The Plant Journal (1999) **18**(1), 43–55

Isolation, characterization and immunolocalization of a novel, modular tomato arabinogalactan-protein corresponding to the *LeAGP-1* gene

Minggeng Gao[1], Marcia J. Kieliszewski[2], Derek T.A. Lamport[3] and Allan M. Showalter[1,*]
[1]Department of Environmental and Plant Biology, Molecular and Cellular Biology Program, [2]Department of Chemistry, Molecular and Cellular Biology Program, Ohio University, Athens, OH 45701–2979, USA, and [3]Emeritus Professor, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824–1312, USA

Summary

Arabinogalactan-proteins (AGPs) are a family of hydroxyproline-rich glycoproteins implicated to function in plant growth and development. This report focuses on a novel, modular AGP found in tomato, LeAGP-1, which was predicted by DNA cloning and herein verified at the protein level as a major AGP component. LeAGP-1 was isolated from tomato suspension-cultured cells and verified to be an AGP by precipitation with (β-D-galactosyl)$_3$ Yariv phenylglycoside and by amino acid composition analysis. Furthermore, LeAGP-1 was determined to correspond to *LeAGP-1* clones based on three criteria: (1) amino acid composition identity, (2) amino acid sequence identity, and (3) specific immunoreactivity of glycosylated and deglycosylated LeAGP-1 with an antibody developed against the highly basic subdomain predicted from *LeAGP-1* clones. The antibody was also used to immunolocalize LeAGP-1 in tomato to the cell surface of suspension-cultured cells, maturing metaxylem elements in young internodes and petioles, and stylar transmitting tissue cells. At the subcellular level, LeAGP-1 immunolocalized to the cell walls of these particular cells as well as to intercellular spaces between stylar transmitting tissue cells. LeAGP-1 now emerges as one of the most comprehensively studied AGPs in terms of (1) characterization at the genomic DNA, cDNA and protein levels, (2) known organ-specific and developmentally regulated mRNA expression patterns, (3) development of an antibody against a unique, peptide subdomain which specifically recognizes LeAGP-1 in its glycosylated and deglycosylated states, and (4) immunolocalization of a single, well-defined AGP molecule at the tissue and subcellular levels.

Received 18 December 1998; accepted 5 February 1999.
*For correspondence (fax +1 740 593 1130; email showalte@ohio.edu).

Introduction

Arabinogalactan-proteins (AGPs) are a family of proteoglycans and part of the superfamily of plant hydroxyproline-rich glycoproteins (HRGPs) (Fincher *et al.*, 1983; Kieliszewski and Lamport, 1994; Nothnagel, 1997; Pennell, 1992; Showalter, 1993). These proteoglycans are widely distributed throughout the plant kingdom and occur in intercellular spaces, cell walls, plasma membranes and certain cytoplasmic vesicles. AGPs conveniently and diagnostically bind to (β-D-glucosyl)$_3$ and (β-D-galactosyl)$_3$ Yariv phenylglycosides, commonly known as Yariv reagents, in a selective and non-covalent manner.

AGPs typically contain high proportions of carbohydrate and only 1–10% protein by weight (Clarke *et al.*, 1979; Nothnagel, 1997; Showalter and Varner, 1989). The carbohydrate moiety consists of mainly arabinose and galactose with minor amounts of other sugars including uronic acids in some AGPs. Studies indicate that a backbone of 1–3-linked β-D-galactopyranose is branched through C(O)6 to (1–6)-linked β-D-galactopyranose side chains which in turn are substituted with arabinofuranose and other less abundant monosaccharides (Bacic *et al.*, 1987; Komalavilas *et al.*, 1991). The precise attachment site(s) and number of attachments of such polysaccharide chains per core protein remain to be determined, although galactosyl-O-hydroxyproline, arabinosyl-O-hydroxyproline and galactosyl-O-serine linkages have been reported for several AGPs (Pope, 1977; Qi *et al.*, 1991).

The protein moieties of AGPs are diagnostically rich in hydroxyproline (Hyp), Ala, Ser, Thr and Gly. Exceptions to this general rule exist as evidenced by the characterization of Hyp-poor AGPs (Baldwin *et al.*, 1993; Hillestad *et al.*, 1977; Mollard and Joseleau, 1994), a His-rich AGP (Kieliszewski *et al.*, 1992) and an Ala-poor gum arabic glycoprotein (Qi *et al.*, 1991).

Recently, molecular cloning of core polypeptides for several AGPs has contributed to a greater understanding of the protein moiety and AGP gene expression (reviewed in Nothnagel, 1997). Under the nomenclature system suggested by Mau *et al.* (1995) and Du *et al.* (1996), these clones encode polypeptides for either 'classical' AGPs or 'non-classical' AGPs. Clones corresponding to classical AGPs encode a polypeptide with at least three distinct

domains, an N-terminal secretion signal sequence, a central domain which contains most of the Pro/Hyp residues, and a C-terminal hydrophobic region which can facilitate membrane attachment via glycosylphosphatidylinositol (GPI) anchoring (Youl *et al.*, 1998). The classical designation is given when Pro/Hyp, Ala, Thr and Ser represent the most abundant residues in the encoded polypeptide excluding the N-terminal signal sequence; otherwise the non-classical designation applies. Clones corresponding to non-classical AGPs specify N-terminal secretion signal sequences and various other domains including Pro/Hyp-rich, Asn-rich and Cys-rich domains, but, to date, do not specify any C-terminal hydrophobic domains.

Exciting work dealing with AGP expression has involved the use of antibodies to immunolocalize AGPs in various plant tissues and organs. Such work corroborates and is consistent with studies which address AGP expression using other approaches (i.e. Yariv reagent staining, RNA blot analysis, biochemical isolation and characterization). The picture which emerges from all such studies is that AGP family members are localized in a variety of organs and tissues and in intercellular spaces, cell walls, plasma membranes and certain cytoplasmic vesicles (reviewed in Nothnagel, 1997). In addition, the antibody work in particular has provided important clues to AGP function as detailed below. Of these AGP antibodies, the monoclonal antibodies directed against AGPs on the plant cell surface are the most well known. These monoclonal antibodies are characterized to varying degrees in terms of the epitope recognized, but invariably the epitopes have corresponded to the carbohydrate moiety. Such findings are eminently logical given that carbohydrate encompasses the bulk of the molecule and probably largely masks display of the core protein during antigen presentation. Aside from the localization patterns and in some cases the carbohydrate structure of the epitope, little is known about the AGPs recognized by these monoclonal antibodies, including the degree to which multiple AGP family members share common carbohydrate epitopes.

While precise functions for AGPs remain to be established, evidence indicates that AGPs play important roles in cell differentiation, development, and cell–cell interactions. For example, AGPs are expressed in organ-specific, tissue-specific and cell-specific fashions. Immunolocalization studies using monoclonal antibodies for AGPs showed the dramatic appearance and disappearance of cell-specific AGP epitopes during development (Knox *et al.*, 1991; Pennell and Roberts, 1990; Pennell *et al.*, 1991; Schindler *et al.*, 1995; Stacey *et al.*, 1990). Such AGP expression patterns can be distinguished and observed *before* differentiation is anatomically visible, indicating that AGPs are clearly markers, or possibly even regulators, of cell development. Moreover, partially purified carrot and spruce AGPs can induce or inhibit plant embryogenesis (Egertsdotter and von Arnold, 1995; Kreuger and van Holst, 1993; Kreuger and van Holst, 1995), depending upon the particular AGPs and cell culture lines used. In a related, novel experiment, the addition of Yariv reagent to rose cell cultures inhibits cell growth (Serpe and Nothnagel, 1994). Similar experiments using Yariv reagent to bind AGPs selectively and hence perturb function resulted in inhibition of lily pollen tube growth *in vitro* (Jauh and Lord, 1996; Roy *et al.*, 1998), in inhibition of *Arabidopsis* root growth (Willats and Knox, 1996), in blocking elongation of suspension-cultured carrot cells (Willats and Knox, 1996), and may even induce cell death in young rose cell cultures (Langan and Nothnagel, 1997). Also, Cheung *et al.* (1995) have demonstrated that pollen tube growth is stimulated and controlled by specific AGPs in tobacco style, although Sommer-Knudsen *et al.* (1998) dispute these findings. Based on the observation that salt-adapted tobacco cells have much lower extractable AGP levels and are much less extensible than unadapted cells, AGPs are hypothesized to participate in wall expansion (Zhu *et al.*, 1993). Furthermore, in the leafy liverwort, AGPs are implicated in regulating localized suppression of cell proliferation (Basile and Basile, 1993). Clearly, these data implicate AGPs in many important stimulatory and inhibitory roles in plant growth and development.

Here, we report on a novel, modular AGP found in tomato, initially predicted by molecular cloning of the tomato *LeAGP-1* gene (Li and Showalter, 1996; Pogson and Davies, 1995) and herein verified at the protein level as a major AGP component. The extraordinary domain structure of this AGP as predicted by cDNA and genomic cloning is shown in Figure 1. While this clone encodes a classical AGP as defined above, it is distinguished from other clones in this grouping by the presence of a highly basic (i.e. Lys-rich) subdomain which interrupts the central Pro/Hyp-rich AGP domain. We present data not only on the isolation and biochemical characterization of this particular AGP corresponding to *LeAGP-1* clones, but also document the development and use of a highly specific antibody directed against the Lys-rich subdomain to localize expression of this individual AGP family member (i.e. the LeAGP-1 core protein and its glycoforms) at the tissue and subcellular levels.

