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ORIGINAL ARTICLE

Expression and localization of AtAGP18, a lysine-rich arabinogalactan-protein in *Arabidopsis*

Jie Yang · Allan M. Showalter

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Abstract Arabinogalactan-proteins (AGPs) are present on the surface of all plant cells. 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 (GPI) anchor addition sequence. Organ- and tissue-specific expression patterns and subcellular localization of AtAGP18 were studied and compared to other Lys-rich AGPs. *AtAGP18* was highly expressed in roots, flowers and stems and weakly expressed in seedlings and rosettes. High *AtAGP18* promoter activity was closely associated with vascular tissues and high in young organs as well as styles. Microarray and massively parallel signature sequencing (MPSS) data were also examined and showed largely consistent transcription profiles of *AtAGP18*. On the protein level, AtAGP18 was most abundant in roots and flowers, moderate in stems, seedlings and siliques and low in rosette leaves. Furthermore, AtAGP18 was localized to the plasma membrane and to Hechtian strands, following plasmolysis of tobacco cultured cells expressing a green fluorescence protein (GFP)–AtAGP18 fusion protein. Localization of AtAGP18 on the plasma membrane was further confirmed by biochemical two-phase fractionation and Western blotting. These expression and localization data further our understanding of AtAGP18 and provide a molecular basis to approach and decipher its function.

Keywords Arabinogalactan-proteins · *Arabidopsis* · Expression · GUS · Subcellular localization · Western blotting

Abbreviations

ABA	Abscisic acid
AGP	Arabinogalactan-protein
GFP	Green fluorescence protein
GPI	Glycosylphosphatidylinositol
GUS	β-Glucuronidase
HRGP	Hydroxyproline-rich glycoprotein
MPSS	Massively parallel signature sequencing
PM	Plasma membrane
TPM	Transcripts per million

Introduction

Arabinogalactan-proteins (AGPs) are a family of hyperglycosylated macromolecules in the hydroxyproline-rich glycoprotein (HRGP) superfamily. AGPs are present in all lower and higher plants studied to date (Nothnagel 1997; Lee et al. 2005). They exist on the plasma membrane (PM), in periplasm, in cell walls and in extracellular secretions (Showalter 2001; Lamport et al. 2006).

Yariv reagent and AGP antibodies are two major tools used to examine AGPs. Yariv reagent (Yariv et al. 1962)

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J. Yang · A. M. Showalter (✉)
Department of Environmental and Plant Biology,
Ohio University, 504 Porter Hall, Athens,
OH 45701-2979, USA
e-mail: showalte@ohio.edu

J. Yang · A. M. Showalter
Molecular and Cellular Biology Program,
Ohio University, Athens, OH 45701-2979, USA

specifically binds and precipitates AGPs. AGPs are expressed in different organs including seedlings, leaves, stems, roots, flowers and seeds. On the tissue level, AGPs are particularly abundant and well documented in xylem, styles and tissue cultures (Nothnagel 1997; Showalter 2001). With available AGP genetic sequences, RT-PCR (Pereira et al. 2006), Northern blotting (Schultz et al. 2000) and in situ hybridization (Acosta-Garcia and Vielle-Calzada 2004; Motose et al. 2004; Ito et al. 2005; Dahiya et al. 2006) are also often utilized to reveal AGP expression on the cell, tissue and organ levels. Searching public EST, microarray and massively parallel signature sequencing (MPSS) databases represents an alternative approach to obtain expression profiles of AGPs in *Arabidopsis* as well as a few other plant species (Zhang et al. 2003; Zimmermann et al. 2004; Faik et al. 2006; Nakano et al. 2006).

Seven lysine-rich AGPs 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). Lysine-rich AGPs have a small Lys-rich region within the AGP domain, which is not glycosylated and allows for peptide-specific antibody production. For example, the PAP and anti-AGPB antibodies recognize the Lys-rich subdomains of LeAGP1 and PtaAGP6, respectively. These antibodies immunolocalize LeAGP1 and PtaAGP6 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 of LeAGP1 identifies an organ-specific expression and glycosylation pattern of LeAGP1 (Gao and Showalter 2000). LeAGP1 possesses a glycosylphosphatidylinositol (GPI) anchor that attaches it to the PM (Sun et al. 2004b), and all other Lys-rich AGPs are predicted to have the GPI anchor.

LeAGP1 and *NaAGP4* have high sequence identity and similar expression patterns. Their transcript levels are high in young stems, flowers and roots and low in leaves (Li and Showalter 1996; Gilson et al. 2001). *CsAGP1* is expressed throughout cucumber seedlings, including in the shoot apices, cotyledons, hypocotyls and roots (Park et al. 2003). In addition, expression of these AGPs is responsive to biotic and abiotic stresses, including wounding, pathogen invasion, heat shock and phytohormones (Li and Showalter 1996; Gilson et al. 2001; Park et al. 2003; Sun et al. 2004a).

Of the three putative Lys-rich AGPs in *Arabidopsis*, AtAGP17 has been experimentally demonstrated to be a bona fide AGP (Sun et al. 2005). 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 two major glycosyl residues; AtAGP17 is precipitated by Yariv reagent; 81% Hyp residues in AtAGP17 are modified by AG polysaccharides, and 12% by oligoarabinosides; and last, AtAGP17 has glycosyl linkages characteristic of AGPs, with the branched 1,3,6-galactose being the most abundant linkage. Like LeAGP1, green fluorescence protein (GFP)–AtAGP17 fusion protein is localized to the PM and Hechtian strands, presumably via the GPI anchor (Sun et al. 2005).

