Chapter 6

Characterization and Localization of a Novel Tomato Arabinogalactan-Protein (LeAGP-1) and the Involvement of Arabinogalactan-Proteins in Programmed Cell Death

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

1. INTRODUCTION

Arabinogalactan-proteins (AGPs) are a family of plant Hyp-rich glycoproteins containing Type II AG side chains (Fincher *et al* 1983, Nothnagel 1997). Several AGPs have been characterized over the years on the (glyco)protein level and, more recently, on the molecular level. Collectively, these studies indicate that AGPs are a diverse family that can be classified in one of two groups: the "classical" and the "non-classical" AGPs. Classical AGPs contain 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 is involved in the attachment of a glycosylphosphatidylinositol (GPI) membrane anchor (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, Cys-rich, and Hyp-poor domains, but, to date, do not specify any C-terminal hydrophobic domains. As additional structural data

are obtained on AGPs, this working classification system will undoubtedly be refined. More importantly, additional structural data and the availability of purified AGPs will help elucidate the function of these extraordinary plant glycoproteins.

Currently, AGPs are believed to function in various aspects of plant growth and development (Nothnagel 1997). Much of this belief is based on studies performed using monoclonal antibodies directed against AGP carbohydrate epitopes, which indicate that AGPs are markers of cellular identity during development. Others have used certain AGPs to induce or inhibit plant embryogenesis. Several other groups have used Yariv reagent to perturb growth and development. For example, Serpe and Nothnagel (1994) treated rose cell cultures with Yariv reagent and observed inhibition of cell growth. Similar experiments using Yariv reagent to bind AGPs and 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 in the death of young rose cells in culture (Langan and Nothnagel 1997).

In this communication we focus on the structure and localization of LeAGP-1, a major AGP expressed in tomato cell cultures and plants. In addition, we present data dealing with the production of transgenic *LeAGP-1* antisense plantlets and the utilization of Yariv reagent to induce programmed cell death (PCD) in cultured cells. These later studies respectively represent our initial assault on elucidating the function of LeAGP-1 and AGPs.

2. RESULTS AND DISCUSSION

2.1 Molecular and Biochemical Characterization of LeAGP-1

The LeAGP-1 was first identified by DNA cloning in tomato (Fig 1) (Pogson and Davies 1995, Li and Showalter 1996). Verification that *LeAGP-1* cDNA and genomic clones encode an AGP core protein was accomplished by precipitating total AGPs from tomato cultured cells and purifying the corresponding core proteins. Yariv reagent was used to precipitate total AGPs from cultured tomato cells following homogenization or sonication. Subsequently, anhydrous hydrofluoric acid was used to deglycosylate these AGPs, and the resulting deglycosylated core proteins were separated by reverse phase HPLC on an PRP-1 column. Each of the resulting core protein fractions was subjected to amino acid composition analysis (Table 1). Although all four fractions had compositions consistent with classical AGPs, one fraction (HC-3) matched the composition predicted by the *LeAGP-1* cDNA clones the best. These fractions were also tested in Western blot and gel blots for their ability to react with an antibody produced from a synthetic peptide containing the Lys-rich subdomain, predicted by the *LeAGP-1* DNA clones, conjugated to keyhole lympet hemocyanin. Only fraction HC-3 reacted with this so-called PAP antibody named with respect to the first three residues in the synthetic peptide (see Fig 1). Fraction HC-3 was then subjected to trypsin digestion, and the

resulting tryptic peptides were separated by reverse phase HPLC chromatography and sequenced. Three peptide sequences were obtained: 1) Hyp-Ala-Ala-Ala-Hyp-Thr-Lys-Pro-Lys, 2) Ala-Hyp-Ala-Ser-Ser-Hyp-Hyp-Val-Gln-Ser-Hyp-Hyp-Ala-Hyp-Ala-Hyp-Glu-Val-Ala-Thr-Hyp-Hyp-Ala-Val..., and 3) Ala-Hyp-Ala-Ser-Ser-Hyp-Hyp-Val-Gln-Ser-Hyp-Hyp-Ala-Hyp-Ala-Hyp-Glu-Val-Ala-Thr-Hyp-Hyp-Val-Ser-Thr-Hyp-Hyp-Ala-Ala-Ala-(Xaa)-Val-Ala-Ala.... These sequences corresponded precisely to the sequence predicted from the *LeAGP-1* clones and further indicated which prolyl residues were hydroxylated (Fig 1). It should be noted that two of these peptide sequences overlap and that a predicted Ala residue is absent in the longer overlapping sequence, indicating that LeAGP-1 isoforms exist or a minor amino acid sequencing error occurred. Taken together, these data indicate that fraction HC-3 represents an AGP core protein that corresponds to that predicted by the *LeAGP-1* clones.