Results

Isolation of LeAGP-1 from tomato cell suspension cultures

RNA blots of tomato suspension-cultured cells hybridized with an *LeAGP-1* gene probe indicated that *LeAGP-1* mRNA accumulated to high levels which were approximately equal to those observed in young stems and flowers of tomato (Li, 1996; Li and Showalter, 1996). Consequently,

Figure 1. Modular organization and sequence of the 215-residue LeAGP-1 protein deduced from tomato *LeAGP-1* cDNA and gene clones.
(a) Modular domain organization for the predicted LeAGP-1 core protein consists of an N-terminal signal peptide (blue), a central 'classical' AGP domain (red) sandwiching a highly basic subdomain (black), and a C-terminal hydrophobic domain (green) which may allow for GPI-mediated plasma membrane association.
(b) Corresponding amino acid sequence of the LeAGP-1 domains. A potential N-glycosylation site (Asn-X-Ser/Thr) appears immediately following the basic domain and is italicized. A synthetic peptide encompassing the basic (Lys-rich) subdomain used here for production of a LeAGP-1 antibody is underlined with a bold line; the peptide and its corresponding antibody are given a 'PAP' designation in accordance with the first three residues of this synthetic sequence. Peptide sequences elucidated in this study by amino acid sequencing are underlined; Pro residues found to be hydroxylated in these sequences are displayed as Hyp residues. Note that the Pro (or Hyp) residue at position 125 was undetermined by amino acid sequencing and hence is indicated with a dotted underline. The predicted site of C-terminal processing and GPI-anchor addition at Ser192 is underlined in red and discussed elsewhere.

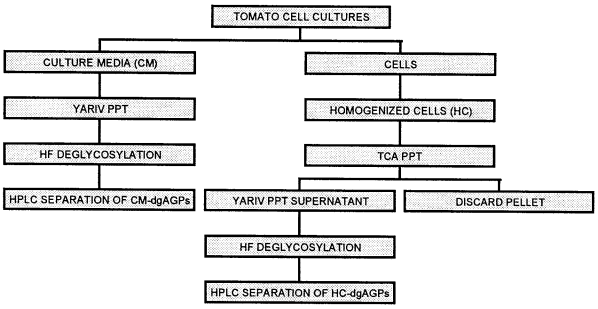

Figure 2. Biochemical separation strategy for purification of LeAGP-1 protein from tomato suspension-cultured cells and culture media.

tomato cell cultures were viewed as a potentially rich and convenient source for isolation of LeAGP-1. The purification strategy involved both suspension-cultured cells and culture medium as depicted in Figure 2. Briefly, this strategy involved isolating total AGPs from homogenized cells (HC) or culture medium (CM) using precipitation with $(\beta\text{-D-galactosyl})_3$ Yariv reagent, which binds and precipitates AGPs specifically, as a key purification step. In homogenized cells, trichloroacetic acid (TCA) precipitation removed most proteins leaving extensins and AGPs in the supernatant. Yariv reagent precipitation followed by deglycosylation with anhydrous hydrofluoric acid (HF) and subsequent HPLC of the resulting polypeptides separated the putative AGP core proteins. Culture medium was handled in a similar fashion to homogenized cells with the omission of the TCA precipitation step since a limited array of proteins were present in the medium. The resulting HC and CM polypeptide profiles following HF deglycosylation are shown in Figure 3. The absorption profile for the HC preparation displayed several peaks, similar but not identical to that of the CM preparation. HC-3 and CM-3 were the major peaks in the two preparations, and eluted from the reverse-phase column at identical times. The various peaks, representing putative AGP core proteins, were then subjected to three separate biochemical/immunological analyses in order to identify the LeAGP-1 core protein. This paper mainly focuses on the HC preparation, although some analysis of the CM preparation is included as well.

Biochemical characterization of LeAGP-1 from tomato cell suspension cultures: LeAGP-1 represents a bona fide AGP and corresponds to LeAGP-1 cDNA and genomic clones

First, amino acid composition analyses of four of the major peaks from the homogenized cells (i.e. HC-1, HC-2, HC-3

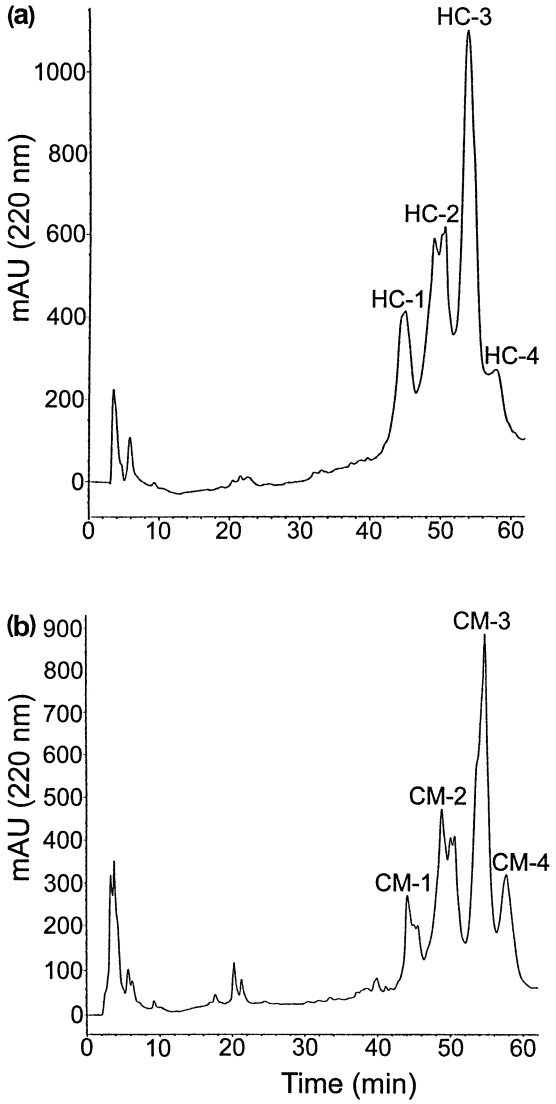

Figure 3. Reverse-phase HPLC separation of (β-D-galactosyl)$_3$ Yariv reagent-precipitable, HF-deglycosylated AGPs from tomato cell cultures.
(a) Homogenized tomato suspension-cultured cells (HC).
(b) Culture medium (CM).
The solid line indicates (peptide bond) absorbance at 220 nm in milli-absorption units (mAU). Labelled peaks were subjected to subsequent analysis as presented in Table 1 and Figures 4 and 5.

and HC-4 in Figure 3a) were performed and compared to the composition of LeAGP-1 predicted from the *LeAGP-1* clones as shown in Table 1. These analyses show that each of the peaks are rich in Hyp, Ala, Ser and Thr, consistent with compositions of classical AGPs and with their precipitation with (β-D-galactosyl)$_3$ Yariv reagent. Furthermore, peak HC-3 has the composition which is consistent with that predicted from *LeAGP-1* clones regardless of whether the C-terminal hydrophobic domain is present. It should be noted that cloned sequences cannot distinguish between Pro and Hyp, since Hyp is a post-translational modification of Pro. In addition to Hyp, Ala, Ser and Thr compositional

Table 1. Amino acid composition[a] of AGPs isolated from tomato suspension-cultured cells (i.e. HC-AGPs)

AA	LeAGP-1[b]	LeAGP-1[c]	HC-3	HC-1	HC-2	HC-4	PPC[d]
Hyp	–	–	29	37	34	30	23.4
Pro	24.4	27.5	1	2	2	2	nd
Asx	3.1	3.5	2	6	3	2	3.8
Thr	7.7	8.8	10	9	8	8	9.1
Ser	11.4	11.7	12	11	11	18	14.3
Glx	3.6	3.5	3	1	3	3	4.5
Gly	4.6	3.5	5	7	9	6	6.8
Ala	22.2	23.4	21	19	19	15	19.6
Val	8.2	8.2	9	3	4	7	5.2
Cys	0.0	0.0	0	0	0	0	3.0
Met	2.6	1.7	0	0	0	0	0.8
Ile	0.0	0.0	1	2	4	2	1.5
Leu	3.6	1.2	1	2	2	3	3.0
Tyr	0.0	0.0	0	0	0	0	0.4
Phe	0.5	0.0	1	0	0	1	0.4
His	0.5	0.6	0	0	0	0	0.4
Lys	6.7	6.4	5	2	2	2	3.0
Arg	0.0	0.0	0	0	0	1	0.8
Trp	1.0	0.0	nd	nd	nd	nd	nd

[a]Expressed in mole percentage.
[b]LeAGP-1 excluding the N-terminal signal sequence (from Li and Showalter, 1996).
[c]LeAGP-1 excluding the N-terminal signal sequence and the C-terminal hydrophobic domain presumably removed during GPI attachment (from Li and Showalter, 1996).
[d]Protein-polysaccharide complex isolated from tomato suspension-cultured cells (Lamport, 1970).
nd, not determined.

consistency, other diagnostic LeAGP-1 matches are seen, particularly with respect to Val and Lys.