Gaspar et al. (2004) reported that *AtAGP17* mRNA was only found in the flowers, and *AtAGP18* transcription was high in flowers, moderate in stems and very low in leaves. In a separate study, *AtAGP17* transcript is found in seedlings, rosette leaves, flowers and stems, but not roots. *AtAGP19* mRNA, on the other hand, is abundant in stems, moderate in flowers and roots and low in leaves (Yang et al. 2007). By using the *β-glucuronidase* (*GUS*) reporter gene and in situ hybridization, *AtAGP18* expression is only found in developing anthers and ovules, in embryos until the globular stage and transiently in vascular tissues. Furthermore, *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 (Acosta-Garcia and Vielle-Calzada 2004).

AGPs play important roles in various aspects of plant growth and development, such as morphogenesis, xylem differentiation, programmed cell death, somatic embryogenesis, reproduction, cell proliferation, cell expansion, cell adhesion and signaling (Majewska-Sawka and Nothnagel 2000; Showalter 2001). However, most roles of AGPs are inferred from their spatially- and temporally-specific expression patterns, and their mode of action is still elusive. One exception is AtAGP18; RNA interference of *AtAGP18* results in ovule abortion and reduced seed set, due to failure of functional megaspores to enlarge and divide (Acosta-Garcia and Vielle-Calzada 2004). AtAGP18 is therefore proposed to be essential for female gametogenesis. This earlier work focuses on expression and function of *AtAGP18* in reproduction, while this paper presents a more complete expression profile of AtAGP18 obtained from comprehensive expression analyses on

its mRNA and protein levels. Subcellular localization of AtAGP18 is also reported here. These data lead to a better understanding of AtAGP18 as well as provide functional insight to AtAGP18 in *Arabidopsis*.

Materials and methods

Plant material and growth conditions

Arabidopsis plants (Columbia-0 ecotype) were grown at 22°C under 16 h light/8 h dark conditions in soil or MS plates consisting of 1× MS (Murashige and Skoog) medium, 1% sucrose and 0.8% agar.

Bioinformatics

The similarities/identities table was generated with MatGAT (Matrix Global Alignment Tool) v2.01 using the BLOSUM62 algorithm (http://www.angelfire.com/nj2/arabidopsis/MatGAT.html) (Campanella et al. 2003). Amino acid sequence alignment of Lys-rich AGPs was rendered with Vector NTI version 6.0 (Invitrogen, Carlsbad, CA).

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 (http://www.ebi.ac.uk/clustalw). The tree was displayed with TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

RNA extraction and Northern blotting

Arabidopsis suspension-cultured cells were collected by filtering at different times after subculturing; aerial parts of 7-day-old *Arabidopsis* seedlings and 4-week-old mature rosette leaves, roots, flowers and inflorescence stems were harvested. Total RNA was extracted with the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA). Northern blotting was carried out according to Sun et al. (2005).

GUS staining

For the $P_{AtAGP18}$:*GUS* construct, the *AtAGP18* promoter (2.5 kb 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′ using the AccuPrime *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR product and pMDC164 binary vector (Curtis and Grossniklaus 2003) were digested by restriction enzymes *Pac*I (TTAATTAA) and *Asc*I (GGCGCGC C) and ligated (New England BioLabs, Beverly, MA). Binary vectors were delivered into *Agrobacterium* strain LBA4404 by electroporation (Weigel and Glazebrook 2002). WT *Arabidopsis* plants were transformed by the floral dip method (Clough and Bent 1998). GUS staining was performed on T2 plants (Yang et al. 2007). At least ten independent transgenic lines harboring the $P_{AtAGP18}$:*GUS* fusion were tested, and representative GUS staining patterns were presented. WT controls were also subjected to the same GUS staining solution and were never stained.

Expression data retrieval

Microarray data for *AtAGP18* expression in WT *Arabidopsis* (Columbia-0) under normal conditions were obtained from Genevestigator (Zimmermann et al. 2004) at http://www.genevestigator.ethz.ch/ and plotted. Expression data of *AtAGP18* in roots were available at the Arabidopsis Gene Expression Database at http://www.arexdb.org. Microarray data of *AtAGP18* 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. Co-expression of *AtAGP18* with other genes was examined at the *A. thaliana* Co-Response Database (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath.html) and the *Arabidopsis thaliana trans*-factor and *cis*-element prediction database (http://www.atted.bio.titech.ac.jp/). Signature MPSS data were obtained from Arabidopsis MPSS Plus Database (http://mpss.udel.edu/at/) (Meyers et al. 2004). Transcripts per million (TPM) values (sums of abundance for classes 1, 2, 5 and 7 signatures) of the 17 and 20 bp signatures were averaged and plotted.

Production of the anti-AtAGP18 peptide antibody

A 20 aa peptide (PAPAPSKHKKTTKKSKKHQA) corresponding to the Lys-rich region of AtAGP18 was synthesized and conjugated to keyhole limpet hemocyanin to promote immune responses in rabbits. The antibody was produced and purified with affinity columns (Genemed Synthesis Inc., South San Francisco, CA). This antibody is referred to either as anti-AtAGP18 peptide antibody or simply as AtAGP18 antibody in this manuscript.

Western blotting

Total proteins were extracted from *Arabidopsis* leaves, roots, stems, flowers and siliques (Weigel and Glazebrook 2002) and quantified with *DC RD* Protein Assay Kit (Bio-Rad, Hercules, CA). 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 were run on 5–10% SDS-PAGE gels and electro-blotted to PVDF membrane (Bio-Rad, Hercules, CA).

