT-PCR showed that \textit{AtAGP17} and \textit{18} mRNA was absent in the T1 generation of their respective RNAi plants. However, in the T2 generation, their transcript levels reverted to normal levels. \textit{AtAGP19}, in contrast, was not silenced even in the T1 transformants. Overbranching phenotypes were shown by RNAi transformants of \textit{AtAGP17}, \textit{18} and \textit{19}. The correlation between phenotypes observed in putative RNAi plants and RNA silencing was discussed.
Introduction

RNAi is a relatively new and powerful technique to suppress expression of targeted gene(s). RNAi is also known as posttranscriptional gene silencing (PTGS) or cosuppression in plants (Matzke and Matzke, 2004). Small double-stranded RNA (dsRNA) or single-stranded self-complementary hairpin RNA (hpRNA) binds to mRNA in a sequence-specific manner, resulting in degradation of mRNA (Fire et al., 1998; Wesley et al., 2001) into 21-25 nt small interfering RNA (siRNA) populations (Hamilton and Baulcombe, 1999).

Introducing dsRNA or hpRNA into plants enables researchers to directly silence genes of interest. By comparing and contrasting the phenotypes of WT plants with RNAi mutants, valuable information can be deduced with respect to gene function. Indeed, RNAi has been successfully used as a functional identification tool in several model genetic systems, such as Caenorhabditis elegans (Fire et al., 1998), Drosophila melanogaster (Kennerdell and Carthew, 1998) and Arabidopsis thaliana (Acosta-Garcia and Vielle-Calzada, 2004).

For plant biologists, the RNAi technique is extremely useful when genes of interest have no null insertion mutants, especially when non-model plant species are being studied. Besides Arabidopsis, RNAi has been successfully applied to petunia (Metzlaff et
al., 1997), tobacco (Waterhouse et al., 1998), moss (Lee et al., 2005) and cultured plant cells (Akashi et al., 2001). Moreover, two AGPs were assigned function by this approach. AGP1 in moss (Physcomitrella patens) is involved in cell expansion because RNAi of AGP1 resulted in reduced cell length (Lee et al., 2005). AtAGP18 is proposed to be essential for female gametogenesis, as functional megaspores in AtAGP18 RNAi mutants failed to enlarge and divide, leading to ovule abortion and reduced seed set (Acosta-Garcia and Vielle-Calzada, 2004).

RNAi can also be used to pheno-copy insertion mutants so as to corroborate the gene function inferred from the insertion mutants. Another advantage of RNAi is that mutants with different levels of RNA silencing (from complete knockout to partial knockdown) can be obtained, so a continuum of phenotypes can be observed. This is very helpful when complete silencing of the gene of interest results in lethality. A chemical inducible RNAi system (Guo et al., 2003) also is useful in this case. Another inducible RNAi system is called virus-induced gene silencing (VIGS), which involves infecting the plants with a virus vector containing an RNAi construct for a gene of interest (Ratcliff et al., 2001). This system enables rapid gene function identification and does not require generation of transgenic plants. Last but not least, RNAi allows for simultaneous silencing of multiple members in the same gene family, providing that they share sufficiently high sequence identities (Burton et al., 2000).
The hpRNA vector used in this study includes a 300-500 bp fragment of the gene in both sense and antisense orientations, separately by a spacer region to allow for efficient gene silencing. The hpRNA vector is transcribed \textit{in vivo} into hpRNA to elicit gene silencing. Many hpRNA constructs were tested in terms of silencing efficacy, and the intron-containing constructs (ihpRNA), in which a functional intron acts as a spacer between the sense and antisense arms of the construct, showed the highest degree of silencing (Wesley \textit{et al.}, 2001). In this research, ihpRNA constructs of \textit{AtAGP17}, \textit{AtAGP18} and \textit{AtAGP19} were introduced into \textit{Arabidopsis} plants. Transformed \textit{Arabidopsis} plants were recovered and investigated genetically and phenotypically.

\section*{Materials and methods}

\subsection*{Growth conditions}

Plants were grown in soil in the \textit{Arabidopsis} growth room under long day conditions. Alternatively, the MS plates were placed in the tissue culture room at 24 \degree C under long day conditions.
Construction of three ihpRNA vectors

One ihpRNA construct was built for each of the three *Arabidopsis* Lys-rich AGP genes, namely *AtAGP17, 18* and *19*. A fragment approximately 500 bp long was amplified from the first exon of each gene by PCR (Figure 4.1). Since the three genes do not share significant nucleotide sequence identities or similarities, the RNAi construct was expected to silence only the gene of interest, but not the other two homologs. Restriction enzyme sites, *Xba*I (TCTAGA) and *Xho*I (CTCGAG), were linked to the 5’ end of the PCR products, and *Cla*I (ATCGAT) and *Kpn*I (GGTACC) were added to the 3’ termini. For PCR amplification, the *AtAGP17* primers were 5’-TCT AGA CTC GAG ATT CTT CAC TCA ACA CTC-3’ and 5’-ATC GAT GGT ACC AGA GAA AGC ATC GCT TGG-3’; the primers for *AtAGP18* were 5’-TCT AGA CTC GAG GAT CGC AAT TTC CTC CTA-3’ and 5’-ATC GAT GGT ACC AAG GAC CGG GA G AAA AAG-3’; and the primers for *AtAGP19* were 5’-TCT AGA CTC GAG CTC TTC CTT TAG TGT AAA-3’ and 5’-ATC GAT GGT ACC TTT TGT GTT TGT GCT TTC-3’.