Second, each of the four HC peaks was tested for their ability to react with the PAP antibody produced against the peptide encompassing the basic subdomain of LeAGP-1 (see Figure 1 for the synthetic peptide sequence). Western gel blots used in this analysis appear in Figure 4(a); Western dot blots (data not shown) were also used and were consistent with the gel blot data. In both gel and dot blots, only peak HC-3 reacted with the PAP antibody. This reacting product was a 48 kDa protein as judged by SDS–PAGE, and was observed to be the sole polypeptide species present in peak HC-3 by Coomassie blue staining. A 48 kDa reacting species was also observed in the Yariv reagent-precipitated HC-AGP preparation following deglycosylation. In addition, crude homogenized cell extracts and Yariv-precipitated homogenized cell preparations, which were not subjected to HF deglycosylation, contained heterogeneous, high molecular weight fractions, which were capable of reacting with the PAP antibody. Such molecular weight heterogeneity is characteristically associated with AGPs as well as other glycoproteins and is due to carbohydrate microheterogeneity, in this case with respect to the LeAGP-1 core protein. Pre-immune sera in all of these Western analyses showed no reactivity.

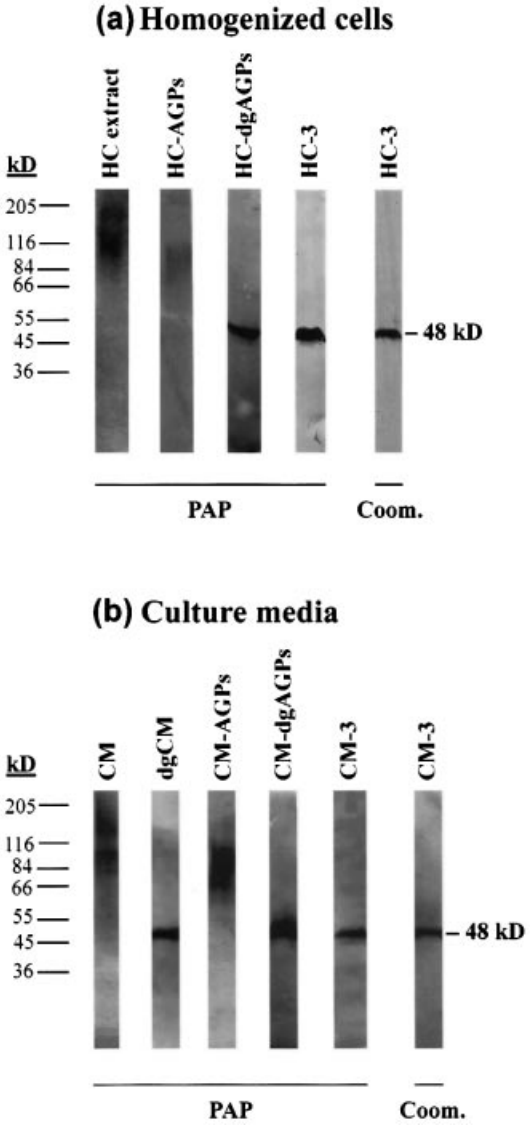

Figure 4. SDS–PAGE analysis of glycosylated and deglycosylated AGPs from tomato suspension-cultured cells subjected to Western blotting with the PAP antibody and Coomassie blue staining.
The molecular masses (kDa) of protein size standards are shown along with the calculated molecular masses of HC-3 and CM-3.
(a) AGPs isolated from homogenized tomato suspension-cultured cells (HC) at various stages of purification are shown. HC extract = crude HC preparation; HC-AGPs = Yariv-precipitated HC extract; HC-dgAGPs = HF deglycosylated HC-AGPs; HC-3 = peak HC-3 from the HPLC separation of HC-dgAGPs. The HC-3 lane was also stained with Coomassie blue (Coom.) to show purity.
(b) AGPs isolated from culture medium (CM) at various stages of purification are shown. CM = crude CM; dgCM = HF deglycosylated CM; CM-AGPs = Yariv-precipitated CM; CM-dgAGPs = HF deglycosylated CM-AGPs; CM-3 = peak CM-3 from the HPLC separation of CM-dgAGPs. The CM-3 lane was also stained with Coomassie blue (Coom.) to show purity.

Western analysis of the culture medium showed that peak CM-3, but not any of the other CM peaks, reacted with the PAP antibody (Figure 4b). It should be noted that peak CM-3 from the culture medium elutes from the PRP-1 HPLC column at virtually the same time as peak HC-3 from homogenized cells. Moreover, Western blots of glycosylated and deglycosylated crude and Yariv-reagent precipitated extracts of culture medium reacted similarly to the homogenized cell preparations, including the appearance of a 48 kDa protein in the deglycosylated preparations (Figure 4b).

Third, based on peak HC-3's tentative identification as LeAGP-1 from amino acid composition analysis and specific immunoreactivity with the PAP antibody, peak HC-3 was subjected to trypsin digestion. Tryptic peptides were separated by HPLC on a PRP-1 HPLC column, resulting in three major peaks designated T1, T2 and T3 (data not shown). Peaks T1, T2 and T3 were used for amino acid sequence analyses and resulted in the sequences presented in Figure 5. Peptide sequences for T1, T2 and T3 corresponded, respectively, to positions 38–46, 94–117 and 94–128 in the predicted LeAGP-1 sequence shown in Figure 1 and also identified Pro residues which were post-translationally modified to Hyp. Notably, trypsin cleaved after Lys-93 to generate the N termini of T2 and T3, but apparently cleaved in a promiscuous fashion after Pro/Hyp-37 to generate the N-terminus for T1. These sequences were wholly consistent with the predicted LeAGP-1 sequence with one exception. The T3 peptide sequence overlapped with the T2 peptide sequence and was identical to the T2 sequence and the predicted LeAGP-1 sequence except for the absence of an Ala residue at position 116. The absence of an Ala residue at position 116 in the T3 sequence and its presence in the T2 sequence indicate that LeAGP-1 occurs in two isoforms.

Immunolocalization of LeAGP-1 at the light microscope level

In order to examine the tissue and general cellular distribution of LeAGP-1, immunolocalization with the PAP antibody was performed at the light microscope level using tomato suspension-cultured cells, stems, petioles and flowers as shown in Figure 6. A fluorescein isothiocynate (FITC)-labelled secondary antibody was used to visualize antibody staining.

Suspension-cultured cells showed strong and generally uniform fluorescent staining at the cell surface with the PAP antibody (Figure 6a). This staining could also be competitively reduced by pre-incubating the PAP antibody with the PAP peptide (data not shown) prior to immunolocalization. Examination of cross-sections of young stem and petiole with the PAP antibody revealed strong surface staining in maturing metaxylem elements (Figure 6b,c). The outer phloem of the petiole (Figure 6b) and the outer phloem, inner phloem and cambial zone of the young stem

```
T1:  HypAlaAlaAlaHypThrLysProLys
T2:  AlaHypAlaSerSerHypHypValGlnSerHypHypAlaHypAlaHypGluValAlaThrHypHypAlaVal...
T3:  AlaHypAlaSerSerHypHypValGlnSerHypHypAlaHypAlaHypGluValAlaThrHypHypValSer
     ThrHypHypAlaAlaAla(-)ValAlaAla...
```

Figure 5. Amino acid sequences elucidated from three tryptic peptides (T1, T2 and T3) generated from HC-3, the deglycosylated AGP corresponding to LeAGP-1.
T2 and T3 are overlapping partial sequences which are identical in the overlapping region except for the underlined Ala residue which occurs in T2 and is predicted by *LeAGP-1* DNA clones. Hyp, hydroxyproline; –, an undetermined amino acid residue.