Duplicate membranes were either stained by 0.1% (w/v) Coomassie blue or immunoblotted. For immunoblotting, the membrane was treated with blocking buffer [5% (w/v) milk, 0.05% (v/v) Tween 20, PBS] and incubated with the GFP antibody (Clontech, Mountain View, CA) diluted 1:400 for 1 h at RT or the purified AtAGP18 antibody diluted 1:500 overnight at 4°C. The membrane was washed in PBS supplemented with 0.05% (v/v) Tween 20 before incubation with alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich, St Louis, MO) or horse radish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL). For Yariv staining of gels, the gel was submerged in Yariv solution (200 μM Yariv, 1% NaCl) overnight at RT and rinsed.

Localization of the GFP-AtAGP18 fusion protein

To construct the GFP-AtAGP18 fusion protein, the *LeAGP1* signal sequence followed by the *enhanced GFP* sequence was amplified from the *pBI121–SS^{tom}–EGFP–AtAGP17* vector (Sun et al. 2005) with a 5′ primer containing a *Xho*I site (CTCGAG) (5′-CCG GCT CGA GGG TCT ATA TTT TCT TTA GCT ACC ATG-3′) and a 3′ primer containing a *Cla*I site (ATCGAT) (5′-AAA TTA ATC GAT GTA CAG CTC GTC CAT GCC GAG AGT GA-3′). The *AtAGP18* coding region without the signal sequence was 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′. The PCR products were successively cloned into the vector pKANNIBAL (Wesley et al. 2001) between the *Xho*I site (CTCGAG) and the *Cla*I site (ATCGAT) and between the *Cla*I site (ATCGAT) and the *Xba*I site (TCTAGA), and the fragment containing the 35 S CaMV promoter, *GFP* and *AtAGP18* was then inserted into the binary vector pART27 (Gleave 1992).

Bright yellow-2 (BY-2, *Nicotiana tabacum*) cells were transformed and maintained following published methods (Persson et al. 2001; Love et al. 2002; Zhao et al. 2002). Transgenic BY-2 cells were observed using a Zeiss confocal laser scanning fluorescence microscope LSM510 (Zeiss, Germany) (Sun et al. 2004b, 2005). Images were processed with the Zeiss LSM image browser. PM vesicles were purified by two-phase fractionation following published procedures (Larsson et al. 1987; Komalavilas et al. 1991; Sun et al. 2004b). The PM and PM-depleted (intracellular membranes) phases were dissolved in ddH_2O, stored at −80°C and used for Western blotting.

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 (Fig. 1a). Amino acid sequences of the Lys-rich AGPs were aligned (Fig. 1b). When compared to other Lys-rich AGPs, AtAGP18 had highest and lowest amino acid similarity/identity with AtAGP17 and AtAGP19, respectively (Table 1). In order to provide some insight on the relationship among Lys-rich AGPs and other AGPs, a phylogeny tree was created (Fig. 1c). All known Lys-rich AGPs clustered together; AtAGP17 and AtAGP18 were closely related. In contrast, AtAGP19 was most closely related to PtaAGP6 and AtAGP9.

Northern blotting

Northern blot analyses revealed that expression of *AtAGP18* in *Arabidopsis* was high in roots, flowers and stems and low in seedlings and rosettes (Fig. 2a). The organ-specific expression pattern of *AtAGP18* was also compared to microarray and MPSS data, as discussed later (Fig. 2b, c). In the *Arabidopsis* cell culture, *AtAGP18* transcript levels were high in exponentially growing cells and decreased with time (Fig. 2a).

Promoter activity of *AtAGP18*

To investigate the expression of *AtAGP18* on the tissue level, expression of the *GUS* gene under the control of the *AtAGP18* promoter was examined in transgenic *Arabidopsis* plants (Fig. 3). *AtAGP18* was widely expressed in *Arabidopsis* tissues at different developmental stages. In light-grown seedlings, cotyledons, hypocotyl and roots were stained. The basal portion of the hypocotyl was more stained than the apical portion.