(a)

1 21 141 153 198 215 aa

signal peptide domain | highly basic subdomain

"classical" AGP domain | C-terminal hydrophobic domain

(b)

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MetAspArgLysPheValPheLeuValSerIleLeuCysIleValValAlaSerValThr    20
GlyGlnThrProAlaAlaAlaProValGlyAlaLysAlaGlyThrThrProHypAlaAla    40
AlaHypThrLysProLysThrProAlaProAlaThrAlaProAlaSerAlaProProThr    60
AlaValProValAlaProValThrAlaProValThrAlaProThrThrProValValAla    80
AlaProValSerAlaProAlaSerSerProProLeuLysAlaHypAlaSerSerHypHyp   100
ValGlnSerHypHypAlaHypAlaHypGluValAlaThrHypHypAlaValSerThrHyp   120
HypAlaAlaAlaProValAlaAlaProValAlaSerGluThrThrProAlaProAlaPro   140
SerLysGlyLysValLysGlyLysLysGlyLysLysHisAsnAlaSerProAlaProSer   160
ProAspMetMetSerProProAlaProProSerGluAlaProGlyProSerMetAspSer   180
AspSerAlaProSerProSerLeuAsnAspGluSerGlyAlaGluLysLeuLysMetLeu   200
GlySerLeuValAlaGlyTrpAlaValMetSerTrpLeuLeuPhe                  215
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Figure 1. (a) Domain organization for the predicted LeAGP-1 core protein consists of an N-terminal signal peptide, a central "classical" AGP domain sandwiching a highly basic subdomain, and a C-terminal hydrophobic domain, which may allow for GPI-mediated plasma membrane association. This modular organization was predicted by *LeAGP-1* cDNA and genomic clones. (b) Amino acid sequence of LeAGP-1 as predicted by *LeAGP-1* DNA clones and embellished with amino acid sequencing data. 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 in medium boldface; 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 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 Ser 192 is underlined in heavy boldface and discussed in the text.

Table 1. Amino acid compositions[a] of AGPs isolated from tomato suspension-cultured cells (i.e., HC-AGPs) compared with that predicted from *LeAGP-1* DNA clones

AA	LeAGP-1[b]	LeAGP-1[c]	HC-3	HC-1	HC-2	HC-4
Hyp	–	–	29	37	34	30
Pro	24.4	27.5	1	2	2	2
Asx	3.1	3.5	2	6	3	2
Thr	7.7	8.8	10	9	8	8
Ser	1.4	11.7	12	11	11	18
Glx	3.6	3.5	3	1	3	3
Gly	4.6	3.5	5	7	9	6
Ala	22.2	23.4	21	19	19	15
Val	8.2	8.2	9	3	4	7
Cys	0.0	0.0	0	0	0	0
Met	2.6	1.7	0	0	0	0
Ile	0.0	0.0	1	2	4	2
Leu	3.6	1.2	1	2	2	3
Tyr	0.0	0.0	0	0	0	0
Phe	0.5	0.0	1	0	0	1
His	0.5	0.6	0	0	0	0
Lys	6.7	6.4	5	2	2	2
Arg	0.0	0.0	0	0	0	1
Trp	1.0	0.0	nd	nd	nd	nd

[a] Expressed in mole %.
[b] LeAGP-1 excluding the N-terminal signal sequence.
[c] LeAGP-1 excluding the N-terminal signal sequence and the C-terminal hydrophobic domain presumably removed during GPI attachment.
nd = not determined.

Several interesting features emerge from the LeAGP-1 core protein sequence (Fig 1). First, its domain structure corresponds to other known classical AGPs; however, it is distinguished from them by the occurrence of the Lys-rich subdomain. This subdomain represents a prime site for intermolecular interactions with negatively charged cell surface molecules such as pectin. Second, this AGP is predicted to be modified by the addition of a GPI anchor near its C-terminus as demonstrated in two known classical AGPs (Youl *et al* 1998). The LeAGP-1 sequence contains the consensus sequence for such a modification. In addition, it is interesting to note that this consensus sequence (i.e., Ser-Gly-Ala) and C-terminal hydrophobic domain reside exclusively in exon 2 of the *LeAGP-1* gene and represent a likely functional exon domain (Fig 1) (Li and Showalter 1996). Third, a potential N-linked glycosylation site is encoded by the *LeAGP-1* clones and is located at the C-terminal side of the Lys-rich subdomain; such sites are not typical for AGPs. We predict that if such an N-linked glycan exists on LeAGP-1, then it will be in addition to numerous Type II AG side chains as well as other short oligoarabinosides as demonstrated for sycamore AGPs, gum arabic glycoprotein, and a maize AGP (Pope 1977, Qi *et al* 1991, Kieliszewski *et*