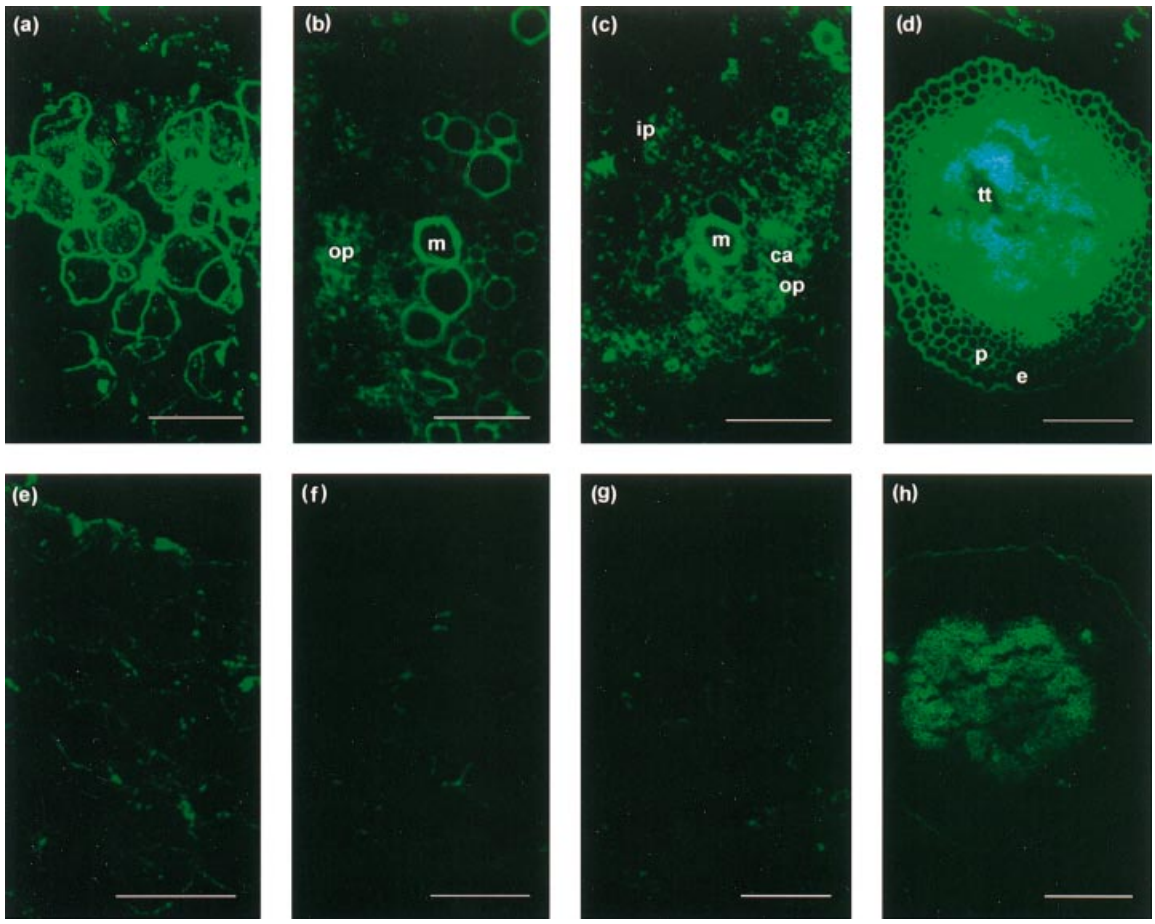

Figure 6. Immunocytochemical localization of LeAGP-1 with the PAP antibody in tomato suspension-cultured cells, petiole, first internode and style at the light microscope level.
(a) and (e) Tomato suspension-cultured cells. (b) and (f) Cross-section of tomato petiole. (c) and (g) Cross-section of tomato first internode. (d) and (h) Cross-section of tomato style. (a)–(d) were immunolocalized with the PAP antibody, while (e)–(h) were reacted with pre-immune serum as a control. m, metaxylem element; ca, cambial zone; ip, inner phloem; op, outer phloem; e, epidermis; p, stylar parenchyma; tt, stylar transmitting tissue. Bars = 100 μm.

(Figure 6c) also showed some staining. Control reactions performed with pre-immune sera for cultured cells as well as stem and petiole cross-sections showed barely detectable fluorescence (Figure 6e,f,g).

Initial examination of floral cross-sections indicated strong fluorescent staining of stylar material with the PAP antibody; closer examination of the stylar staining pattern showed that transmitting tissue stained most strongly, with surrounding parenchyma tissue showing a lesser but still appreciable degree of staining (Figure 6d). Staining of the transmitting tissue was so intense that it was impossible to determine whether staining was limited to the cell surface. In contrast, parenchyma tissue showed clear staining at the cell surface. Control reactions performed with pre-immune sera in the style showed a somewhat higher degree of background staining attributable to autofluorescence, particularly in transmitting tissue, than in other tissue samples examined, but was still orders of magnitude less intense than staining revealed with the PAP antibody (Figure 6h). Pre-immune sera also showed

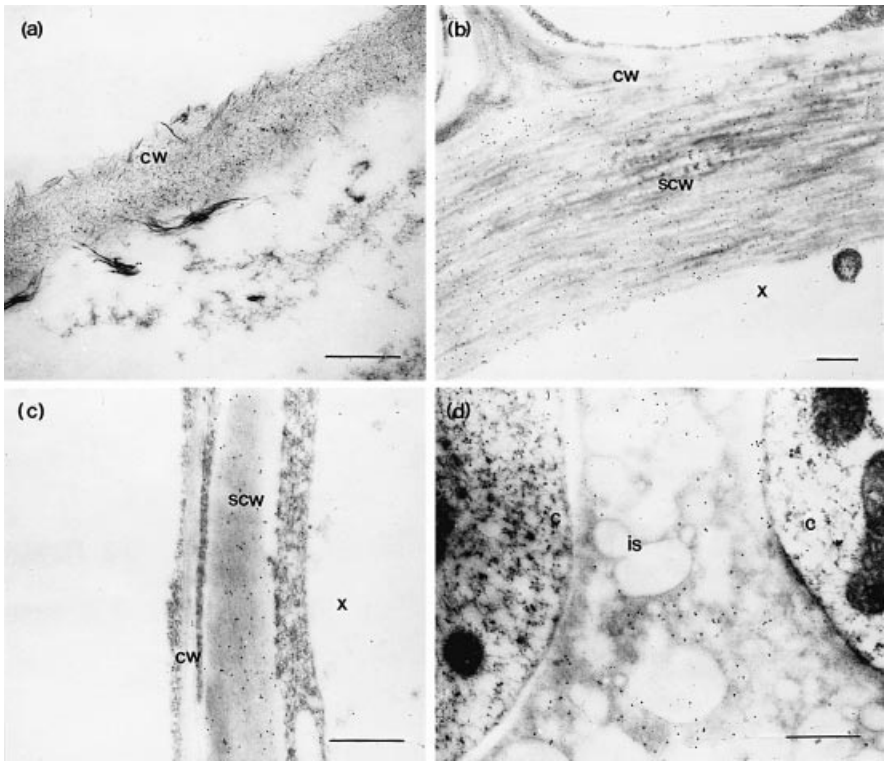

Figure 7. Immunocytochemical localization of LeAGP-1 with the PAP antibody in tomato suspension cultured cells, first internode, petiole and style at the transmission electron microscopic level.
(a) Tomato suspension-cultured cell.
(b) Cross-section of the tomato first internode showing a xylem element.
(c) Cross-section of tomato petiole showing an immature xylem element.
(d) Cross-section of tomato style showing the intercellular space between two transmitting tissue cells.
cw, primary cell wall; scw, secondary cell wall; x, xylem element; c, cytoplasm; is, intercellular space. Bars = 500 nm.

staining of the outer surface of the style, but was completely attributable to autofluorescence as determined by observing untreated sections.

Immunolocalization of LeAGP-1 at the transmission electron microscope level

In order to follow-up on the cell surface staining detected in the light microscopy study and ascertain the cellular or subcellular location of LeAGP-1 as detected by PAP antibody staining, immunolocalization at the transmission electron microscope level was performed using gold-labelled secondary antibody as shown in Figure 7. Cultured cells showed PAP antibody staining in their primary cell walls (Figure 7a). This staining was seen throughout the cell wall; no particular wall localization pattern was discerned. Examination of maturing metaxylem elements in young stem and petiole revealed PAP antibody staining primarily in the thickening secondary cell walls and to a lesser extent in the thin primary cell wall (Figure 7b,c). Moreover, adjacent xylem parenchyma cells and mature metaxylem elements also showed some staining in their cell walls (data not shown). Staining was found throughout the wall of these xylem elements, and control sections reacted with pre-immune sera showed negligible staining.

In the style, PAP antibody staining was observed in both the cell wall and in the intercellular spaces of both transmitting tissue cells (Figure 7d) and adjacent parenchyma cells (data not shown). Control sections of stylar cells treated with pre-immune sera showed negligible background staining.

Discussion

LeAGP-1 represents a bona fide *AGP and corresponds to cDNA and genomic clones*

Molecular characterization of cDNA and genomic clones predicts the existence of a novel, modular AGP as illustrated in Figure 1. In order to confirm this prediction, it was necessary to isolate and characterize the protein encoded by these DNA sequences. Verification of this prediction is manifested in three distinct sets of experiments. First, the amino acid composition of a Yariv reagent-precipitable fraction (i.e. peak HC-3) corresponded to that predicted by the cloned LeAGP-1 sequences. This correspondence is arguably greatest when the N-and C-terminal hydrophobic domains are eliminated from the clone sequence as opposed to just eliminating the N-terminal signal sequence (see Table 1 and processing section below). Second, an antibody developed against the Lys-rich subdomain predicted from the cloned sequences reacted specifically with the same peak fraction (i.e. peak HC-3) showing the corroborating amino acid composition data. Third, when this peak fraction was digested with trypsin and the tryptic peptides subjected to sequence analysis, identity was irrefutably confirmed. Thus, LeAGP-1 is a *bona fide* AGP and confirms predictions from the DNA data; moreover, as

seen in Figure 3, LeAGP-1 is the major core protein and hence AGP present in these cells, both in homogenized cell preparations (i.e. peak HC-3) as well as culture medium (i.e. peak CM-3), with the latter being subjected to less rigorous biochemical analysis at this point. Significantly, a 'protein–polysaccharide complex' isolated from tomato cultures and described in 1970 (Lamport, 1970) has an amino acid composition remarkably similar to LeAGP-1 (Table 1) and also a carbohydrate composition that showed molar ratios of hydroxyproline, galactose and arabinose of 1:24:17, typical of an arabinogalactan-protein although that term was not then in use. Interestingly, similar material isolated from sycamore-maple cell suspension cultures led to the discovery that the arabinogalactan polysaccharide was attached via the Hyp residues (Pope, 1977).