The cloning vector pKANNIBAL (Figure 4.2) was composed of a 35S CaMV promoter, an *octopine synthase* (*OCS*) terminator, two polylinkers separated by the *pyruvate orthophosphate dikinase* (*Pdk*) intron. One polylinker contained the *Xho*I and *Kpn*I sites, and the other contained the *Cla*I and *Xba*I sites (Wesley *et al.*, 2001). The
Figure 4.1 Illustration of primers used for ihpRNA vector construction.
Blue, primer. Brown, intron. Red, exon. Arrow indicates 5’ to 3’ direction of PCR amplification. The total length of the gene is labeled below the gene. The number in the parenthesis indicates the position of 5’ nucleotide of the gene included in the primer. The amplified fragments for AtAGP17, 18 and 19 were 517 bp, 509 bp and 496 bp in length (not including the additional restriction sites), respectively.
Figure 4.2 Cloning vector pKANNIBAL (Genebank accession number AJ311873). Arrows denote the 5’-3’ orientation.

35S promoter, cauliflower mosaic virus 35S promoter. Kan, kanamycin resistance gene (50 μg/ml kanamycin was used for *E.coli* colony selection). Pdk intron, pyruvate orthophosphate dikinase intron (present for high silencing efficiency). OCS terminator, octopine synthase terminator. PCR fragments were inserted between the *XhoI* and *KpnI* sites in the sense orientation and between the *ClaI* and *XbaI* sites in the antisense orientation.
PCR products from above were cloned into pKANNIBAL between the XhoI and KpnI sites in the sense orientation and between ClaI and XbaI sites in the antisense direction. Thus, the sense and antisense fragments were separated by the Pdk intron, and the constructs were referred to as the ihpRNA constructs. The presence of the PCR fragments in the ihpRNA constructs was confirmed by enzyme digestions and sequencing. To verify the inserts by sequencing, two sequencing reactions were carried out for each vector; one amplified from the XhoI site towards the KpnI site, and the other amplified from the XbaI restriction site towards the ClaI site.

The verified constructs were subsequently subcloned into the NotI site in the binaryvector pART27 (Gleave, 1992). Double antibiotic resistance (25 μg/ml kanamycin and 25 μg/ml streptomycin) as well as blue-white screening were used for colony selection.

Preparation of chemically competent Agrobacterium cells

To prepare Agrobacterium competent cells for freeze-thaw transformation, 100 μl Agrobacterium strain LBA4404 (frozen stock) was cultivated in 5 ml YEP medium (1% yeast extract, 1% peptone, 0.5% NaCl, 1.5% agar, pH 7.2) containing 200 μg (40 μg/ml) streptomycin overnight at 28 °C. Two ml of the overnight culture was added to 50 ml
YEP in a 250 ml flask and shaken at 250 rpm until the culture reached an OD$_{600}$ of 0.5 to 1.0. The culture was chilled on ice and centrifuged at 3,000g for 5 min at 4 °C. The pellet was resuspended in 1 to 1.5 ml of ice-cold 20 mM CaCl$_2$, and 0.1 ml aliquots were dispensed into prechilled Eppendorf tubes.

**Transformation of Agrobacterium and Arabidopsis**

Purified pART27 binary vectors harboring verified ihpRNA fragments were introduced into *Agrobacterium* competent cells using the freeze-thaw procedure (An *et al.*, 1988), and the plates were cultivated at 28 °C for 2 to 3 days. Double antibiotic resistance (25 μg/ml kanamycin and 25 μg/ml streptomycin) was used for colony selection. Colony PCR was performed on selected LBA4404 liquid cultures to confirm the presence of the desired constructs (Weigel and Glazebrook, 2002) with Taq PCR Master Mix (QIAGEN, Valencia, CA). *Arabidopsis* transformation was then performed as described in Chapter 2, and *Agrobacterium* was grown in YEP medium instead of LB medium.
**Screening of kanamycin resistant Arabidopsis plants**

Seeds from dipped plants were dried at RT for at least 2 weeks and stored at 4 °C. For screening, seeds were surface sterilized and germinated on MS plates (without sucrose) supplemented with 25-50 μg/ml kanamycin (Weigel and Glazebrook, 2002). After 10 to 14 d, kanamycin resistant plants were green and developed true leaves and long roots; kanamycin sensitive plants did not have true leaves and were pale. Kanamycin resistant seedlings were transferred to soil and allowed to grow and set seed.

**Genetic analyses of transformed plants**

PCR was performed on individual kanamycin-resistance plants (T1 generation) to confirm the integration of the ihpRNA construct into the plant genome using the Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, St. Louis, MO). RNA extraction and RT-PCR were performed as described in Chapter 2. Total RNA was extracted from inflorescence stems of individual transformants or kanamycin resistant seedlings. One to two μg of total RNA was used in each RT-PCR, and 25-28 cycles were performed.
**Calli induction**

Leaves and inflorescence stems were cut into 1 cm segments, sterilized in 30% bleach for 30 min and rinsed. The sterilized stems were placed on callus-growth medium (MS medium, 30 g/L sucrose, 0.2 g/L Myo-Inositol, 0.5 mg/L BAP, 1 mg/L NAA, 1 mg/L IAA, 1 mg/L 2,4-D, 8 g/L agar, pH 5.7) and incubated in the dark (Encina et al., 2001). The hormone stock solutions were prepared at 1 mg/mL in ddH₂O and stored at 4 °C. The calli were subcultured every month and maintained on the same medium.

**Search for small RNAs in the Arabidopsis genome**

Small RNA hits as well as putative small RNA targets in and around AtAGP17, 18 and 19 were identified using the ASRP (Arabidopsis Small RNA Project) Database (http://asrp.cgrb.oregonstate.edu/db/) (Gustafson et al., 2005) and Arabidopsis Small RNA MPSS Database (http://mpss.udel.edu) (Nakano et al., 2006).
Results

First-time transformation into the gl1 background

For the first experiment, Columbia gl1 (Col-6 glabra1, CS8155) Arabidopsis plants were transformed. An easily identified characteristic of these plants was that they did not have trichomes on their leaf surfaces due to the knockout of GL1. At least 10 independent transgenic lines were obtained for each of the three Lys-rich AGP genes.

