Expression analyses of AtAGP17 and AtAGP19, two lysine-rich arabinogalactan proteins, in Arabidopsis

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ABSTRACT
AtAGP17 and AtAGP19 are members of the lysine-rich arabinogalactan protein (AGP) subfamily in Arabidopsis. Detailed anatomical analysis of promoter activity of the AtAGP19 gene was carried out using transgenic Arabidopsis plants expressing a PAGP19-GUS fusion. AtAGP19 promoter activity was tissue-specific and associated with vascular bundles, particularly differentiating xylem elements. Peptide-specific antibodies were raised against the Lys-rich regions of AtAGP17 and AtAGP19 and used to study the organ-specific expression patterns of these two AGPs. AtAGP17 and AtAGP19 were most abundant in roots and flowers, moderately abundant in stems, seedlings and siliques and virtually absent in leaves. Antibodies specific for AtAGP17 and AtAGP19, as reported here, represent valuable tools for understanding the biology of these two AGPs.

INTRODUCTION
Arabinogalactan proteins (AGPs) are a family of hyperglycosylated proteins within the hydroxyproline-rich glycoprotein (HRGP) superfamily. Protein backbones of AGPs are rich in Pro/Hyp (hydroxyproline), Ser, Ala and Thr and are modified by the addition of type II arabinogalactan polysaccharides and arabinose oligosaccharides to Hyp residues. AGPs are present in all lower and higher plants studied to date (Nothnagel 1997; Gaspar et al. 2001; Showalter 2001; Seifert & Roberts 2007; Ellis et al. 2010).

AGP antibodies are valuable tools to probe the expression and function of AGPs. Given that AGPs are typically 10% protein and 90% carbohydrate by weight, most of the protein backbone of a typical AGP is coated with sugar. Consequently, most of antibodies for AGPs developed against native AGPs recognise the carbohydrate epitopes on AGPs. The JIM and MAC antibodies, JIM4, JIM8, JIM13, JIM15 and MAC207, are examples of such AGP antibodies (Knox 1997). These antibodies are useful for examining organ-, tissue- and cell-specific expression of AGPs sharing common carbohydrate epitopes; however, they cannot discriminate among individual AGPs and may cross-react with other macromolecules bearing the same carbohydrate epitopes, such as pectin (Kreuger & van Holst 1996; Showalter 2001).

AGPs are divided into several classes: classical AGPs, lysine-rich classical AGPs, AGP peptides, fasciclin-like AGPs (FLAs) and other chimeric AGPs (Schultz et al. 2002; Showalter et al. 2010). Classical AGPs typically consist of an N-terminal signal peptide, a Pro/Hyp-rich AGP central domain and a C-terminal glycosylphosphatidylinositol (GPI) lipid anchor addition sequence (Schultz et al. 1998). Lysine-rich AGPs are a subclass of the classical AGPs and contain a short Lys-rich region within the Pro/Hyp-rich AGP central domain. This Lys-rich region is not glycosylated and allows for production and recognition of peptide-specific antibodies (Gao et al. 1999; Zhang et al. 2003; Yang & Showalter 2007).

Seven Lys-rich AGPs were identified from different plant species: LeAGP1 in tomato (Li & Showalter 1996; Gao et al. 1999), 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). Expression of LeAGP1, PtaAGP6 and AtAGP18 was characterised on the protein level (Gao & Showalter 2000; Zhang et al. 2003; Yang & Showalter 2007). Antibodies were raised against peptides encompassing the unique Lys-rich subdomains of LeAGP1 (PAPA-PKGGKVKGGKGGKHA), PtaAGP6 (HHKRKGGKGGKHHH) and AtAGP18 (PAPAPSKHKKTCKSKKSKQA). Western analyses revealed similar expression patterns of LeAGP1 and AtAGP18; protein levels of both AGPs were high in roots, flowers, stems and young fruits and low in leaves. LeAGP1 and PtaAGP6 were both localised to differentiating xylem elements; LeAGP1 was also abundant in staminal transmitting tissues (Gao et al. 1999; Gao & Showalter 2000; Zhang et al. 2003; Yang & Showalter 2007).