Clearly, the homogenized cell preparation contains several AGPs as judged by amino acid composition analyses of several peak fractions following HPLC separation of deglycosylated Yariv reagent-precipitated material. Culture medium contains a similar, but not identical, AGP profile, and LeAGP-1 is present. The presence of LeAGP-1 in the culture medium is indicated by (1) reactivity of the PAP antibody to the major culture medium peak fraction (i.e. CM-3) with the same HPLC retention time on the PRP-1 column as LeAGP-1 (peak HC-3) from homogenized cells (Figures 3 and 4), and (2) deglycosylation of culture medium followed by Western blotting resulting in the same size polypeptide being recognized as in the homogenized cell preparations. Based on AGP quantification with Yariv reagent and LeAGP-1 peak area calculations, nearly twice as much total AGPs and LeAGP-1 exists in the culture medium as compared to the homogenized cells.

Characteristics of the LeAGP-1 core protein

Based on migration in SDS–PAGE gels, the LeAGP-1 protein core is estimated to be 48 kDa. This size is approximately three times the calculated 16.7 kDa molecular mass of the predicted core protein assuming that 97% of the Pro residues are hydroxylated and the N and C-termini are removed. Two possibilities exist to account for this apparent anomaly. First, LeAGP-1 is migrating as a trimer. Second, as others have reported, some HRGPs migrate anomalously in SDS–PAGE gels, most likely due to their high content of secondary amino acids, namely Pro and Hyp, which may bind less SDS and thereby retard migration (John and Keller, 1995; Kieliszewski *et al.*, 1990; Stiefel *et al.*, 1988). Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis is underway on deglycosylated LeAGP-1 in order to obtain an accurate molecular weight and distinguish between the two possibilities. If the trimer idea is supported by MALDI-TOF MS analysis, it is likely that an oxidative cross-link such as that suggested by Kjellbom *et al.* (1997) or a novel cross-link such as one catalysed by a transglutaminase is present, with the latter consistent with the occurrence of Gln and Lys residues in LeAGP-1 (Serafini-Fracassini *et al.*, 1995). In contrast, the absence of Cys and Tyr residues in LeAGP-1 precludes the possibility of disulphide or isodityrosine cross-links. In this context, it should also be noted that deglycosylation appears to be complete given the sharp protein band observed by SDS–PAGE/Western blotting and given that the harsh conditions used to strip off the carbohydrate are identical to those used to remove virtually all carbohydrate from other HRGP molecules, including AGPs (M.J. Kieliszewski, unpublished data).

Based on the finding that the T3 tryptic peptide sequence lacks an Ala residue at position 116 which is found in the overlapping T2 peptide and predicted by the *LeAGP-1* clones, it appears that two isoforms of LeAGP-1 exist. Such isoforms may account for the asymmetrical nature of peak HC-3 observed in the HPLC profile of the Yariv reagent-precipitable material from homogenized cells which may represent an unresolved doublet polypeptide peak. It is however unlikely that great differences will appear between such isoforms, given that multiple mRNA or protein bands are not detected in RNA and protein gels, respectively.

Composition analysis clearly shows that approximately 97% of the Pro residues are hydroxylated to form Hyp, and amino acid sequencing has revealed the positions of several of these Hyp and one of the Pro residues. Examination of these prolyl hydroxylation patterns and those elucidated for other AGPs are consistent with established hydroxylation rules and observed hydroxylation patterns for HRGPs (Kieliszewski and Lamport, 1994). Indeed, based on the 97% hydroxylation data as well as the known hydroxylation rules and patterns, the confirmed Pro residue at position 45 may be the only Pro residue that is not hydroxylated in LeAGP-1. This lack of hydroxylation is wholly consistent with the hydroxylation rule that Pro residues preceded by a Lys residue are not hydroxylated and perhaps indicates an important functional region that would be obscured by hydroxylation and/or subsequent glycosylation (Kieliszewski and Lamport, 1994).

Production of a highly specific antibody against LeAGP-1

The LeAGP-1 antibody generated here is highly specific. Its specificity is indicated by (1) its ability to recognize a single 48 kDa protein out of crude extracts of culture medium following deglycosylation, (2) its ability to recognize a high molecular weight AGP fraction in crude extracts of homogenized cells and culture medium, with the size range of this fraction accounted for by differential glycosylation given that deglycosylation results in reactivity to a single 48 kDa species, (3) its ability to recognize specifically glycosylated and deglycosylated LeAGP-1 in Western dot

and gel blot assays when tested against a variety of other glycosylated and deglycosylated HRGP molecules including deglycosylated tomato extensin P2, carrot extensin P1b, carrot extensin P1, deglycosylated carrot extensin P1 and deglycosylated maize THRGP, and (4) its ability to react with a 48 kDa protein in crude style extracts following deglycosylation. Such specificity is further reflected in the PAP antibody's ability to react with particular cell types in tomato.

This antibody was purposely designed based on a peptide epitope which encompasses the Lys-rich subdomain (KGKVKGKKGKKH) sandwiched between two Hyp-rich AGP modules (Figure 1), as this basic region cannot be glycosylated given the absence of hydroxy amino acid and Asn residues, which are reported attachment sites for carbohydrate side chains. Thus, the PAP antibody was predicted, and based on all observations is able, to recognize both glycosylated and deglycosylated versions of LeAGP-1. Based on our Western dot and gel blot assays, the antibody has somewhat greater reactivity with the deglycosylated form, indicating that carbohydrate may partially obscure or alter conformation of the antigenic site in glycosylated LeAGP-1.

Some of the most useful antibodies directed against AGPs have arisen from monoclonal antibodies produced against plant cell surface components. Such antibodies have been characterized to varying degrees and used to follow developmental expression of AGPs, but it is possible that such monoclonal antibodies may recognize more than a single AGP molecule since they are directed against carbohydrate epitopes, which are likely to be shared by two or more AGPs (Nothnagel, 1997; Yates *et al.*, 1996). Another limitation is that these monoclonal antibodies recognize only AGPs in their glycosylated states and therefore are not useful in analysis of the protein core *per se*.

Immunolocalization of LeAGP-1 to the cell walls of cultured cells, xylem and transmitting tissue

As already mentioned, some of the most specific data on AGP localization have emerged from the immunolocalization work with monoclonal antibodies against AGPs. Unfortunately, the AGPs which correspond to these monoclonal antibodies are not well characterized. There are also several excellent reports on the isolation and characterization of specific AGPs showing their biochemical localization to be either plasma membrane, cell wall or culture medium; however, corresponding localization data at the tissue and organ levels largely do not exist and no clones for these particular AGPs exist to provide the complete primary sequence (Nothnagel, 1997).

In this system, LeAGP-1 is now well characterized in terms of its primary sequence, core protein, RNA expression and immunolocalization. Immunolocalization data show that LeAGP-1 is prominently deposited in tomato in suspension-cultured cells, maturing metaxylem elements of young stems and petioles, and in the transmitting tissue cells of the style (Figure 6). LeAGP-1 also exists in the inner and outer phloem as well as the cambial zone of young stems and petioles (Figure 6). At the subcellular level, LeAGP-1 localizes to the cell wall in each of these cells as well as to the intercellular spaces between transmitting tissue cells (Figure 7). In addition, immunochemical evidence indicates that tomato cell culture medium also contains a substantial quantity of LeAGP-1. These data are consistent with the tissue and cellular locations reported for other AGPs (Nothnagel, 1997). Thus, the experimental contribution here is not just the localization *per se*, but more significantly is the precise tissue and cellular distribution profile which is revealed for a particular, well characterized AGP family member.

Processing of LeAGP-1 (GPI anchoring, C-terminal tail, medium location)

Like other classical AGP clones, *LeAGP-1* clones predict a C-terminal hydrophobic tail. This tail is hypothesized to play a role in membrane anchoring and subsequent processing of classical AGPs so that they might be released into cell walls, culture medium and intercellular spaces. Evidence indicating that two classical AGPs, AGP*Na*1 from tobacco styles and AGP*Pc*1 from pear cell culture medium, are glycosylphosphatidylinositol (GPI)-modified proteins which lack the C-terminal domain, has recently appeared (Youl *et al.*, 1998). This finding indicates that classical AGPs can be attached to the plasma membrane via a GPI anchor and moreover that the GPI anchor can be processed, probably by the action of a phospholipase, to release the GPI-modified AGP into the extracellular milieu. Similarly, LeAGP-1 is probably a GPI-anchored protein which undergoes processing to be released into the cell wall and medium (or intercellular spaces). Our existing data are consistent with this interpretation. First, LeAGP-1 is found in cell walls, culture medium and intercellular spaces. Second, the predicted C-terminal tail of LeAGP-1 may actually be missing from the purified LeAGP-1 core protein given that its composition perhaps most closely matches the composition of LeAGP-1 predicted from clones when the N-terminal signal peptide as well as the C-terminal domain is missing as opposed to when the C-terminal domain is present (Table 1). Consistent with this observation, no 280 nm absorption is detected from the LeAGP-1 protein (i.e. HC-3) as expected if the C-terminus containing LeAGP-1's two exclusive, aromatic Trp residues is absent. Third, as pointed out by Youl *et al.* (1998), LeAGP-1 contains a consensus sequence for GPI modification and concomitant elimination of the C-terminal hydrophobic domain. Interestingly, this consensus sequence (i.e. Ser-Gly-Ala)

and C-terminal hydrophobic domain reside exclusively in exon 2 of the *LeAGP-1* gene (Figure 1) (Li and Showalter, 1996). It should be noted that the PAP antibody has not detected LeAGP-1 associated with the plasma membrane as predicted in this GPI-anchor model. Two possibilities may account for the apparent absence of LeAGP-1 from the plasma membrane: (1) LeAGP-1 is associated with the plasma membrane but the antigenic site is masked in this location so as not to be recognizable, and/or (2) LeAGP-1 is only transiently associated with the plasma membrane so that the majority of its pool is found in the cell wall and cell medium. Additional biochemical analysis of LeAGP-1 will have to be performed to verify the presence of a GPI anchor and to distinguish between these two possibilities. Furthermore, we speculate that the multiple cell surface locations for LeAGP-1 are determined in a given cell type by the degree of processing of its putative GPI anchor and by its subsequent interactions with cell wall components.