RNA levels of targeted genes were not studied in T1 transformants. The T2 generation was examined, and similar levels of mRNAs were found in both WT and putative RNAi plants (Figure 4.3). One-week-old kanamycin resistant seedlings as well as inflorescence stems from individual kanamycin resistant plants showing overbranching phenotypes (discussed in detail below) were used for RNA extraction and RT-PCR. PCR analyses of these same plants with three pairs of ihpRNA vector specific primers indicated that the ihpRNA constructs were still present in these plants (data not shown).
Figure 4.3 *AtAGP18* mRNA was present in the T2 *AtAGP18* RNAi plants. Transgenic plants were in the *gl1* background, and they contained the RNAi construct. 
(a) Kanamycin resistant seedlings (1-week-old) from two independent lines were examined with RT-PCR.
(b) Three individual kanamycin resistant plants of line 4 showing overbranching phenotypes expressed *AtAGP18*. 
Second-time transformation into the WT Columbia-0 background

Target RNA was absent in the T1 generation

For the second attempt, WT Columbia-0 *Arabidopsis* plants were transformed with the same *AtAGP17, 18* and *19* RNAi constructs used above. At least 20 independent transgenic lines were obtained for each of the three Lys-rich AGP genes. In contrast to the first experiment, several independent T1 lines were examined. *AtAGP17* and *18* were silenced in their respective RNAi plants (Figure 4.4a,b), but this was not the case for *AtAGP19* (Figure 4.4c). Since *AtAGP19* mRNA was still present in the T1 generation, its T2 generation was not further studied.

Targeted RNA reappeared in the T2 generation

Similar to the results obtained form the first experiment, WT levels of mRNA were detected in the T2 *AtAGP17* and *18* RNAi plants (Figure 4.5 and data not shown). PCR analyses indicated that the ihpRNA constructs were present in these plants (data not shown), eliminating the possibility that reappearance of the target mRNA was due to loss of the RNAi constructs in the T2 plants.
Figure 4.4 RT-PCR analysis of RNAi plants (T1 generation).
Total RNA was extracted from Columbia-0 WT and RNAi plants.
(a) \textit{AtAGP17} mRNA was absent in T1 \textit{AtAGP17} RNAi plants.
(b) \textit{AtAGP18} mRNA was absent in T1 \textit{AtAGP18} RNAi plants.
At least 14 independently transformed lines were tested, and all showed no \textit{AtAGP18}
transcript.
(c) \textit{AtAGP19} mRNA was still present in T1 \textit{AtAGP19} RNAi plants.
Figure 4.5 *AtAGP18* mRNA reappeared in the T2 *AtAGP18* RNAi plants.
Four independent lines in the T2 generation (WT Columbia-0 background) containing the *AtAGP18* RNAi construct were tested. Lines 29 and 36 were confirmed not to have *AtAGP18* mRNA in the T1 generation. Lines 34 and 36 had overbranching phenotypes. Lines 12, 29 and 36 showed segregation of the kanamycin resistance marker, and line 34 plants were all resistant to kanamycin selection. RNA was extracted from leaves and stems of individual plants.
Segregation of T2 RNAi plants

Segregation of kanamycin resistance was studied in the T2 plants. Here, only segregation of *AtAGP18* RNAi progeny is reported. Different lines of T2 seeds were germinated on separate MS plates containing kanamycin. Most lines showed different segregation rates; one line was homozygous for the insertion (all progeny were resistant to kanamycin); 3 lines lost kanamycin resistance; and 1 line did not germinate at all.

Phenotypic analyses of kanamycin resistant plants

Kanamycin resistance plants (also referred to as RNAi transformants or putative RNAi plants) harboring the *AtAGP17, 18* or *19* ihpRNA construct were transferred from the MS plates to soil and examined under normal growth conditions over 2 generations, with the exception that *AtAGP19* RNAi transformants from the second experiment were only observed for one generation. The plants from both experiments, in spite of their different genetic backgrounds and different constructs they were transformed with, exhibited similar phenotypes. Particularly, although *AtAGP19* putative RNAi plants expressed normal levels of *AtAGP19* in the second experiment, they resembled *AtAGP17*...
and 18 RNAi plants. Only selected T1 AtAGP18 RNAi transformants from the first experiment are shown here. A small proportion of the kanamycin resistant plants (less than 20%) were dwarf and flowered early, while most transformed plants were of normal size compared to WT (Figure 4.6a,b).

While approximately 10% AtAGP18 RNAi T1 plants were completely sterile, producing only short and sterile siliques, most T1 plants produced long and fertile siliques (Figure 4.6c,d); no significant reduction in seed production was observed in these fertile plants. AtAGP19 transformants plants did not show phenotypes of atagp19 (see Chapter 3); they were perhaps smaller and had fewer seeds, but they were not paler green. Furthermore, stems of RNAi transformants tended to recline and lay on their sides (Figure 4.6c,d).

Overbranching phenotypes

A prevalent and consistent phenotype observed in two generations of normal sized RNAi plants was overbranching of the inflorescence stems. There were variations in the overbranching phenotypes: two or more branches, cauline leaves, siliques or a mixture of all could initiate from a single node on an inflorescence stem (Figure 4.7), which was absent in the WT. In fact, some RNAi transformants of AtAGP17, 18 and 19 produced more seeds than WT due to extensive overbranching (Figure 4.6d). These phenotypes
Figure 4.6 Phenotypes of RNAi transformants.
Plants shown here were the T1 generation in the *gl1* background.
(a) A dwarf RNAi plant flowered early (there were only 6 rosette leaves when the plant flowered).
(b) A RNAi plant with size similar to WT.
(c) A completely sterile *AtAGP18* RNAi plant.
(d) A fertile *AtAGP18* RNAi plant.
Figure 4.7 Overbranching phenotypes of RNAi transformants.
These plants were the T1 generation of the gl1 background. Circles indicate some of the overbranching locations. Only *AtAGP18* RNAi transformants were shown, and putative RNAi plants of *AtAGP17* and 19 looked similarly.
(a) A single RNAi plant showing multiple branches and cauline leaves coming from the same internode.
(b) A RNAi plant had two siliques form at the same point.
(c) An overbranching RNAi plant inflorescence stem. Two normal auxillary branches and one with only a terminal flower branched out together.
were observed in RNAi transformants for all three AGP genes and inheritable. Moreover, overbranching was often accompanied by delayed senescence of the plant (Figure 4.7a).