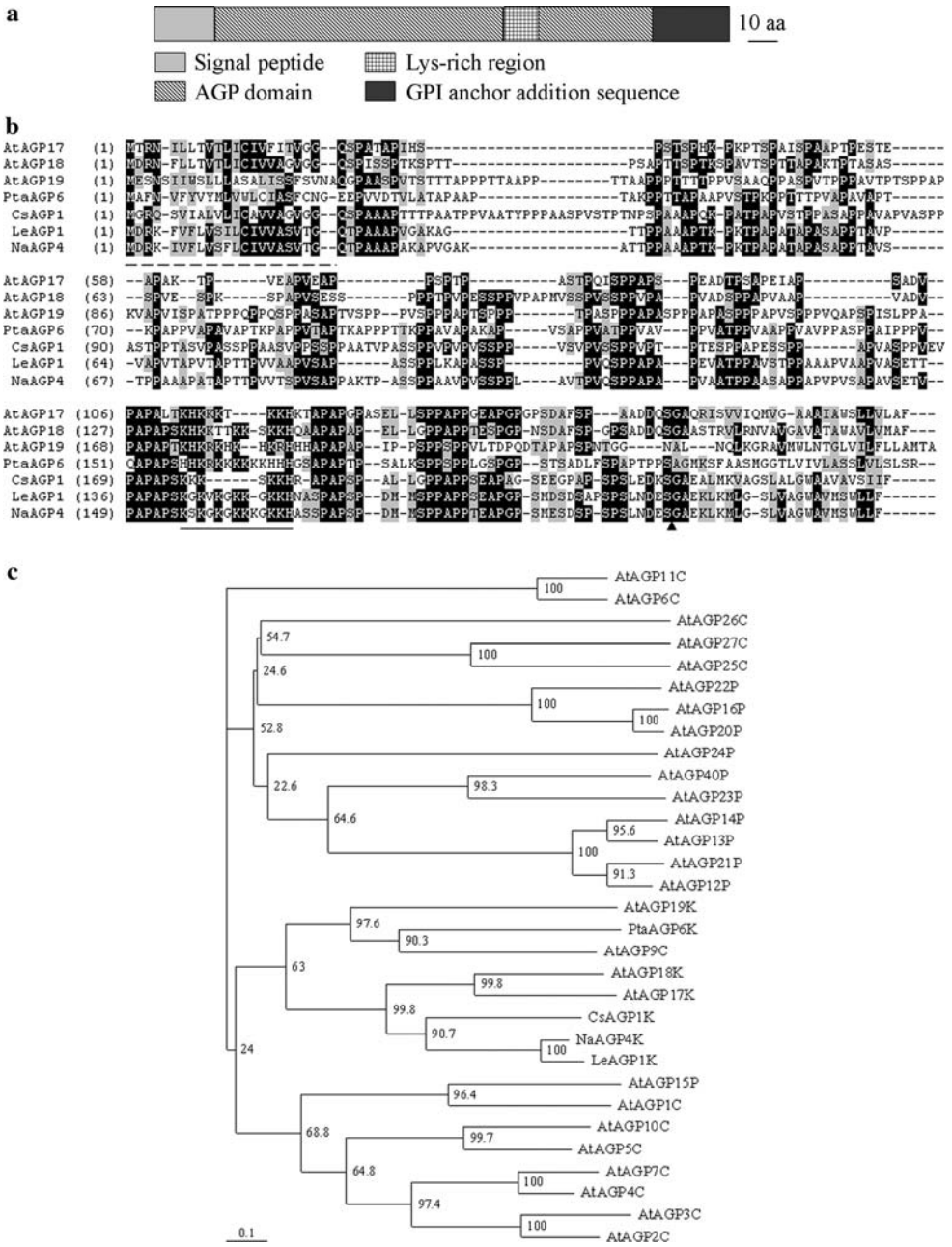

Fig. 1 Lys-rich AGPs. **a** Protein backbone organization of AtAGP18. **b** Amino acid sequence alignment of seven Lys-rich AGPs. Identical amino acids are in a *black background*, while similar amino acids are in a *gray background*. Signal peptide sequences are *dash underlined*. Lys-rich regions are *underlined*. A *triangle* indicates the predicted cleavage sites for GPI anchor addition. **c** Phylogeny tree of all classical AGPs and AG peptides in *Arabidopsis* as well as Lys-rich AGPs from other plants. *Capitalized letters* after the protein names indicate the classes of AGPs: *C* classical AGPs; *K* Lys-rich AGPs; *P* AG peptides. Bootstrap values from 1,000 trials are indicated at *branch points*. The *scale* indicates the branch length. Gene identification No. (either *Arabidopsis* AGI locus No. or Genbank accession No.) are: *AtAGP1*, At5g64310; *AtAGP2*, At2g22470; *AtAGP3*, At4g40090; *AtAGP4*, At5g10430; *AtAGP5*, At1g35230; *AtAGP6*,At5g14380; *AtAGP7*, At5g65390; *AtAGP9*, At2g14890; *AtAGP10*, At4g09030; *AtAGP11*, At3g01700; *AtAGP12*, At3g13520; *AtAGP13*, At4g 26320; *AtAGP14*, At5g56540; *AtAGP15*, At5g11740; *AtAGP16*, At2g46330; *AtAGP17*, At2g23130; *AtAGP18*, At4g37450; *AtAG P19*, 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

Roots were generally stained, including the vasculature, root hairs and other root cells, but not the root tip. In 3-day-old dark-grown seedlings, staining was throughout the seedlings; in 5-day-old dark-grown seedlings, the upper portion of hypocotyl was strongly stained. Strong *AtAGP18* promoter activity was found in young rosette

Table 1 Amino acid similarities/identities among the seven Lys-rich AGPs

Similarities and identities are indicated in the left and right hand sides of the diagonal, respectively

	AtAGP17	AtAGP18	AtAGP19	PtaAGP6	CsAGP1	LeAGP1	NaAGP4
AtAGP17		53.7	27.9	32.9	34.5	40.3	38.8
AtAGP18	64.1		33.6	34.9	46.2	45.2	43.8
AtAGP19	37.7	44.9		40.0	38.3	36.1	34.7
PtaAGP6	41.9	47.9	55.5		37.5	38.7	39.8
CsAGP1	48.6	58.8	51.8	51.0		47.6	49.8
LeAGP1	57.2	62.3	45.7	50.4	58.8		79.6
NaAGP4	55.7	60.1	47.8	51.3	64.2	82.5	