al 1992). Clearly, we now hope to verify the existence of a GPI anchor and conduct a comprehensive carbohydrate analysis on LeAGP-1.

2.2 Localization of LeAGP-1

The PAP antibody was also used to immunolocalize LeAGP-1 in tomato cultured cells and various plant organs at the light and electron microscope levels (Fig 2). These data not only reveal where LeAGP-1 is located but also provide clues as to its functions. In tomato cultured cells, LeAGP-1 was detected at the cell surface (Fig 2a). Immunolocalization at the electron microscope level revealed that LeAGP-1 is located in the cell wall (Fig 2b). It should be noted that additional biochemical analysis indicated that LeAGP-1 exists as the major AGP in the media of cultured cells. The LeAGP-1 was also immunolocalized in tomato stems and floral styles. In stems, LeAGP-1 is prominently localized to developing metaxylem elements but is also found in outer phloem and inner phloem (Fig 2c). Ultrastructurally, LeAGP-1 is located primarily in the secondary cell wall of such developing metaxylem elements; however, some staining of the primary cell wall was also observed (Fig 2d). The most intense staining for LeAGP-1, however, was observed in the transmitting tissue of the style (Fig 2e). Immunolocalization at the electron microscope level showed LeAGP-1 to be copiously deposited in the intercellular spaces of transmitting tract cells as well as in their thin cell walls (Fig 2f).

Thus, LeAGP-1 is found in and outside tomato cell walls. The question of whether LeAGP-1 is also associated with the plasma membrane remains unanswered. Given that LeAGP-1 contains a putative site for GPI anchoring, it should be at least temporarily associated with the plasma membrane. We envision that LeAGP-1 moves from the endoplasmic reticulum to the Golgi and then to the plasma membrane, where processing of the GPI anchor occurs to release LeAGP-1 into the cell wall and space beyond. The inability of our antibody to immunolocalize LeAGP-1 to the plasma membrane may be due to a small pool of plasma membrane associated LeAGP-1 or to the antigenic epitope being blocked or inaccessible in this microenvironment. We also speculate that the amount of LeAGP-1 found beyond the wall is governed by the particular composition and amounts of cell wall components that may serve as a saturable affinity column, as opposed to a more elaborate trafficking scheme. In this regard, it will be extremely interesting to identify the cell surface components that interact with LeAGP-1.