**Discussion**

Dwarfism and sterility are not considered as RNAi-induced phenotypes. Since RNAi transformants of different genetic backgrounds, constructs or levels of gene silencing display similar phenotypes, they are discussed here as a group. Some RNAi transformants are dwarfs, they also flower early and produce fewer flowers. However, most RNAi plants look healthy, have a normal size and do not flower early. In addition, dwarfism and less seed production are not always correlated. *AtAGP18* RNAi mutants were reported to suffer from ovule abortion and less seed production (Acosta-Garcia and Vielle-Calzada, 2004). However, this was not seen here in the RNAi plants; 90% of T1 plants (without detectable *AtAGP18* expression) did not show significantly decreased fertility. Furthermore, calli were induced from the completely sterile *AtAGP18* RNAi plants, but no decrease of mRNA intensity was observed with RT-PCR (data not shown). The reasons for such a discrepancy were not pursued.

Therefore, dwarfism, early flowering and compromised fertility that only a small number of kanamycin resistant plants suffered from might be simply due to stress derived
from transgene expression and kanamycin selection. These transgenic plants might be sensitive to little fluctuations in the growth conditions. It was also possible that insertion of the ihpRNA constructs disrupt function of some important genes, resulting in the above phenotypes. These observations were not treated as RNAi phenotypes.

**Overbranching was likely an artifact**

Two generations of putative RNAi plants of all three AGP genes manifested heritable overbranching phenotypes. However, after careful evaluation, the overbranching phenotypes were not considered to be derived from RNAi of *AtAGP17, 18* or *19* for the following three reasons. First, RNA analyses of T2 RNAi plants revealed WT levels of targeted AGP gene expression in plants showing overbranching phenotypes, suggesting there was no connection between gene silencing and overbranching phenotypes. Second, in transgenic plants harboring other constructs, i.e. GUS and GFP constructs, overbranching was also occasionally observed (data not shown), although to a lesser degree than in RNAi plants. Therefore, overbranching was probably only an artifactual effect of transformation and antibiotic selection at the seedling stage. Third, overbranching was not apparent in SALK T-DNA mutants, further implying there was no correlation between the knockout of individual AGP transcripts and the overbranching
phenotypes.

**RNAi in this study was not inheritable**

Although RNAi constructs were stably integrated into the *Arabidopsis* genome, they did not result in knockout or knockdown of targeted genes in the T2 generation. The reasons for this are not known. Protein amounts of AtAGP17, 18 and 19 in their respective RNAi plants were not compared to WT because the antibodies were not yet produced at the time of this study.

**Other types of RNA silencing**

Besides RNAi, there are two other types of RNA silencing, mediated by microRNAs (miRNAs) and RNA-directed DNA methylation. These three types of RNA silencing are not mutually exclusive, and regulation of gene expression by RNA silencing occurs on transcriptional or posttranscriptional levels.
miRNAs

The exciting discovery of small RNAs in nature broadens the area of RNA silencing. Small RNAs are present in a wide variety of eukaryotic organisms and play important regulatory roles, but only a small proportion of these molecules have been identified (Lu et al., 2005). There are two classes of endogenous plant small RNAs (20-25 nt): miRNAs and siRNAs, depending on their origins, structure and processing. miRNAs (20-22 nt) negatively regulate endogenous targeted gene expression mainly by pairing to target mRNA and inducing its cleavage. miRNAs are important for normal growth and development of plants, including stress responses (Baulcombe, 2004; Gustafson et al., 2005).

Comprehensive plant small RNA or miRNA databases are available at ASRP (http://asrp.cgrb.oregonstate.edu/db/) (Gustafson et al., 2005), Arabidopsis Small RNA MPSS Database (http://mpss.udel.edu) (Nakano et al., 2006) and miRBase (http://microrna.sanger.ac.uk/sequences/) (Griffiths-Jones et al., 2006). Potential miRNAs and miRNA target sites can be searched at miRNA Precursor Candidates for Arabidopsis thaliana Database (http://sundarlab.ucdavis.edu/mirna/), based purely on computational predictions of miRNAs in Arabidopsis thaliana (Adai et al., 2005). No potential small RNA targets were identified within 3 kb of the genomic sequences of AtAGP17, 18 and
including their promoters. Small RNAs, however, were identified in the downstream intergenic region of *AtAGP17* (Table 4.1). These small RNAs are far away from *AtAGP17* genetic sequence (location: 9851430 - 9852465): ASRP #927 is approximately 5 kb from the 3' end of *AtAGP17*, while ASRP #193, 416 and 130 are more than 15 kb downstream of *AtAGP17*. The functional significance of these small RNAs remains unknown.

DNA methylation

A third pathway of RNA silencing in plants, besides RNAi and miRNA pathways, involves site-specific DNA methylation and transcription suppression. This methylation of DNA is directed by siRNA. In contrast to RNAi/PTGS that degrades mRNA, RNA-guided genome modification is regarded as transcriptional gene silencing (TGS), which prevents mRNA synthesis by the assembly of heterochromatin. RNAi, by producing siRNA, can induce TGS (Baulcombe, 2004; Matzke and Matzke, 2004).