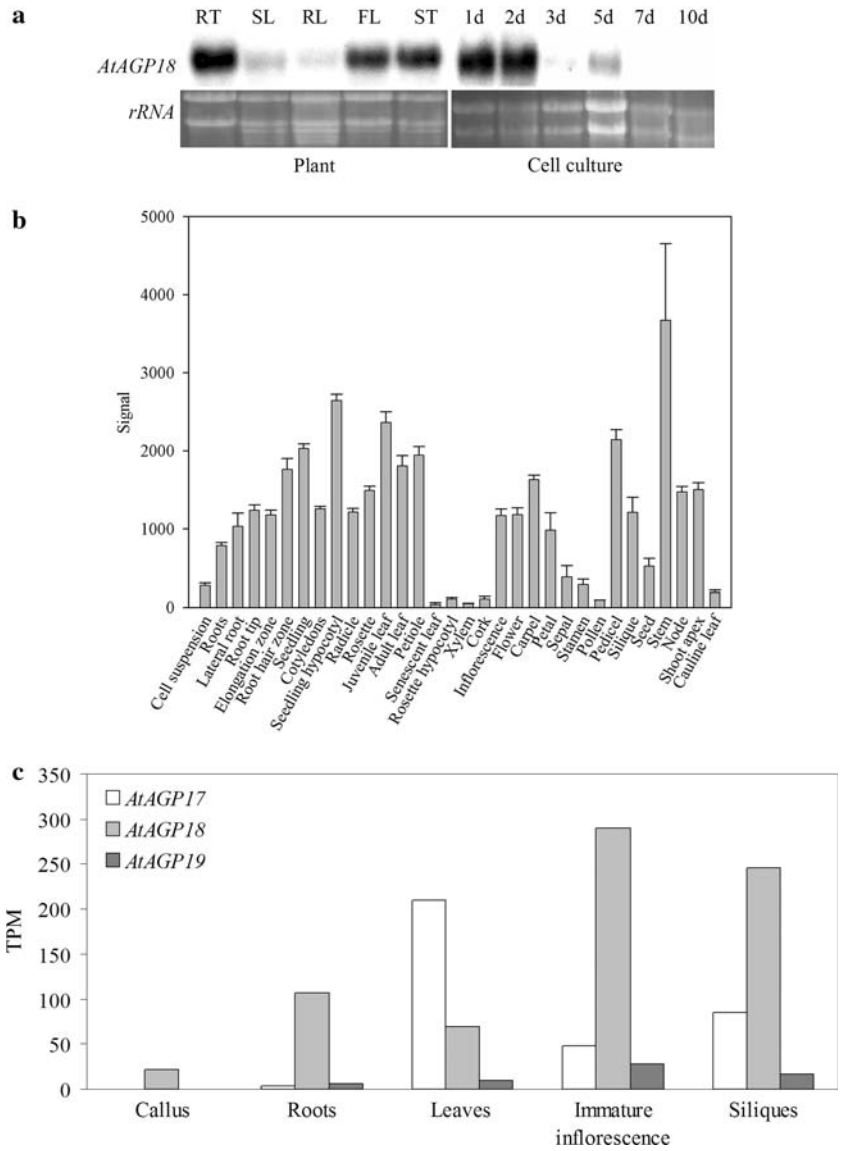

Fig. 2 Expression analyses of *AtAGP18*. **a** Northern blotting of *AtAGP18* in *Arabidopsis* plants and cell cultures. Aerial parts of 10-day-old seedlings (*SL*), 4-week-old roots (*RT*), rosette leaves (*RL*), flowers (*FL*) and stems (*ST*) as well as cultured cells of different ages after subculturing were examined. rRNAs were stained with ethidium bromide to show equal loading. **b** Expression of *AtAGP18* in *Arabidopsis* detected by microarray experiments (compiled by Genevestigator). *Signal* indicates expression intensities. **c** Expression of *AtAGP17*, *18* and *19* detected by the signature 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

leaves and decreased as the leaf matured. Stomata, trichomes and trichome basal cells displayed GUS staining. Staining diminished from the midvein region in older leaves and persisted in the hydathodes (epidermal structures specialized for water secretion). All staining disappeared in old and senescent leaves. Young stems, flowers (especially the styles) and elongating siliques also exhibited strong *AtAGP18* promoter activity. In addition to styles, GUS staining was found in vascular tissues of sepals, petals and stamens, but not pollen. In older siliques, *AtAGP18* expression was restricted to seeds, but not seed pods. From the above, *AtAGP18* promoter activity was tissue-specific and developmentally-regulated; vascular tissues and styles as well as young organs (i.e., leaves, stems and siliques) were preferentially stained by GUS.