AtAGP17 and AtAGP19 share 37.7% amino acid sequence similarity and 27.9% amino acid sequence identity. Genetic
expression of AtAGP17 and 19 was previously characterised at the organ level via Northern blotting and RT-PCR (Gasper et al. 2004; Sun et al. 2005; Yang et al. 2007). Interestingly, although the AtAGP17 transcript was barely detectable in roots, a T-DNA knockdown mutant of AtAGP17 displayed abnormal phenotypes only in the roots (i.e., reduced Agrobacterium binding). The tissue-specific expression patterns of these two genes and accumulation of the corresponding proteins at the organ level was never characterised, until now.

Here, we report on the tissue-specific promoter activity of AtAGP19 and the production and use of peptide-specific antibodies for AtAGP17 and AtAGP19 to examine accumulation of these AGPs at the protein level. The AtAGP17- and AtAGP19-specific antibodies reported here represent new additions to the small family of antibodies specific for single AGP family members in their glycosylated forms and will facilitate further analyses to probe the biology and function of these two Lys-rich AGPs.

**MATERIALS AND METHODS**

**Bioinformatics**

Peptide sequences used for antibody production were aligned with ClustalW (http://www.ebi.ac.uk/clustalw). AGP gene identification numbers (either Arabidopsis AGI locus numbers or Genbank accession numbers) are as follows: AtAGP17, At2g23130; AtAGP18, At4g37450; AtAGP19, At1g68725; LeAGP1, X99147.

**Plant material and growth conditions**

Arabidopsis thaliana (ecotype Columbia-0) 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⁻¹ MS basal salts (Caisson Laboratories, Logan, UT, USA), 1% sucrose and 0.8% agar (pH 5.8), unless indicated otherwise. The plates were kept at 24 °C under long-day conditions.

**Histology**

Leaves, hypocotyls and stems of T2 transgenic Arabidopsis plants harbouring the P_{AAGP17;GUS} reporter gene construct were stained for GUS activity as described previously (Yang et al. 2007). At least 10 independent transgenic lines were tested. WT samples were included as negative controls, and no staining of WT samples was observed. Immediately following GUS staining, samples were fixed overnight in Safe-fix II (Fisher Scientific, Hampton, NH, USA), dehydrated in an ethanol series (30%, 50%, 60%, 70%, 80%, 90%, 95%, and 100%, each for 2 h) and embedded in paraffin. Sections 8–25-μm thick were cut with a rotary microtome, depending on the intensity of staining. Sections were dried for 24 h, dewaxed in Citrisolv and rehydrated. Rehydrated sections were either mounted directly in Permount 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.

**Production of AtAGP17 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; Zhang et al. 2003; Yang & Showalter 2007). The same approach was used to produce antibodies against AtAGP17 and 19. Before peptide injection, rabbit pre-immune sera were screened, and rabbits with the lowest immune responses to Arabidopsis total leaf proteins were chosen for production of antibodies. Peptides (20 amino acids in length) were synthesised (Genemed Synthesis, San Francisco, CA, USA) encompassing the Lys-rich regions of the AGPs (Fig. 1).

The synthetic peptides were conjugated to keyhole limpet haemocyanin (KLH) to promote immune responses in rabbits. Peptide-specific antibodies were purified by passing antisera through affinity columns conjugated to the synthetic peptides, and they are referred to as (anti-peptide) antibodies. The flow-through portions from the affinity columns were also collected and used in analyses; these were designated as antisera. Purified antibodies in PBS buffer, antisera and pre-immune sera were stored in aliquots at −80 °C; they were also stable at 4 °C for at least 1 month.

**Western blotting**

Total proteins were extracted from Arabidopsis seedlings, leaves, roots, stems, flowers and siliques according to an established method (Weigel & Glazebrook 2002). 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, USA). Protein samples were run on 5–10% SDS-PAGE gels with Precision Plus Protein All Blue Standards (Bio-Rad) and electro-blotted to a PVDF membrane (Bio-Rad).