RNAi represents a powerful reverse genetics tool in functional genomics. However, its application in the present work was not successful. Because *AtAGP17* and 18 were still expressed at similar levels compared to WT in their respective T2 RNAi plants, and no obvious phenotypes were observed besides overbranching, which as discussed above
Table 4.1 Small RNAs downstream of *AtAGP17* from ASRP.

<table>
<thead>
<tr>
<th>ASRP #</th>
<th>Length (nt)</th>
<th>Sequence</th>
<th>Starting site</th>
</tr>
</thead>
<tbody>
<tr>
<td>193</td>
<td>23</td>
<td>AAATGGCTGCAAAAAATGTATATA</td>
<td>9867567</td>
</tr>
<tr>
<td>416</td>
<td>22</td>
<td>TGGCTGCAAAAAATGTATATA</td>
<td>9867570</td>
</tr>
<tr>
<td>927</td>
<td>24</td>
<td>ATGTACATCAGAACACGACGACA</td>
<td>9857383</td>
</tr>
<tr>
<td>1303</td>
<td>20</td>
<td>AAAATCCAGTGCAACCATT</td>
<td>9867513</td>
</tr>
</tbody>
</table>
may not be related to these AGP genes. Consequently, these mutants were not studied further, and attention was focused instead on T-DNA mutants, as reported in Chapter 3.
Chapter 5 CONCLUSIONS AND FUTURE WORK
Conclusions

Summary of previous relevant work in the lab

AGPs are a large family of HRGPs found in all higher plants in abundance without clearly defined function. In contrast, the expression and biochemistry of many AGPs, are relatively well known. For example, LeAGP1 is characterized in terms of its genetic and protein sequences, expression patterns, cellular localization, glycosyl composition, Hyp glycosyl profile, glycosyl linkages and possession of a GPI anchor (Pogson and Davies, 1995; Li and Showalter, 1996; Gao et al., 1999; Gao and Showalter, 2000; Zhao et al., 2002; Sun et al., 2004b). However, with respect to the function of LeAGP1, relatively little was known when this dissertation research was initiated. Later, Dr. Wenxian Sun studied transgenic tomato plants expressing high levels of LeAGP1 and proposed its involvement in cytokinin signaling. Notably, it was also found that the GPI anchor of LeAGP1 was crucial for its function (Sun et al., 2004a).

To date, AtAGP17 is the only member of the Arabidopsis Lys-rich AGP subfamily that has been experimentally confirmed to be a bona fide AGP. AtAGP17 has a biased amino acid composition typical of AGPs, with 84% of the Pro residues being hydroxylated to Hyp; AtAGP17 contains 86% carbohydrate by dry weight, with
arabinose and galactose being the two major glycosyl residues present; AtAGP17 is precipitated by Yariv reagent; 81% of the Hyp residues in AtAGP17 are modified with AG polysaccharides, and 12% with oligoarabinoses; and lastly, AtAGP17 has glycosyl linkages characteristic of AGPs, with the branched 1,3,6-galactose\textsubscript{p} being the most abundant linkage (Sun et al., 2005).

**Conclusions of the present dissertation work**

In order to determine the function of Lys-rich AGPs as well as to provide insight to the function of other AGPs, *Arabidopsis thaliana* was chosen as the research subject because powerful genetics tools were available and ready to use with this model plant. The ultimate goal of this dissertation research was to identify the expression and function of the three Lys-rich AGP homologs of LeAGP1 in *Arabidopsis* (Schultz et al., 2002). These three Lys-rich AGPs, namely AtAGP17, 18 and 19, were studied with regard to their expression, cellular localization and function. Publicly available microarray and MPSS data provided comparisons for expression analyses of *AtAGP17, 18* and *19*; collections of insertion mutants greatly facilitated functional analysis of these three AGPs.

Genetic expression of *AtAGP17, 18* and *19* was developmentally controlled and
organ- and tissue-specific. In Arabidopsis plants, AtAGP17 mRNA accumulated in leaves, flowers and stems but not in roots. AtAGP18 and 19 had similar expression profiles; their mRNA levels were high in roots, flowers and stems and low in leaves. In cell cultures, AtAGP17 and 18 were highly expressed in cells in the exponential growth stage, but this expression decreased with time and disappeared in cells in the stationary stage. Promoter activities of AtAGP18 and 19 were preferentially detected in young organs, closely associated with the vascular tissues and high in seedlings, styles, stems, elongating siliques and young leaves. These results were largely consistent with publically available microarray and MPSS data. On the protein level, AtAGP17, 18 and 19 were all rich in roots and flowers, followed by stems and siliques, and of low quantities in leaves. The protein expression patterns of AtAGP18 and 19 were similar to their genetic expression patterns, while the protein and gene expression profiles of AtAGP17, in leaves and roots in particular, were different. Data presented in this dissertation indicated some cross-reactivity of AtAGP17, 18 and 19 antisera and a high degree of specificity of affinity column purified anti-peptide antibodies.

In addition, an identical localization pattern as to LeAGP1 and AtAGP17 (Zhao et al., 2002; Sun et al., 2004b; Sun et al., 2005) was obtained for AtAGP18 and 19. EGFP-AtAGP18/19 fusion proteins were also localized to the PM and Hechtian strands.

The main part of this dissertation research was functional identification of AtAGP17,
18 and 19, and two reverse genetics approaches, RNAi and insertional mutagenesis were taken. RNAi experiments were not successful, and no mutant phenotypes were conclusively observed. Three SALK T-DNA insertion lines, one for each AGP gene, were chosen for detailed analyses. atagp17 and atagp19 were null knockout mutants, while the mutant of AtAGP18 expressed a truncated AtAGP18 transcript at a level similar to WT.