Function of LeAGP-1 and future work

Several possible roles for AGPs have been suggested, including roles in cell differentiation, development, cell–cell interactions, cell–cell recognition and as glues, lubricants and humectants (Fincher *et al*., 1983; Kieliszewski and Lamport, 1994; Nothnagel, 1997; Pennell, 1992; Showalter, 1993). Our previous observations on the organ-specific, developmentally regulated expression of LeAGP-1 coupled with the present immunolocalization data indicate that this AGP may play a role in xylem development and in guiding pollen tubes or serving as a nutrient source for growing pollen tubes. Further, we know that this AGP, which is clearly localized in the cell wall and secreted into the medium of cultured cells and the intercellular spaces of transmitting tissue cells and which is likely localized to the plasma membrane via a GPI anchor, may in some way participate in intermolecular cell surface interactions. Indeed there are a wealth of potential sites/domains on LeAGP-1 for interaction with other cell surface molecules such as itself and pectin. These sites include the polysaccharide side chains and the oligoarabinoside side chains which are predicted but not yet characterized, the lysine-rich domain, and even the potential for an N-linked glycan as a potential N-glycosylation site is found uniquely in this AGP (Figure 1). Such sites may also serve as extracellular receptors for some as yet unidentified ligand(s) and consequently control some as yet undefined response, perhaps programmed cell death in the case of xylem or release of glycosidase activity in the case of pollen tubes. Such interactions may include, but are not limited to, intercellular attachment or plasma membrane–cell wall adhesion.

In conclusion, LeAGP-1 represents one of the best characterized AGPs with respect to the vast array of information including cDNA and genomic clones, regulatory studies by RNA blotting, isolation and characterization of the core protein, development of a specific antibody, and immunolocalization of this AGP at the tissue and subcellular levels. Thus, the detailed knowledge and tools to characterize this major AGP in terms of its carbohydrate component, potential intermolecular partners, and function are now in place.

Experimental procedures

Plant materials

Tomato (*Lycopersicon esculentum*, cv. Bonnie Best) suspension-cultured cells were subcultured in Murashige and Skoog medium at a ratio of 1:10 every 8–10 days in 250 ml flasks. These flasks were shaken at 120 rev min^{-1} on a orbital shaker at 25°C under indirect fluorescent lighting in a tissue culture room. Tomato (*Lycopersicon esculentum*, cv. UC82B) plants were grown in pots from seed in Sunshine Mix I (Sungro Horticulture Inc., Bellezue, Washington State, USA), a soiless mix consisting of peat, shredded bark and perlite, watered regularly with tap water, and fertilized with Plant Marvel (Plant Marvel Laboratory, Chicago Heights, Illinois, USA), a water-soluble 20–20–20 fertilizer, in the greenhouse at Ohio University.

Isolation of AGPs from tomato cell cultures

After 8–10 days of subculturing, cultures were filtered through a Pyrex brand Buchner-type filtering funnel with fritted disc (coarse pore size: 40–60 μm) in order to separate cells from culture medium (CM). CM was dialysed against distilled H_2O for 2 days at 4°C and then lyophilized. Cells were washed twice with distilled H_2O, homogenized in ice-cold 100 mM $AlCl_3$ with a polytron and centrifuged at 23 430 **g** for 20 min at 4°C. The supernatant was dialysed against distilled H_2O and the cellular debris was discarded. Following dialysis, the homogenized cell (HC) extract was lyophilized, redissolved in distilled H_2O, and brought to a final concentration of 10% TCA using a 100% stock solution. After incubation overnight at 4°C, the extract was spun at 23 430 **g** for 20 min at 4°C to separate the TCA precipitate containing most cellular proteins from the supernatant containing AGPs and extensins. The supernatant was then dialysed against distilled H_2O and lyophilized.

The freeze-dried CM and HC preparations were dissolved in distilled H_2O, and the amount of AGPs present in each preparation was estimated by using (β-D-galactosyl)$_3$ Yariv reagent as follows. A 100 μl aliquot was removed from each preparation (and from a dilution series of an AGP (gum arabic) standard) and added to an equal volume of (β-D-galactosyl)$_3$ Yariv reagent, which was prepared as described by Yariv *et al*. (1962) using *p*-aminophenyl-β-D-galactopyranoside (Sigma Chemical Co., St Louis, Missouri, USA), at a concentration of 1 mg ml^{-1} in 2% NaCl in glass tubes. After a 1 h incubation at room temperature, tubes were centrifuged for 2 min at high speed in a bench-top centrifuge. The pellet was washed and re-centrifuged twice with 2 ml of 1% NaCl. The pellet was then dissolved in 2 ml of 0.02 N NaOH, and the absorbance at 420 nm was determined and compared to the AGP standards.

CM-and HC-AGPs were precipitated by (β-D-galactosyl)$_3$ Yariv reagent added in a ratio (w/w) of 1:2.5 (Yariv reagent:AGPs). After overnight incubation at 4°C, the solutions were spun at 5000 **g** for

15 min at 4°C to pellet the AGP–Yariv reagent complex. Pellets were washed with 1% NaCl, then redissolved in distilled H_2O; any undissolved solid residue was discarded. The complex was then quickly re-precipitated at room temperature by addition of 10% NaCl to a final concentration of 1% NaCl. Pellets were washed, redissolved and reprecipitated one or two more times. Finally, the complex was dissolved in a minimum volume of distilled H_2O, and dissociated with 10% sodium dithionite at 50°C under N_2 gas. When the solutions became colourless, they were dialysed immediately against distilled H_2O for 2 days at 4°C, and then spun briefly in order to discard any pellet prior to lyophilization.

Anhydrous hydrogen fluoride (HF) deglycosylation of AGPs

Anhydrous HF (containing 10% anhydrous methanol) was added to completely dried CM- and HC-AGPs in 2 ml Sarstedt screw cap microtubes at a concentration of 20 mg AGPs ml^{-1} following the protocol previously described by Kieliszewski *et al.* (1994) and initially devised by Mort and Lamport (1977). Briefly, after 1 h of HF treatment at 4°C, the mixtures were frozen in liquid nitrogen, and quenched by the addition of ice-cold distilled H_2O to achieve a final HF concentration of 10%. The quenched mixture was dialysed against distilled H_2O at 4°C overnight and then freeze-dried.

Reverse-phase HPLC separation of AGP core proteins from tomato cell cultures

CM- and HC-deglycosylated AGPs (1 mg) were dissolved in 150 μl 0.1% TFA, and spun briefly at 9 500 ***g***. The supernatant was subjected to reverse-phase HPLC by injection into a Hamilton PRP-1 column (250 mm, 4.1 mm internal diameter), using gradient elution with the solvents A (0.1% TFA) and B (0.1% TFA in 80% acetonitrile (MeCN) (aqueous)). Gradient elution involved 100% A for 1 min, then up to 40% B by 100 min, followed by a return to 100% A by 110 min using a flow rate of 0.5 ml min^{-1}. Absorbance was monitored at 220 nm; data capture was by a Hewlett Packard 1050 HPLC equipped with Chem Station software. All peaks were collected manually, frozen at –80°C, and freeze-dried prior to further analysis.

Amino acid analysis of HPLC-separated core proteins

For amino acid analysis of acid hydrolysates, we used the AccQ Tag column, reagents and buffer system from the Waters Corporation (Milford, Massachusetts, USA) (Diaz *et al.*, 1996). To enable the resolution of Hyp we used the gradient conditions recommended by Waters Corporation for analysing collagen hydrolysates and adjusted the initial buffer to be within the range pH 5.1–5.15. We used the sample preparation improvements described by Crimmins and Cherian (1997) and monitored the column at 256 nm.