Duplicate PVDF membranes were either stained in 0.1% (w/v) Coomassie blue R-250 or immunoblotted. For immunoblotting, the membrane was treated with blocking buffer (5% non-fat dry milk, 0.05% Tween 20, PBS) for 1 h at room temperature (RT) and then incubated with primary antibodies for at least 3 h at RT or overnight at 4 °C. Purified AtAGP17 and 19 antibodies were diluted to between 1:500 and 1:1000; pre-immune sera and antisera were diluted to between 1:100 and 1:500. Membranes were washed (0.05% Tween 20, PBS) before incubation with goat anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) or goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, San Francisco, CA, USA) or goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA).

**Fig. 1.** Alignment of AtAGP17 and AtAGP19 peptides used for antibody production and their homologous sequences from AtAGP18 and LeAGP1. Peptide sequences used for production of PAP antibodies (for LeAGP1) and AtAGP18 antibodies are from previous reports (Gao et al. 1999; Yang & Showalter 2007). ‘*’ indicates identical amino acids. ‘.’ indicates strongly conserved residues. ‘.’ indicates weakly conserved residues.
expression was not detectable in old displayed GUS staining (Fig. 2d). Eventually, AtAGP19 promoter activity was studied at the tissue level. AtAGP19 promoter was confined to vascular tissue, and was later restricted to the periderm on both sides of the leaf as well as to the vascular bundles (Fig. 2c). Both xylem and phloem displayed GUS staining (Fig. 2d). Eventually, AtAGP19 expression was not detectable in old/senescent leaves.

In hypocotyls undergoing secondary cell wall thickening, AtAGP19 GUS activity was found in the xylem, vascular cambium, phloem and periderm, substantiating a previous report on the presence of AGPs in the periderm (Dolan & Roberts 1995). The collapsing outer layers, including the epidermis, cortex and endodermis, did not show GUS activity (Fig. 3a and b). In secondary xylem, xylem parenchyma cells and developing xylem vessels were stained, but differentiated vessels containing lignified secondary cell walls were not stained (Fig. 3c-e). Staining in the periderm decreased in hypocotyls at later developmental stages (Fig. 3e).

In young inflorescence stems where high AtAGP19 expression was observed, virtually all cells were stained (Fig. 3f and g). Vascular bundles, phloem, procambium, xylem parenchyma cells and developing AtAGP17 and AtAGP19.

**RESULTS**

*AtAGP19* expression is specific and developmentally controlled

AtAGP19 promoter activity was studied at the tissue level. Consistent with whole mount GUS staining in leaves (Yang et al. 2007), all leaf cells were stained in newly developed leaves (Fig. 2a). As the leaf matured, AtAGP19 expression decreased in the adaxial half of the leaf (Fig. 2b) and was later restricted to the epidermis on both sides of the leaf as well as to the vascular bundles (Fig. 2c). Both xylem and phloem displayed GUS staining (Fig. 2d). Eventually, AtAGP19 expression was not detectable in old/senescent leaves.

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Specificity and reactivity of antibodies

Antibodies were generated against the Lys-rich regions of AtAGP17 and 19 (Fig. 1) and purified by affinity chromatography against their respective synthetic peptides. The flow-through from the affinity column still contained unremoved antibodies and was referred to as antiserum. The purified antibodies were first tested for their specificity against synthetic peptides encompassing the Lys-rich regions of four homologous AGPs, namely AtAGP17, 18 and 19 and LeAGP1. The AtAGP17 antibodies only recognised the AtAGP17 peptide and did not recognise the peptides for AtAGP18, AtAGP19 or LeAGP1. Similarly, the AtAGP19 antibodies did not bind to peptides for AtAGP17, AtAGP18 or LeAGP1, but only recognised the peptide it was raised against (Fig. 4). Therefore, both anti-peptide antibodies were specific for their respective AGP Lys-rich regions. In addition to being specific, the antibodies exhibited strong reactivity against the peptides. Similar to the antibodies, the AtAGP17 and 19 antisera were specific against their respective peptides. Although the AtAGP19 antiserum recognised the LeAGP1 peptide at 100 μM, this did not cause a cross-reactivity problem since AtAGP17 and LeAGP1 are found in different plant species. Furthermore, the corresponding pre-immune sera did not produce any signals against any of the peptides.