An intensive effort was made to characterize atagp19, a pleitropic mutant. Compared to WT, the atagp19 mutant had: 1) smaller, rounder and flatter rosette leaves with shorter petioles, 2) lighter green leaves containing less chlorophyll, 3) delayed growth and flowering, 4) fewer lateral roots, 5) shorter hypocotyls, 6) shorter and thinner inflorescence stems, 7) fewer siliques and less seed production and 8) reduced secondary growth. Reduction in cell expansion, cell division, cell packing and xylem development was noted in the mutant. Therefore, AtAGP19 plays important roles in various aspects of plant growth and development, including cell division and expansion, leaf formation, lateral root initiation, reproduction and secondary xylem development.

**Implications of the AGP mutants**

Knockout of AtAGP19 in atagp19 results in adverse effects in different stages of plant growth and development; abnormal phenotypes are found in certain tissues, but not
the others, suggesting specificity of AtAGP19 function. Cell division and cell expansion are two fundamental processes contributing to plant growth, and it appears that defects in cell division lead to many downstream/secondary effects. For example, altered leaf and stem morphology in atagp19 is derived from reduced cell division and fewer cells in leaves and stems. In addition, decreased lateral root initiation can reflect failure of root pericycle cells to divide as the first step in lateral root development. Slower cell division in atagp19 can also result in slower vegetative growth, delayed flowering and even reduced secondary thickening and xylem development in atagp19. Specifically, reduced dividing activities in procambium and vascular cambium result in fewer xylem elements before plants finish the life cycle.

Despite the importance of cell division, it is certain that not all the mutant phenotypes in atagp19 can be attributed to less and slower cell division and that AtAGP19 also participates in other fundamental biological processes besides cell division, such as cell elongation. Indeed, repressed cell elongation can account for shorter hypocotyls and at least partially for smaller leaf sizes in atagp19. Furthermore, it is not clear whether cell division is the ultimate primary effect of the AtAGP19 knockout, considering lower chlorophyll levels and compact spongy mesophyll layers might cause less efficient photosynthesis and thus a lower cell division and growth rate.

Not much is known at this stage about the mode of action of AGPs. The AGP
mutants provide invaluable insight to AGP function and represent a big leap in AGP research, but little direct evidence is available for speculating the precise mechanism(s) by which AGPs function in plant growth and development. Microarray analyses on AGP mutants and protein-protein interaction assays (i.e., cross-linking experiments) may be resorted to solve this problem.

Overall, the present work implies that AtAGP19 participates in cellular signaling pathways for the following reasons. First, \textit{atagp19} has multiple abnormal phenotypes, and the disruption of one or more signaling pathways in this mutant would provide a plausible explanation to account for these pleiotropic effects. Second, AtAGP19 has a predicted GPI anchor, and GPI-anchored proteins have putative roles in cellular signaling and communication (Schultz \textit{et al.}, 1998; Youl \textit{et al.}, 1998). Third, many of the phenotypes observed are related to alterations of phytohormone pathways, especially those involving auxin. Auxin is a candidate of particular interest because auxin is essential for cell division and expansion, vascular differentiation and lateral root development, and \textit{atagp19} has repressed cell division and expansion, under-developed xylem and fewer lateral roots. Moreover, auxin is synthesized in leaf hydathodes (Aloni, 2001), where pronounced \textit{AtAGP19} expression is observed via GUS staining. In particular, AGPs have been proposed to interact with auxin to regulate cell wall loosening during expansion (Schopfer, 1990). Consistent with this wall loosening idea, it has been
reported that AGPs are able to increase the activity of xyloglucantransglycosylases which cut and rejoin xyloglucan polymers to allow for cell expansion (Takeda and Fry, 2004).

Similar to AtAGP19, possible signaling roles were proposed for AtAGP17 and 18 (Acosta-Garcia and Vielle-Calzada, 2004; Gaspar et al., 2004). Although the exact underlying molecular mechanism(s) by which AtAGP19, as well as other Lys-rich AGPs or indeed any AGP, function remains to be elucidated, a hypothetical model is presented here for GPI-anchored AGPs (Figure 5.1). GPI-anchored AGPs, as PM co-receptors, work in concert with transmembrane receptor proteins in the phospholipids bilayer. By delivering ligands to the transmembrane receptors and activating their kinase activities, intracellular signaling is initiated (Showalter, 2001; Lee et al., 2006; Sardar et al., 2006).

**Future work**

Although expression, subcellular localization and some functional implications were revealed for AtAGP17, 18 and 19, more work is needed to further elucidate protein expression patterns, biochemical properties and biological roles of these three Lys-rich AGPs.
Figure 5.1 A hypothetic model for the signaling role of GPI-anchored Lys-rich AGPs. TM receptor, transmembrane receptor.

GPI-anchored Lys-rich AGPs (including AtAGP19) are localized to lipid raft portions of the PM. They act as PM co-receptors and initiate intracellular signaling by trapping ligands and delivering them to transmembrane receptor kinases. The receptor kinases are localized in the phospholipids bilayer and are brought into proximity with the lipid raft by GPI-anchored Lys-rich AGPs. The kinases are activated by ligand binding and phosphorylate downstream substrates (green oval).
**Immunolocalization of AtAGP17, 18 and 19**

With the antibodies generated against the Lys-rich region of AtAGP17, 18 and 19, detailed protein expression profiles can be obtained on the organ, tissue and cellular levels by using immunolocalization and be compared to genetic expression patterns (especially the ones revealed by GUS staining). The *atagp17* and *atagp19* null mutants should be included in immunolocalization experiments as negative controls and to test antibody specificity.