Tryptic digestion and HPLC peptide mapping of LeAGP-1 core protein

Freeze-dried LeAGP-1 core protein (i.e. HC-3) was dissolved in deionized, distilled H_2O at 10 mg ml^{-1} in a 3 ml microvial. The sealed vial was heated in boiling water for 5 min, cooled, and then an equal volume of freshly made 2% ammonium bicarbonate containing 20 mM calcium chloride was added. Trypsin (Calbiochem, San Diego, California, USA) was added to the mixture at a ratio of 1 mg for each 100 mg of substrate and stirred using a spin bar overnight (18 h) at approximately 27°C. The digest was spun at 9 500 ***g*** and fractionated on a reverse-phase HPLC by injection into a Hamilton PRP-1 column as already described. Gradient elution here involved 100% A for 1 min, then up to 50% B by 100 min, followed by a return to 100% A by 110 min using a flow rate of 0.5 ml min^{-1}. Absorbance was monitored at 220 nm; data capture was by a Hewlett Packard 1050 HPLC equipped with Chem Station software. All peaks were collected manually, frozen at –80°C, and freeze-dried prior to peptide sequencing.

Peptide sequencing by automated Edman degradation

Tryptic peptides were sequenced at the Michigan State University Biochemistry Department's Macromolecular Facility on a 477-A Applied Biosystems Inc. gas phase sequencer.

LeAGP-1 antibody preparation and Western blot analysis

Since the LeAGP-1 core protein is expected to be heavily glycosylated, it was important to choose a non-glycosylated, yet accessible, region of the protein when designing a specific antibody against LeAGP-1. One such region is the basic domain (KGKVKGKKGKKH) sandwiched between two AGP modules (Figure 1). Synthesis of PAPAPSKGKVKGKKGKKHNA, a peptide encompassing this basic region, and production of an antipeptide antibody was performed by BAbCO (Berkeley Antibody Company, Berkeley, California, USA). In designing this peptide (referred to as the PAP peptide) with specialists from BAbCO, we added flanking domain sequences in order to ensure a more natural presentation of the basic domain. BAbCO coupled this peptide to keyhole limpet haemocyanin (KLH) and used this conjugate to immunize rabbits (Gullick, 1994). The peptide was also coupled to bovine serum albumin (BSA) and successfully used as a test antigen in ELISAs to establish serum titres of the peptide/KLH antisera. Pre-immune sera was also collected from these rabbits for use as controls.

In order to circumvent any potential non-specific binding of the antisera, possibly due to KLH glycosylated epitopes (Wirguin *et al.*, 1995), BAbCo immunoadsorbed KLH antibodies from the peptide/KLH antisera (Page *et al.*, 1994). Such immunoadsorbed PAP antibody reacted identically in Western blots and immunolocalizations to the PAP antiserum. Furthermore, we also successfully used the synthetic PAP peptide to compete for PAP antibody binding in immunolocalization experiments.

Western gel and dot blotting was performed essentially as described previously (Wisdom, 1994) with immunodetection facilitated with secondary antibody (goat anti-rabbit IgG) conjugated to alkaline phosphatase. Briefly, all samples were dissolved in 1× treatment buffer (0.0625 M Tris–HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8) at concentrations of 0.5–12 mg ml^{-1}, and boiled for 3 min. The samples were then cooled to room temperature before dot blotting or loading a 10% SDS–PAGE gel for subsequent electroblotting and Coomassie blue staining. Blots were incubated with PAP (primary) antibody (1:2500 dilution) or pre-immune (control) sera (1:2500 dilution) followed by secondary antibody (1:2500 dilution). The PAP antibody was also examined for cross-reactivity to other purified and biochemically characterized HRGPs such as tomato extensin P2 (Smith *et al.*, 1986), carrot extensin P1 and P1b (Schnabelrauch *et al.*, 1996), and a maize AGP known as THRGP (Kieliszewski *et al.*, 1992).

Immunolocalization of LeAGP-1 at the light microscope level

Stems and petioles from 1-month-old plants and flowers from 2-month-old plants were fixed in 2% paraformaldehyde and 1% glutaraldehyde (in 50 mM citrate-phosphate buffer, pH 7.4) for 2 h at 4°C, then heated in a 1.25 KW microwave oven for 12 sec. After samples were washed in 50 mM citrate-phosphate buffer for 5 × 10 min, they were dehydrated in an ethanol series (30, 50, 70, 85, 95, 100, 100, 100%, each for 10 min), and then infiltrated with absolute ethanol/xylene mixtures (3:1, 1:1, 1:3, each for 30 min) and xylene (2 × 30 min). Subsequently, samples were infiltrated with increasing concentrations of wax (paraplast embedding medium, Sigma) in xylene, continuously. Finally, samples were infiltrated at 60°C for 48 h with pure wax. Six hours later, all materials were embedded in fresh wax. Sections 11 μm thick were cut and attached onto poly L-lysine covered slides. After sections were dewaxed and rehydrated, they were blocked in blocking solution consisting of 1% BSA in TBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl) for 2 h, rinsed with TBS for 5 × 5 min, then incubated with the PAP antibody or pre-immune sera (1:2000 dilution) overnight. Sections were then washed with TBS for 3 × 10 min and incubated with the FITC-conjugated sheep anti-rabbit IgG (whole molecule) antibody (1:160 dilution) in blocking solution for 1 h. After rinsing with TBS for 5 × 5 min, slides were mounted in 50% glycerol in PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 136.9 mM NaCl, 2.7 mM KCl, pH 7.2), and observed under a Nikon Labophot-2 fluorescence microscope.

Immunolocalization of LeAGP-1 at the transmission electron microscope level

Suspension-cultured cells (on the 6th day after subculturing), stems and petioles from 1-month-old plants, and styles from 2-month-old plants were fixed and dehydrated as described above and then infiltrated with ethanol/LR White (Sigma, St Louis, Missouri, USA) mixtures (3:1, 1:1, 1:3, ethanol:LR White, each for 30 min) and LR White (2 × 60 min, 1 × 2 days). All materials were embedded in LR White resin in gelatin capsules and polymerized at 60°C for 24 h. Semi-thin sections (2 μm thick) of suspension-cultured cells were cut and attached onto poly L-lysine covered slides for immunolocalization at the light microscope level as described above, except that sections did not need to be dewaxed and rehydrated. Ultra-thin sections were cut and collected on nickel grids (200 mesh) for immunolocalization at the TEM level by the same method as the semi-thin sections, except that: (1) the PAP antibody and pre-immune sera were diluted 1:100, (2) the secondary antibody was gold (10 nm)-conjugated goat anti-rabbit IgG (whole molecule) diluted 1:250, and (3) the specimens were further stained with 2% uranyl acetate (in 50% ethanol), and observed under the TEM (Zeiss EM 109 electron microscope).

Acknowledgements

This work was supported by NSF grant IBN-9727757, by research funds from the Molecular and Cellular Biology Program, and by an Ohio University Research Challenge Grant to A.M.S. and M.J.K. The authors are especially grateful to Drs Gar Rothwell and Robert Hikida at Ohio University, Athens, Ohio, USA, for helpful advice and suggestions during the course of this work and in the preparation of this manuscript.

References

Bacic, A., Churms, S.C., Stephen, A.M., Cohen, P.B. and Fincher, G.B. (1987) Fine structure of the arabinogalactan-protein from *Lolium multiflorum*. *Carbohydr. Res.* **162**, 85–93.

Baldwin, T.C., McCann, M.C. and Roberts, K. (1993) A novel hydroxyproline-deficient arabinogalactan protein secreted by suspension-cultured cells of *Daucus carota*. Purification and partial characterization. *Plant Physiol.* **103**, 115–123.

Basile, D.V. and Basile, M.R. (1993) The role and control of the place-dependent suppression of cell division in plant morphogenesis and phylogeny. *Memoirs Torrey Bot. Club*, **25**, 63–83.

Cheung, A.Y., Wang, H. and Wu, H.-M. (1995) A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell*, **82**, 383–393.

Clarke, A.E., Anderson, R.L. and Stone, B.A. (1979) Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry*, **18**, 521–540.

Crimmins, D.L. and Cherian, R. (1997) Increasing the sensitivity of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate amino acid analysis: a simple solution. *Anal. Biochem.* **244**, 407–410.

Diaz, J., Lliberia, J.L., Comellas, L. and Broto-Puig, F. (1996) Amino acid and amino sugar determination by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate followed by high-performance liquid chromatography and fluorescence detection. *J. Chromatogr. A*, **719**, 171–179.

Du, H., Simpson, R.J., Clarke, A.E. and Bacic, A. (1996) Molecular characterization of a stigma-specific gene encoding an arabinogalactan-protein (AGP) from *Nicotiana alata*. *Plant J.* **9**, 313–323.

Egertsdotter, U. and von Arnold, S. (1995) Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). *Physiol. Plant.* **93**, 334–345.

Fincher, G.B., Stone, B.A. and Clarke, A.E. (1983) Arabinogalactan-proteins: structure, biosynthesis, and function. *Annu. Rev. Plant. Physiol.* **34**, 47–70.

Gullick, W.J. (1994) Production of antisera to synthetic peptides. In *Basic Protein and Peptide Protocols* (Walker, J.M., ed.). Totowa, New Jersey: Humana Press, pp. 389–399.

Hillestad, A., Wold, J.K. and Engen, T. (1977) Water-soluble glycoproteins from *Cannabis sativa* (Thailand). *Phytochemistry*, **16**, 1953–1956.

Jauh, G.Y. and Lord, E.M. (1996) Localization of pectins and arabinogalactan-proteins in lily (*Lilium longiflorum* L.) pollen tube and style, and their possible roles in pollination. *Planta*, **199**, 251–261.