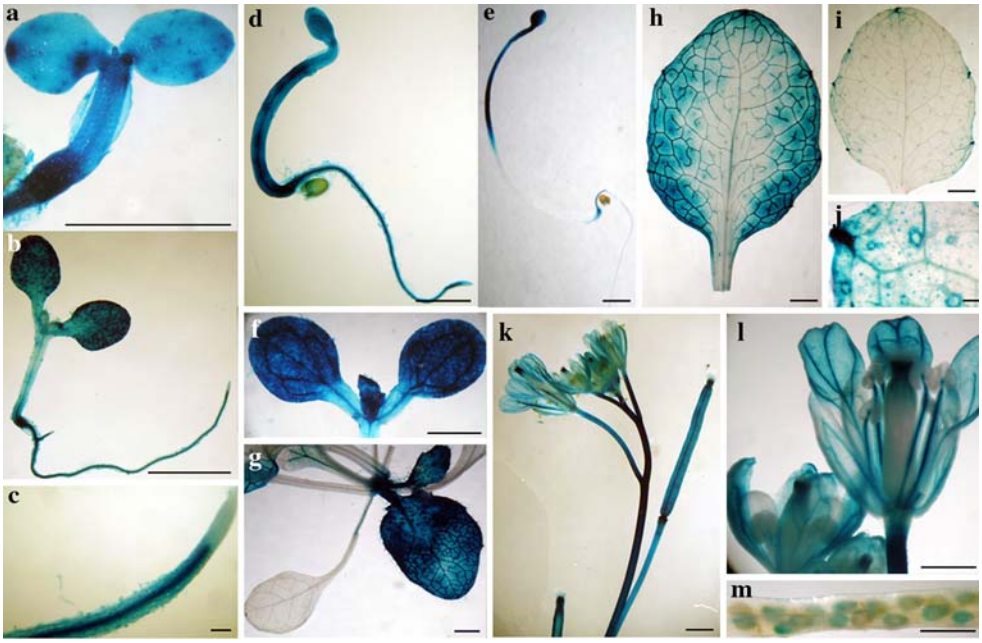

Fig. 3 GUS staining in transgenic *Arabidopsis* plants harboring the $P_{AtAGP18}$:*GUS* construct. **a**, **b** Three- and seven-day-old seedling grown in light, respectively. **c** Root of 7-day-old seedling grown in light. **d**, **e** Three- and five-day-old seedling grown in dark, respectively. **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**, **i** Maturing rosette leaves. Staining started to disappear from the midvein. **j** Magnified view of a hydathode in **i**. Trichome basal 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**–**i** and **k**, 100 μm in **c** and **j** and 500 μm in **l** and **m**

Microarray and MPSS expression data

In addition to Northern blotting and GUS analysis, *AtAGP18* expression was also examined by accessing publicly available microarray data at Genevestigator (Zimmermann et al. 2004). *AtAGP18* expression was detected in cell suspension cultures and a variety of plant tissues and organs (Fig. 2b) in microarray experiments with Affymetrix GeneChips 25 k chips (ATH1 chips covering all *Arabidopsis* genes). Moreover, tissue comparison experiments using cDNA chips detected 5–6 times more *AtAGP18* transcript in flowers relative to leaves (data not shown). The abundance of the *AtAGP18* transcript in vascular tissues, as revealed by the promoter-GUS analysis, was also corroborated by *AtAGP18* expression data in *Arabidopsis* roots from the Arabidopsis Expression Database. In addition to high expression in the root vasculature, *AtAGP18* expression was detected in other root tissues, such as endodermis, cortex and epidermis (Supplemental Fig. S1).

Another approach to analyze gene expression is signature MPSS (Meyers et al. 2004). *AtAGP18* was the only Lys-rich AGP whose expression was detected by MPSS in callus (Fig. 2c). Its transcript levels were high in inflorescence and siliques and moderate in roots and leaves.

AtAGP18 expression levels in *Arabidopsis* treated with various abiotic and biotic stress were also examined. Some abiotic treatment microarray data were summarized at Arabidopsis Membrane Protein Library (http://www.cbs.umn.edu/arabidopsis/). *AtAGP18* transcript levels decreased in the presence of cold, osmotic and UV stress, but not wounding (data not shown).

Expression data in biotic stress experiments were found via TAIR Microarray Expression Search (http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression) and manually analyzed. *AtAGP18* signals were downregulated by ABA, upregulated by brassinosteroid and did not significantly change in response to other hormones (data not shown). Suppression of *AtAGP18* expression by ABA was corroborated by Northern blot analysis (Sun 2004). Pathogen-mediated regulation of *AtAGP18* expression was also examined. *AtAGP18* transcription was significantly suppressed by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), but not by a nonpathogenic mutant version *Pst* DC3000 *hrcC*$^{-}$ that had a mutation in the *hrcC* gene (Supplemental Fig. S2).

Two databases, the *A. thaliana* Co-Response Database and the *Arabidopsis thaliana trans*-factor and *cis*-element prediction database, were used to examine genes co-expressed with *AtAGP18*. Both databases

showed that *AtAGP18* was co-regulated with genes belonging to various families, including those involved in cell wall biosynthesis, hormone pathways and signaling. A list of the top 50 genes that showed highest expression correlation with *AtAGP18* from both websites is provided in Supplemental Table S1. *AtAGP18* was also co-expressed with *AtAGP17* (data not shown) as well as other AGPs genes, such as *AtFLA2* and *AtAGP21*. Interestingly, some glycosyltransferases were observed to be co-expressed with *AtAGP18*, leading to the speculation that AtAGP18 is the acceptor substrate for these glycosyltransferases. Co-regulation of *AtAGP18* with other genes, however, needs to be pursued more completely to understand the significance.

Western blotting with the anti-AtAGP18 peptide antibody

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 the antibody (data not shown). The purified anti-AtAGP18 peptide antibody was tested for its specificity. When synthetic peptides (20 aa) corresponding to Lys-rich regions of AtAGP17, 18 and 19 were used in dot blotting, the AtAGP18 antibody did not recognize the AtAGP17 and 19 peptides but exhibited strong reactivity against the AtAGP18 peptide (Supplemental Fig. S3a). Additionally, the AtAGP18 antibody did not react with the purified GFP–AtAGP17 fusion protein (Supplemental Fig. S3b). These results indicated that the AtAGP18 antibody was specific towards the targeted peptide, and in particular, did not show detectable cross-reactivity with AtAGP17.

Organ-specific expression of AtAGP18 was obtained with Western blotting using the purified anti-AtAGP18 peptide antibody against total protein preparations (Fig. 4). AtAGP18 protein levels were highest in roots and flowers, followed by stems and seedlings. Low abundance of AtAGP18 protein was found in siliques and rosette leaves.