Immunohistochemistry can be done on light and electron microscopy levels with tissue samples at different growth stages. The immunolocalization data of AtAGP17, 18 and 19 can be compared to those of LeAGP1 (Gao and Showalter, 2000), PtaAGP6 (Zhang et al., 2003) and AGP monoclonal antibodies, i.e., the JIM series of antibodies, to gain functional insight of these AGPs. Moreover, immunolocalizing these three AGPs to the PM of *Arabidopsis* protoplasts can substantiate the prediction that AtAGP17, 18 and 19 are GPI-anchored.
Biochemical characterization

The *EGFP-AtAGP18/19* fusion constructs that were used for subcellular localization of AtAGP18 and 19 in transgenic BY-2 cultured cells can be transformed again into BY-2 cells to isolate EGFP fusion proteins from culture media for biochemical characterization. The same analyses used to study the LeAGP1 and AtAGP17 fusion proteins can be used to study AtAGP18 and 19 in terms of their amino acid composition, sugar composition, Hyp glycosyl profiles, glycosyl linkages and Yariv binding capacities (Zhao et al., 2002; Sun et al., 2004b; Sun et al., 2005). Moreover, presence of a GPI anchor on the three AGPs can be directly confirmed by the presence of ethanolamine, a residue attached to the C-terminus of GPI-anchored proteins, even after cleavage of the GPI anchor.

Organ-specific glycosylation

Organ-specific glycosylation may be critical for organ-specific function (Gao and Showalter, 2000); therefore, it will be worthwhile examining organ-specific glycosylation (with respect to Hyp glycosyl profiles, glycosyl composition and glycosyl linkages) of AtAGP17, 18 and 19 in *Arabidopsis* plants and cell cultures. To realize this goal, EGFP fusion protein constructs (with both the 35S promoter and the endogenous promoters) of
AtAGP17, 18 and 19 can be expressed in *Arabidopsis* plants and cell cultures, and the fusion proteins can then be purified and analyzed. Specifically, glycosylation patterns in different organs and cell cultures under the control of the 35S and endogenous promoters can be compared. This effort also serves to address two major concerns exist with regards to our published method involving overexpressing and characterizing GFP tagged AGPs in tobacco cell cultures: one concern is that glycosylation in BY-2 cells does not reflect what actually happens in *Arabidopsis* plants or cell cultures; another concern is that due to the abundance of overexpressed AGPs, polysaccharides of smaller sizes are added to AGPs since the glycosylating enzymes may be overwhelmed with the substrate.

In addition, the purified fusion proteins would allow for testing reactivity and cross-reactivity of the antibodies and antisera. Moreover, GFP tagged AGP expression under the control of endogenous promoter can be utilized to elucidate tissue- and cell-specific distribution of the AGPs.

To exclude the possibility that the big EGFP tag may alter glycosylation of fused AGPs, a smaller 6xHis tag can be alternatively used, and the resulting fusion proteins can be characterized biochemically to determine the impacts of EGFP on glycosylation. Hyp glycosyl profiles from the above experiments also serve to further corroborate and refine the Hyp contiguity hypothesis.
Function dissection of *AtAGP17, 18 and 19*

More mutant analyses should be performed in order to identify the molecular mechanisms by which AtAGP17, 18 and 19 function. Understanding biological roles of AtAGP17, 18 and 19 will hopefully further our understanding of other Lys-rich AGPs as well as AGPs in general.

*AtAGP17*

The SALK *atagp17* null mutant did not show any abnormal phenotypes under normal conditions. The deficiency in *Agrobacterium* binding in the well characterized *rat1* mutant was not conclusively observed in SALK *atagp17* mutant. A conundrum associated with the *rat1* mutant is that genetic expression of *AtAGP17* was too low to be detected in roots where the phenotypes were observed. To better address this problem, AtAGP17 in roots can be tracked with either immunohistochemistry or GFP localization in transgenic *Arabidopsis* plants harboring the $P_{AtAGP17}$:GFP-AtAGP17 fusion (which was already constructed in this dissertation research and introduced into *Arabidopsis* plants).
**AtAGP18**

*AtAGP18* RNAi mutants showed a dramatic decrease in seed production, corresponding to a high percentage of ovule abortion (Acosta-Garcia and Vielle-Calzada, 2004). Although multiple lines of *AtAGP18* RNAi mutants generated in this research did not have detectable accumulation of *AtAGP18* mRNA in the T1 generation, they seemed to have normal fertility, contradicting the published report for unknown reasons. *AtAGP18* mRNA reappeared in my RNAi plants in the T2 generation, making detailed analyses of AtAGP18 RNAi plant unfeasible.

Moreover, the SALK and CSHL insertion lines of AtAGP18 are not null mutants, with normal AtAGP18 transcript levels compared to WT, although the transcript in SALK_117268 was truncated. In any case, these mutants do not show any visible phenotypes. In order to better understand the function of AtAGP18, *AtAGP18* mutants from other mutant collections (see Tables 3.1 and 3.2) should be screened for a null knockout mutant. When a null knockout mutant is obtained, special attention should be given to reproduction of the mutant, since female reproductive growth was greatly impaired in AtAGP18 RNAi plants (Acosta-Garcia and Vielle-Calzada, 2004).
For the pleiotropic *atagp19* mutant, it is necessary to address why and how knockout of *AtAGP19* led to multiple phenotypes in the mutant. Further research can be performed in the following directions. First, use deletion constructs to determine the essential module(s) of AtAGP19 for its function. AtAGP19 can be divided into 3 major domains: the AGP domain, Lys-rich region and GPI anchor addition sequence. Overexpression of deletion constructs of *LeAGP1* indicated that the GPI anchor is indispensable for its normal function. The importance of the GPI anchor may be conserved in the three homologs of LeAGP1 in *Arabidopsis*. In contrast, the functional importance of the Lys-rich region is more elusive (Sun *et al.*, 2004a). The AGP domain can be further divided into several smaller regions to be sequentially deleted in the deletion constructs.