John, M.E. and Keller, G. (1995) Characterization of mRNA for a proline-rich protein of cotton fiber. *Plant Physiol.* **108**, 669–676.

Kieliszewski, M.J. and Lamport, D.T.A. (1994) Extensin: repetitive motifs, functional sites, post-translational codes and phylogeny. *Plant J.* **5**, 157–172.

Kieliszewski, M.J., Leykam, J.F. and Lamport, D.T.A. (1990) Structure of the threonine-rich extensin from *Zea mays*. *Plant Physiol.* **92**, 316–326.

Kieliszewski, M.J., Kamyab, A., Leykam, J.F. and Lamport, D.T.A. (1992) A histidine-rich extensin from *Zea mays* is an arabinogalactan protein. *Plant Physiol.* **99**, 538–547.

Kieliszewski, M.J., Showalter, A.M. and Leykam, J.F. (1994) Potato lectin: a modular protein sharing sequence similarities with the extensin family, the hevein lectin family, and snake venom disintegrins (platelet aggregation inhibitors). *Plant J.* **5**, 849–861.

Kjellbom, P., Snogerup, L., Stohr, C., Reuzeau, C., McCabe, P.F. and

Pennell, R.I. (1997) Oxidative cross-linking of plasma membrane arabinogalactan proteins. *Plant J.* **12**, 1189–1196.

Knox, J.P., Linstead, P.J., Peart, J., Cooper, C. and Roberts, K. (1991) Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. *Plant J.* **1**, 317–326.

Komalavilas, P., Zhu, J.-K. and Nothnagel, E.A. (1991) Arabinogalactan-proteins from the suspension culture medium and plasma membrane of rose cells. *J. Biol. Chem.* **266**, 15956–15965.

Kreuger, M. and van Holst, G.-J. (1993) Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. *Planta*, **189**, 243–248.

Kreuger, M. and van Holst, G.-J. (1995) Arabinogalactan-protein epitopes in somatic embryogenesis of *Daucus carota* L. *Planta*, **197**, 135–141.

Lamport, D.T.A. (1970) Cell wall metabolism. *Annu. Rev. Plant Physiol.* **21**, 235–270.

Langan, K.J. and Nothnagel, E.A. (1997) Cell surface arabinogalactan-proteins and their relation to cell proliferation and viability. *Protoplasma*, **196**, 87–98.

Li, S. (1996) *Isolation and characterization of genes and complementary DNAs encoding tomato arabinogalactan-protein.* PhD Dissertation, Ohio University, Athens, Ohio.

Li, S. and Showalter, A.M. (1996) Cloning and developmental/stress-regulated expression of a gene encoding a tomato arabinogalactan protein. *Plant Mol. Biol.* **32**, 641–652.

Mau, S.-L., Chen, C.-G., Pu, Z.-Y., Moritz, R.L., Simpson, R.J., Bacic, A. and Clarke, A.E. (1995) Molecular cloning of cDNAs encoding the protein backbones of arabinogalactan-proteins from the filtrate of suspension-cultured cells of *Pyrus communis* and *Nicotiana alata. Plant J.* **8**, 269–281.

Mollard, A. and Joseleau, J.-P. (1994) *Acacia senegal* cells cultured in suspension secrete a hydroxyproline-deficient arabinogalactan-protein. *Plant Physiol. Biochem.* **32**, 703–709.

Mort, A.J. and Lamport, D.T.A. (1977) Anhydrous hydrogen fluoride deglycosylates glycoproteins. *Anal. Biochem.* **82**, 289–309.

Nothnagel, E.A. (1997) Proteoglycans and related components in plant cells. *Int. Rev. Cytol.* **174**, 195–291.

Page, M., Baines, M.G. and Thorpe, R. (1994) Preparation of purified immunoglobulin G (IgG). In *Basic Protein and Peptide Protocols* (Walker, J.M., ed.). Totowa, New Jersey: Humana Press, pp. 407–432.

Pennell, R.I. (1992) Cell surface arabinogalactan proteins, arabinogalactans and plant development. In *Perspectives in Plant Cell Recognition* (Callow, J.A. and Green, J.R., eds). Cambridge: Cambridge University Press, pp. 105–121.

Pennell, R.I. and Roberts, K. (1990) Sexual development in the pea is presaged by altered expression of arabinogalactan protein. *Nature*, **334**, 547–549.

Pennell, R.I., Janniche, L., Kjellbom, P., Scofield, G.N., Peart, J.M. and Roberts, K. (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. *Plant Cell*, **3**, 1317–1326.

Pogson, B. and Davies, C. (1995) Characterization of a cDNA encoding the protein moiety of a putative arabinogalactan protein from *Lycopersicon esculentum. Plant Mol. Biol.* **28**, 347–352.

Pope, D.G. (1977) Relationships between hydroxyproline-containing proteins secreted into the cell wall and medium by suspension-cultured *Acer pseudoplatanus* cells. *Plant Physiol.* **59**, 894–900.

Qi, W., Fong, C. and Lamport, D.T.A. (1991) Gum arabic glycoprotein is a twisted hairy rope. *Plant Physiol.* **96**, 848–855.

Roy, S., Jauh, G.Y., Hepler, P.K. and Lord, E.M. (1998) Effects of Yariv phenylglycoside on cell wall assembly in the lily pollen tube. *Planta*, **204**, 450–458.

Schindler, T., Bergfeld, R. and Schopfer, P. (1995) Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extension growth. *Plant J.* **7**, 25–36.

Schnabelrauch, L.S., Kieliszewski, M., Upham, B.L., Alizedah, H. and Lamport, D.T.A. (1996) Isolation of pI 4.6 extensin peroxidase from tomato cell suspension cultures and identification of Val-Tyr-Lys as putative intermolecular crosslink site. *Plant J.* **9**, 477–490.

Serafini-Fracassini, D., Del Duca, S. and Beninati, S. (1995) Plant transglutaminases. *Phytochemistry*, **40**, 355–365.

Serpe, M.D. and Nothnagel, E.A. (1994) Effects of Yariv phenylglycosides on *Rosa* cell-suspensions: evidence for the involvement of arabinogalactan-proteins in cell proliferation. *Planta*, **193**, 542–550.

Showalter, A.M. (1993) Structure and function of plant cell wall proteins. *Plant Cell*, **5**, 9–23.

Showalter, A.M. and Varner, J.E. (1989) Plant hydroxyproline-rich glycoproteins. In *The Biochemistry of Plants* (Marcus, A., ed.). New York: Academic Press, pp. 485–536.

Smith, J.J., Muldoon, E.P., Willard, J.J. and Lamport, D.T.A. (1986) Tomato extensin precursors P1 and P2 are highly periodic structures. *Phytochemistry*, **25**, 1021–1030.

Sommer-Knudsen, J., Lush, W.M., Bacic, A. and Clarke, A.E. (1998) Re-evaluation of the role of a transmitting tract-specific glycoprotein on pollen tube growth. *Plant J.* **13**, 529–535.

Stacey, N.J., Roberts, K. and Knox, J.P. (1990) Patterns of expression of the JIM4 arabinogalactan-protein epitope in cell cultures and during somatic embryogenesis in *Daucus carota* L. *Planta*, **180**, 285–292.

Stiefel, V., Perez-Grau, L., Albericio, F., Giralt, E., Ruiz-Avila, L., Ludevid, M.D. and Puigdomenech, P. (1988) Molecular cloning of cDNAs encoding a putative cell wall protein from *Zea mays* and immunological identification of related polypeptides. *Plant Mol. Biol.* **11**, 483–493.

Willats, W.G.T. and Knox, J.P. (1996) A role for arabinogalactan-proteins in plant cell expansion: evidence from studies on the interaction of β-glucosyl Yariv reagent with seedlings of *Arabidopsis thaliana. Plant J.* **9**, 919–925.

Wirguin, I., Suturkova-Milosevic, L., Briani, C. and Latov, N. (1995) Keyhole limpet hemocyanin contains Gal(beta 1–3)-GalNAc determinants that are cross-reactive with the T antigen. *Cancer Immunol. Immunother.* **40**, 307–310.

Wisdom, G.B. (1994) Protein blotting. In *Basic Protein and Peptide Protocols* (Walker, J.M., ed.). Totowa, New Jersey: Humana Press, pp. 207–213.

Yariv, J., Rapport, M.M. and Graf, L. (1962) The interaction of glycosides and saccharides with antibody to the corresponding phenylazo glycosides. *Biochem. J.* **85**, 383–388.

Yates, E.A., Valdor, J.-F., Haslam, S.M., Morris, H.R., Dell, A., Mackie, W. and Knox, J.P. (1996) Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies. *Glycobiology*, **6**, 131–139.

Youl, J.J., Bacic, A. and Oxley, D. (1998) Arabinogalactan-proteins from *Nicotiana alata* and *Pyrus communis* contain glycosylphosphatidylinositol membrane anchors. *Proc. Natl Acad. Sci. USA*, **95**, 7921–7926.

Zhu, J.-K., Bressan, R.A. and Hasegawa, P.M. (1993) Loss of arabinogalactan-proteins from the plasma membrane of NaCl-adapted tobacco cells. *Planta*, **190**, 221–226.