Subcellular localization of AtAGP18

In order to localize AtAGP18 at the cellular level, a GFP–AtAGP18 fusion protein was expressed in transgenic tobacco BY-2 cells under the control of the 35 S CaMV promoter. *GFP* was inserted between the signal sequence and the rest of *AGP18* coding sequences. While WT BY-2 cells showed no background fluorescence, green fluorescence was observed on the surfaces

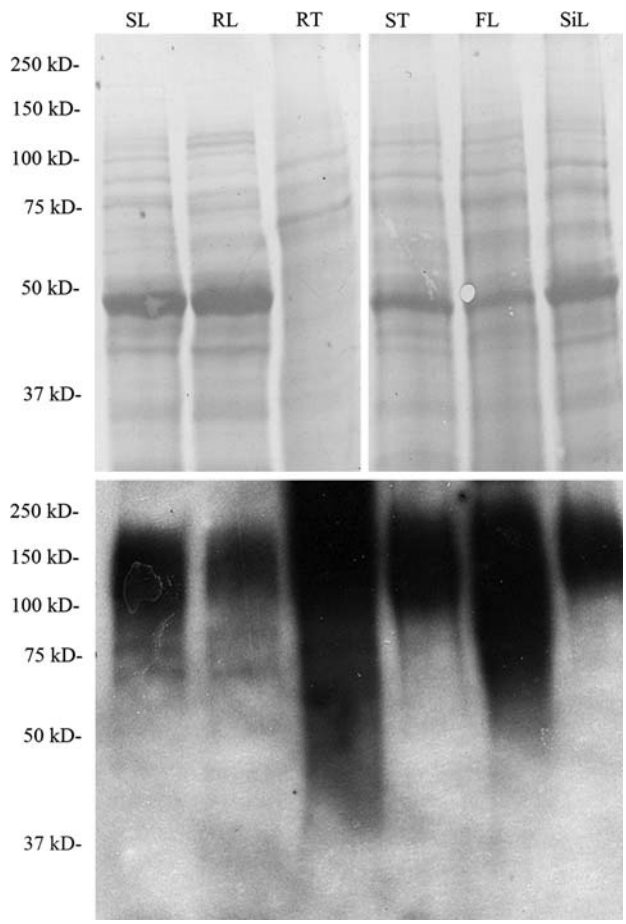

Fig. 4 Organ-specific expression pattern of AtAGP18 revealed by Western blot analysis. Total protein (30 μg) from *Arabidopsis* 12-day-old seedlings (*SL*), 35-day-old rosette leaves (*RL*), roots (*RT*), stems (*ST*), flowers (*FL*) and siliques (*SiL*) were loaded in each lane in duplicate gels. Identical membranes were either stained by Coomassie blue (*top panel*) or incubated with the AtAGP18 antibody (*bottom panel*). Sizes of protein markers are labeled on the *left*

(the PM and the cell wall) of transgenic BY-2 cells (Fig. 5a). When cells were plasmolyzed with 4% NaCl for 15 min, green fluorescence was localized to both the PM and Hechtian strands (Fig. 5b). The above results indicated that AtAGP18 was localized to the PM, presumably via a GPI lipid anchor, and in Hechtian strands, adhesion sites between the PM and cell wall.

To confirm the PM localization of AtAGP18, biochemical two-phase fractionation and Western blotting were employed. Two phases were obtained from BY-2 cells expressing the GFP–AtAGP18 fusion protein: one phase contained purified PM, and the other phase was depleted of PM. Equal amounts of proteins from the two fractions were used for Western blotting, and the GFP antibody revealed the majority of GFP–AtAGP18 in the purified PM fraction, but not in the

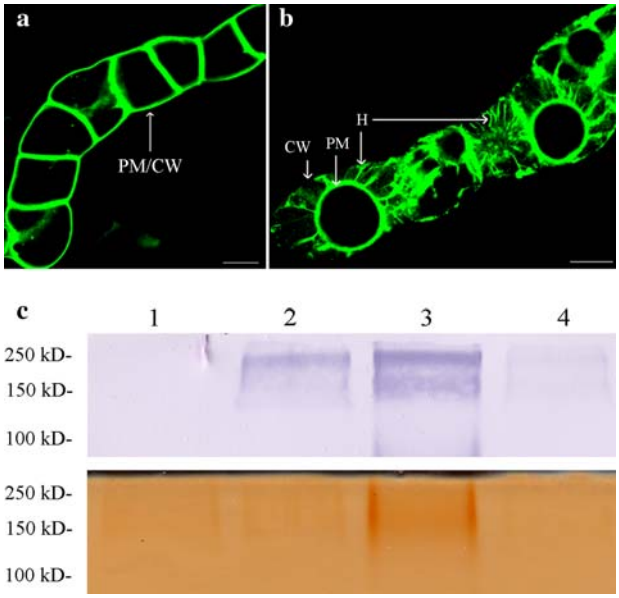

Fig. 5 AtAGP18 protein was localized to the PM. **a** GFP fluorescence was observed on the surfaces of transgenic BY-2 cells expressing the GFP–AtAGP18 fusion protein before plasmolysis. **b** The GFP–AtAGP18 fusion protein was localized to the PM and Hechtian strands of transgenic BY-2 cells after plasmolysis with 4% NaCl for 15 min. *CW*, cell wall. *H*, Hechtian strands. *PM*, plasma membrane. *Scale bars* 20 μm. **c** Western blot analysis of GFP–AtAGP18 in transgenic BY-2 cells. *Top panel* Western blotting with the GFP antibody. *Bottom panel* Yariv staining of a duplicate gel. *1*, WT BY-2 cell lysate. *2–4*, biochemical protein preparations from transgenic BY-2 cells expressing GFP–AtAGP18 fusion protein. *2*, microsomes. *3*, purified PM fraction. *4*, PM-depleted fraction. Ten micrograms of proteins were loaded in each lane. Sizes of protein markers are labeled on the *left*

fraction depleted of PM, indicating that AtAGP18 was indeed present on the PM (Fig. 5c).