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**Fig. 2.** Expression of AtAGP19 in Arabidopsis leaves. Leaves of transgenic Arabidopsis plants harbouring the P<sub>AtAGP19</sub>:GUS reporter gene construct were stained and sectioned. a: A transverse section of a young leaf (7 days old) showing strong GUS staining in leaf epidermal and mesophyll cells. b: In developing leaves, GUS staining gradually diminished from the adaxial portion but was 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 AtAGP19 promoter was confined to vascular tissues in mature leaves. d: A transverse section of a 4-week-old petiole showing that GUS activity was associated with both xylem and phloem. X = xylem; P = phloem; (b) and (d) were counter-stained with Safranin O after GUS staining and sectioning. Bars = 100 μm.
The AtAGP17 antibodies and antiserum were also tested against purified EGFP-AtAGP17 fusion protein obtained previously (Fig. 5a). Pre-immune serum of AtAGP17 did not recognise the fusion protein, while its antiserum and antibodies did. The AtAGP17 antiserum could detect as little as 250 ng of EGFP-AtAGP17 (Fig. 5b). The smears above 250 kD in most lanes were expected, given that AGPs mobility in gels is retarded and diffuse due to carbohydrate microheterogeneity. In addition, a series of dilutions of the AtAGP17 antibodies and antiserum was tested for reactivity against 1 µg of purified EGFP-AtAGP17. Minimal dilutions of 1:1000 for AtAGP17 antibodies and 1:500 for the antise-
rum generated detectable colorimetric signals (data not shown). The purified anti-AtAGP19 antibodies did not react with EGFP-AtAGP17, consistent with its known specificity (data not shown).

Western blotting with AtAGP17 and 19 antibodies and antisera

Total proteins were extracted from Arabidopsis seedlings and various organs, and the organ-specific expression of AtAGP17 and AtAGP19 was examined using their specific antibodies.

DISCUSSION

AtAGP17, 18 and 19 are Lys-rich classical AGPs in Arabidopsis (Schultz et al. 2002; Sun et al. 2005). These three genes are homologous to four other AGP genes characterised to varying extents in other plant species (Gao et al. 1999; Gilson et al. 2001; Park et al. 2003; Zhang et al. 2003). Here, we report on the tissue-specific promoter activity of AtAGP19 as well as the protein expression patterns of AtAGP17 and 19.

Expression of AtAGP19 on the tissue level, as revealed by anatomical analysis of transgenic plants harbouring the P_{AtAGP19}:GUS fusion, corroborates our earlier finding at the organ level that AtAGP19 expression was spatially and developmentally controlled. Notably, vasculature and young organs displayed the highest amounts of GUS staining. In
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In this study, we extend our analysis to show that AtAGP19 promoter-directed GUS activity was high in young cells and diminished as the cells matured; this phenomenon is consistent with AGP19's proposed role in cell expansion (Yang et al. 2007).

AtAGP19 promoter activity is consistently associated with the vascular tissues throughout the plant. In particular, AtAGP19 promoter activity is found in vascular cambium, procambium, xylem parenchyma cells and differentiating xylem vessels in hypocotyls and stems. Similar to AtAGP19, many other AGP genes show xylem-specific or preferential expression patterns. Two such examples are provided by in situ hybridisation of two homologous FLA genes, AtFLA11 and ZeFLA11. The AtFLA11 transcript is restricted to Arabidopsis stem and silique sclerenchyma cells and co-regulated with secondary cell wall cellulose synthase genes (Brown et al. 2005; Ito et al. 2005; Persson et al. 2005), whereas ZeFLA11 is specifically expressed in differentiating xylem elements with reticulate type wall thickenings in Zinnia (Dahiya et al. 2006). In addition to the above correlations between AGP expression and xylem differentiation, xylogen, a chimeric AGP, is unequivocally established as a mediator in xylem differentiation (Motose et al. 2001). In Zinnia stems, xylogen is expressed in procambium and immature xylem cells. A double xylogen mutant in Arabidopsis (atxyp1 atxyp2) has discontinuous leaf venation patterns (Motose et al. 2004).