Second, promoter swapping experiments can be performed to test if *AtAGP17* and *18* expressed under the control of *AtAGP19* promoter can rescue the *atagp19* mutant. *AtAGP9*, a classical AGP without a Lys-rich subdomain should also be tested since it is closely related to *AtAGP19*, according to the phylogeny tree (Figure 2.3).

Third, microarray analyses of *atagp19* can identify putative signaling pathways and interactions involving AtAGP19. Such an experiment has already been performed with total RNA isolated from aerial parts of 2-week-old WT and *atagp19* seedlings using
ATH1 chips (Y. Zhang and A. Showalter, unpublished results). Upregulated and downregulated genes should be verified by QRT-PCR, and interesting candidates should be further studied. For example, two ethylene biosynthetic enzymes (ACC synthase and ACC oxidase) were upregulated and downregulated in atagp19, respectively, suggesting altered ethylene synthesis or levels in the mutant. After confirming gene expression alterations with QRT-PCR, it is reasonable to test ethylene production levels by the mutant as well as responses of the mutant to ethylene and ACC. In addition, microarray experiments of samples harvested at earlier time points and of roots should be carried out. Examining gene expression in younger seedlings should allow for focusing on fewer and more direct candidate genes interacting with or regulated by AtAGP19. Testing gene expression changes in roots, on the other hand, should provide clues to explain why atagp19 produced fewer lateral roots and allows for analyzing alterations in auxin production, transport and redistribution and responses in atagp19. In summary, the above microarray analyses should render valuable information and guidance to further approach molecular mechanisms of AtAGP19 function.

A fourth direction of future research involves testing auxin transport and auxin responses in atagp19. AtAGP19 is likely to be involved in auxin signaling (see Discussion of Chapter 3). Alterations in auxin transport in atagp19 can be determined by three approaches. One approach is to measure the amount of polar transport of $^3$H-IAA in
atagp19 roots and stems. A second approach is to genetically cross atagp19 with plants harboring $P_{DR5}:GUS$ and $P_{GH3}:GUS$ fusion constructs to generate double mutants. $DR5$ and $GH3$ promoters are responsive to auxin. GUS staining can then be used to analyze asymmetric redistribution of auxin in the roots and shoots of the double mutants. Another indirect way to assess auxin transport is to study gravitropism in atagp19. WT and atagp19 with inflorescence stems can be laid on their sides, and their responses to gravity can be compared by curvatures after certain time intervals. Responses of atagp19 to the auxin transport inhibitor naphthylphthalamic acid is also worth examining.

A fifth future research direction can be further characterization of stress responses of atagp19. Data gathered in this dissertation research suggested that atagp19 was more resistant to drought and ABA stress. This needs to be confirmed by more experiments, and possible involvement of phytohormones (i.e., ABA) should be carefully evaluated. It would also be interesting to test the responses of atagp19 to pathogens, since microarray data (Y. Zhang and A. Showalter, unpublished results) indicated that WAK2 was downregulated in atagp19, and WAKs are involved in pathogen resistance. Thus, it is likely that atagp19 is more sensitive to certain pathogen infections.

A sixth direction involves determining how AtAGP19 affects reproductive growth in Arabidopsis. Reciprocal crosses indicated that atagp19 suffered from defective female reproduction, while impaired stamen development was also observed sometimes in
atagp19. To address this problem, both male (in vitro and in vivo pollen germination and in vivo pollen tube growth) and female (female gametophyte development) reproduction should be studied in atagp19. The defects in atagp19 reproduction were apparently related to growth conditions. More fertility was observed in plants grown in the growth chamber than in the growth room where temperature, humidity and light intensity were not carefully controlled. In contrast, fertility of WT plants did not change noticeably in the two environments.

Seventh, cell wall composition of atagp19 should be analyzed. Such an analysis would conceivably shed light on connection between AtAGP19 and cell wall biosynthesis and assembly. Addressing this issue may also help to explain how small fluctuations in growth conditions resulted in substantial changes in seed production of this mutant.

Studies of overexpression mutant

In contrast to loss-of-function mutants, Arabidopsis plants overexpressing AtAGP17, 18 and 19 provide an alternative way to reveal the function of AtAGP17, 18 and 19 (Sun et al., 2004a). Furthermore, characterizing overexpression mutants can complement T-DNA mutant analyses. It was shown in this dissertation research that introducing the P$_{35S}$:EGFP-AtAGP17 construct that was used to localize AtAGP17 in transgenic BY-2
cells (Sun et al., 2005) led to *AtAGP17* overexpression (Figure 5.2). Overexpression plants of AtAGP18 and 19 can be generated in the same way.

**Glycosyltransferases (GTs)**

Relative little is known about GTs in AGP biosynthesis, and efforts underway to characterize GTs include studying co-expression patterns between GTs and AGPs, examining GT mutants and analyzing enzymatic activities of GTs. The complete *Arabidopsis* genome sequence and public microarray databases make it possible to narrow down the number of GTs that are co-expressed with AGPs. Due to complexity of carbohydrates in AGPs, various classes of GTs are expected to work in concert during AGP biosynthesis. Organ-dependent differential glycosylation analyses as described above should provide additional information on these GTs. Since AGPs are decorated with identical or similar sugars and sugar linkages, it stands to reason that at least some GTs will be responsible for glycosylating more than one particular AGP. Consequently, phenotypes for mutants in such multifunctional GT genes are likely to be readily observable as multiple AGPs should be affected.

In the present dissertation research, three Lys-rich AGPs, namely AtAGP17, 18 and 19, were characterized in terms of their genetic and protein expression patterns,
Figure 5.2 Seedlings harboring $P_{35S}$:EGFP-AtAGP17 overexpressed AtAGP17.
subcellular localization and biological function. In conclusion, these three AGPs have developmentally-regulated and organ- and tissue-specific expression patterns. They are localized to the PM, presumably through the GPI anchors. Furthermore, their function is indispensable for normal plant growth and development.
References


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