Discussion

AtAGP17, 18 and *19* are Lys-rich classical AGP genes in *Arabidopsis* and are predicted to be anchored to the PM by a GPI anchor (Schultz et al. 2002). Here, the GFP–AtAGP18 fusion protein produced an identical PM localization pattern as GFP–LeAGP1 and GFP–AtAGP17 in transgenic BY-2 cells (Zhao et al. 2002; Sun et al. 2004b, 2005). Furthermore, the GFP antibody localized GFP–AtAGP18 to the purified PM fraction, corroborating the GPI anchor prediction in AtAGP18 and making it a candidate for participating in cell signaling (Schultz et al. 1998).

In the present work, expression of AtAGP18 was examined by Northern blotting, P_{AtAGP18}:*GUS* study, microarray and MPSS data as well as Western blotting. These methods produced largely consistent results, despite small discrepancies with respect to relative transcript abundance in different organs. For example, only microarray experiments with ATH1 chips detected higher levels of *AtAGP18* mRNA in rosettes than flowers. A likely reason for such differences is that *Arabidopsis* plants used for different analyses were grown under different conditions and might not be harvested at identical stages. Indeed, microarray signals were averaged from a pool of microarray experiments performed by multiple labs. As *AtAGP18* was the main focus of this study, we are confident that our results accurately reflect the endogenous expression pattern of *AtAGP18*. Moreover, the Northern blotting profile of *AtAGP18*, as reported by Gaspar et al. (2004), was similar to the one presented here. Low accumulation of *AtAGP18* mRNA in leaves was also observed with RT-PCR (Pereira et al. 2006). *AtAGP18* promoter activity was not found in pollen in this study, contradictory to results in Acosta-Garcia and Vielle-Calzada (2004) and might be attributed to different promoter sequences used. In the earlier study, in situ hybridization did not detect *AtAGP18* mRNA in other aerial parts of *Arabidopsis* besides developing anthers and ovules and clusters of companion cells closely associated with stem vascular elements (Acosta-Garcia and Vielle-Calzada 2004).

The genetic and protein expression patterns of AtAGP18 resemble those of LeAGP1 (Li and Showalter 1996; Gao and Showalter 2000). mRNA and protein levels of both AGPs were high in roots, flowers, stems and young fruits and low in leaves. In particular, expression was stronger in younger expanding organs, such as suspension-cultured cells, young leaves and siliques. 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, high *AtAGP18* promoter activity was observed in the style and vascular tissues. Moreover, like *CsAGP1* (Park et al. 2003), *AtAGP18* transcripts were found throughout seedlings. Despite similar endogenous expression profiles under normal conditions, *AtAGP18*, *LeAGP1* and *NaAGP4* responded differently to wounding. *AtAGP18* did not change its mRNA level, while *LeAGP1* and *NaAGP4* transcripts rapidly decreased. On the other hand, transcription of *AtAGP18* and *NaAGP4* were both down-regulated by pathogens. Resembling *LeAGP1*, *AtAGP18* expression was suppressed by ABA; unlike *LeAGP1* and *CsAGP1*, it did not change in the presence of exogenous cytokinin, gibberellin or auxin.

AtAGP18 and 19 have relatively low amino acid sequence identity/similarity, and AtAGP18 is more distantly related to AtAGP19. However, genetic expres-

sion patterns of *AtAGP18* and *19*, obtained by Northern blotting and GUS staining, were similar (Yang et al. 2007) although *AtAGP18* promoter activity was stronger. For example, both *AtAGP18* and *AtAGP19* promoter activities were associated with vascular tissues and styles, but not root tips; they were also developmentally regulated, with young organs being more highly stained. An interesting difference between expression patterns of *AtAGP18* and *19* was that *AtAGP18* is preferentially expressed in the basal region of the hypocotyl, while *AtAGP19* GUS staining is more abundant towards the apical region. Another difference was that *AtAGP18* was expressed in root vasculature as well as other root cells, while *AtAGP19* was only expressed in root vascular tissues. Moreover, according to the Arabidopsis Expression Database, which provides detailed root expression data for *Arabidopsis* genes, *AtAGP18* is expressed in the vasculature, endodermis, cortex and epidermis, consistent with the GUS staining pattern reflecting *AtAGP18* promoter activity in roots.

The expression patterns of AGPs have provided guidance in elucidating their biological roles. For instance, based on higher expression of these Lys-rich AGPs in young and expanding tissues, it was proposed that they, in coordination with expansins and xyloglucan endotransglucosylases/hydrolases, contribute to cell wall expansion (Gilson et al. 2001). Co-expression analysis of *AtAGP18* provides limited support for this idea and illustrates another potential approach to understanding the function of AtAGP18 (and its biosynthesis) by identifying candidate genes that network with *AtAGP18*. Similarly, the precisely controlled presence of AGPs can presage, and hence are implicated in, developmental changes and pattern formation (Knox et al. 1989, 1991; Pennell and Roberts 1990; Pennell et al. 1991). RNA interference of *AtAGP18* led to functional megaspores that failed to enlarge and divide, resulting in ovule abortion and reduced seed set (Acosta-Garcia and Vielle-Calzada 2004). This phenotype was not observed in two insertion mutant lines of *AtAGP18*, namely GT_6565 (Cold Spring Harbor Laboratory) and SALK_117268 (Alonso et al. 2003). In these two mutants, insertions were in the C-terminal hydrophobic sequence, and *AtAGP18* was expressed at similar levels relative to WT, although in SALK_117268, the *AtAGP18* transcript was truncated (J. Yang and A. Showalter, unpublished results). Understanding gene and protein expression can be the first step towards deciphering the function. Since AtAGP18 expression is not limited to floral organs, it is likely that its function is required for processes other than female gametogenesis in reproduction, including vascular development and cell division.

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