Important progress in characterising AGPs has been made with AGP antibodies. AGPs are implicated in pattern formation and vascular development, with their spatially and temporally controlled expression in different stages of xylem differentiation (Knox et al. 1989, 1991; Stacey et al. 1990, 1995a,b; Dolan & Roberts 1995; Dolan et al. 1995; Schindler et al. 1995; Casero et al. 1998). While most antibodies recognise the carbohydrate epitopes on AGPs and are not able to discriminate among individual AGPs, a limited number of antibodies against AGP core proteins are also available, allowing the examination of individual AGPs. PAP and anti-AGPB are antibodies against the Lys-rich regions of LeAGP1 and PtaAGP6, respectively. These antibodies allow the recognition and detection of specific, glycosylated AGPs, since the Lys-rich region imparts specificity and also allows accessibility of the antibody to this non-glycosylated region of the native molecule (Gao et al. 1999; Zhang et al. 2003). With peptide-specific antibodies, LeAGP1 and PtaAGP6 were immunolocalised to differentiating xylem elements and functionally associated with secondary cell wall thickening and xylem differentiation (Gao & Showalter 2000; Zhang et al. 2003).

The AtAGP17 and 19 antibodies and antisera generated using synthetic peptides, demonstrate sufficient specificity
with respect to the synthetic peptides and the EGFP-AtAGP17 fusion protein. However, AtAGP17 antibodies are not able to detect AtAGP17 in total protein preparations. In contrast, AtAGP17 antisera and pre-immune serum allow specific antigen detection in total protein preparations; pre-immune sera either do not react at all or reacted only to a minimal degree. Furthermore, the AtAGP19 antibodies and antisera produced Western blotting results that were similar, but not identical, to those obtained with the AtAGP17 antisera, adding to the validity of the data and specificity of the two antisera.

Protein expression patterns obtained in this study are very similar for AtAGP17 and AtAGP19, despite the relatively low sequence similarity/identity between these two homologous proteins. Both are very abundant in roots and flowers, moderate in stems and siliques and low in leaves. Furthermore, the organ-specific expression patterns of AtAGP17 and AtAGP19 are reminiscent of LeAGP1 and AtAGP18 (Gao & Showalter 2000; Yang & Showalter 2007). Moreover, it appears that AGP17 and 19 as well as AGP18 undergo similar glycosylation reactions in any given organ, leading to the hypothesis that the extent of AGP glycosylation is organ-specific and independent of the AGP core protein. Such organ-specific AGP glycosylation may be critical for biological functions unique to a particular organ. Indeed, it would be informative to tag and express different AGP genes under the control of various organ-specific promoters and examine the extent of glycosylation, carbohydrate composition and glycosidic linkages associated with such AGPs.

Although the reported pattern of expression for AtAGP19 is largely in line with its genetic expression data (Yang et al. 2007), this is not the case for AtAGP17. Surprisingly, little AtAGP17 was detected in leaves, while a large amount of AtAGP17 was found in roots; this is opposite to what would be expected based on Northern blotting data reported in different studies, all of which found that the AtAGP17 transcript level was barely detectable in roots (Gaspar et al. 2004; Sun et al. 2005; Yang et al. 2007). Clearly, the amount of mRNA does not always agree with the amount of protein, and the inconsistency between the AtAGP17 protein and mRNA levels here is particularly noteworthy considering that rat1, an AtAGP17 mutant, displayed altered phenotypes only in the roots, but not in other parts of the plant (Nam et al. 1999; Gaspar et al. 2004). Consequently, future work on AtAGP17 protein distribution in Arabidopsis roots at the tissue level would be worthwhile.

With the research convenience and specificity offered by anti-peptide antibodies and antisera, work can be performed in future to detect the distribution of these individual Lys-rich AGPs at the cell and tissue levels in order to elucidate their detailed expression patterns. Additionally, these antibodies may be useful in isolating and identifying interacting molecular partners for these AGP molecules. Both of these approaches can contribute to a better functional understanding of these particular AGP molecules.

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REFERENCES


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