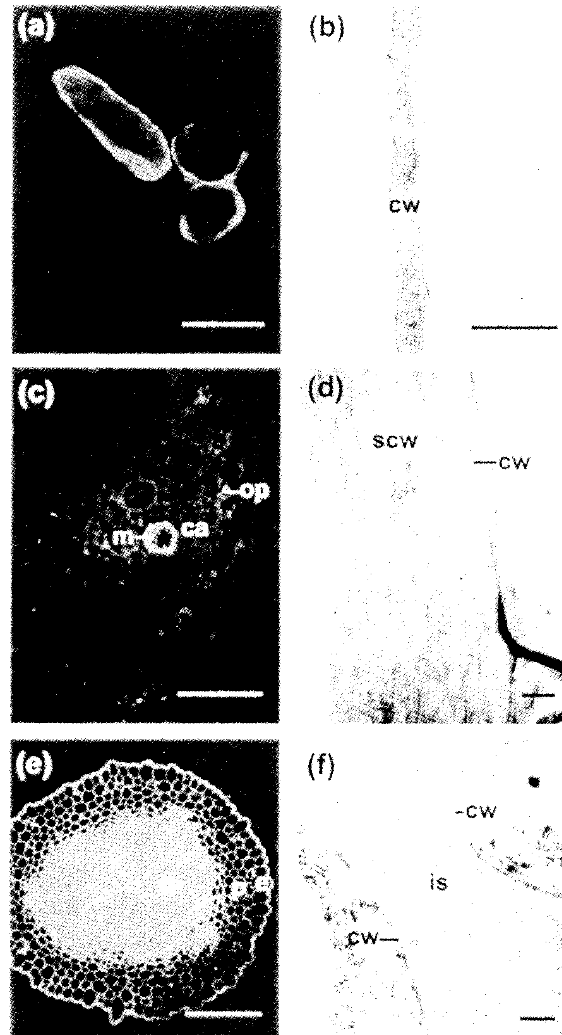

Figure 2. Immunolocalization of LeAGP-1 with the PAP antibody in tomato suspension-cultured cells, young stem, and style at the light and electron microscope levels. (a) Whole mount of suspension-cultured cells; (b) a portion of a suspension-cultured cell; (c) a portion of a cross-section of a young stem; (d) a portion of a maturing metaxylem element in young stem; (e) cross-section of a style; (f) neighboring transmitting tissue cells and their intercellular space; (a), (c), and (e) were immunolocalized at the light microscope level using FITC-conjugated secondary antibody, whereas (b), (d), and (f) were immunolocalized at the electron microscope level using 10 nm gold-conjugated secondary antibody. m, metaxylem element; ca, cambial zone; op, outer phloem; e, epidermis; p, stylar parenchyma; tt, stylar transmitting tissue. cw, (primary) cell wall; scw, secondary cell wall; is, intercellular space. Bars in (a), (c), and (e) = 100 μm. Bars in (b), (d), and (f) = 500 nm.

2.3 Production of Transgenic Anti-Sense *LeAGP-1* Tomato Plantlets

As one approach to elucidate the function of LeAGP-1, we have produced transgenic *LeAGP-1* antisense tomato plantlets. Here we used the 35S cauliflower mosaic virus promoter to drive expression of a nearly full-length *LeAGP-1* cDNA in an antisense orientation. The T-DNA transformed and untransformed tomato plantlets were used as controls. We obtained only 15 independent antisense transformed plantlets in contrast to hundreds of T-DNA transformed plantlets. Northern analysis and immunocytochemical analysis indicated that *LeAGP-1* mRNA and LeAGP-1 protein were severely down-regulated in comparison to the T-DNA transformed and untransformed control plantlets (data not shown). Visual analyses of the antisense plantlets showed them to be dramatically smaller than the control plantlets. Moreover, these antisense plantlets would not root, and all but six have now died. Clearly, this experiment needs to be repeated; however, if these initial results are correct, then the use of an inducible promoter to drive antisense expression warrants serious consideration.

2.4 AGPs and Programmed Cell Death

Previous experiments by Serpe and Nothnagel (1994) and Langan and Nothnagel (1997) demonstrated that Yariv reagent can cause growth inhibition and cell death in cultured cells. However, it was not known whether these cells died by necrosis or by PCD. Our initial studies in 1996 using tomato seedlings treated with 30 μM $(\beta\text{-D-Gal})_3$ Yariv reagent showed ultrastructural changes characteristic of apoptosis in some cells at the root tip. Apoptosis, a type of PCD, was first identified in animals (Wyllie 1980). These changes included cytoplasmic shrinkage, nuclear membrane blebbing, and chromatin condensation; such changes were not observed in untreated control cells. Subsequently, we hypothesized that Yariv reagent causes cultured cells to die a PCD. A precisely controlled form of cellular suicide, PCD is characterized by internuclear DNA cleavage and changes in cell morphology. To test our hypothesis, we used cell suspension cultures of tomato and *Arabidopsis thaliana* and treated them for various times and with various concentrations $(\beta\text{-D-Gal})_3$ and $(\alpha\text{-D-Gal})_3$ Yariv reagents before scoring them for PCD. Three criteria were used to determine PCD: 1) terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of DNA 3'-OH groups, 2) internucleosomal DNA cleavage, and 3) structural changes in cell morphology characteristic of PCD.

Although tomato cells were initially used in the TUNEL experiments and demonstrated appreciable TUNEL positive nuclei, *Arabidopsis* was rapidly adopted as our model system for this study, given the more robust nature of the TUNEL response in these cells (Table 2). For example, 100% of the *Arabidopsis* cells showed TUNEL positive nuclei when treated with 80 μM $(\beta\text{-D-Gal})_3$ Yariv reagent for 72 hours in comparison with about 50% of the tomato cells. Note that untreated cells and cells treated with $(\alpha\text{-D-Gal})_3$ Yariv reagent [which does not react with

AGPs but is nearly identical to (β-D-Gal)$_3$ Yariv reagent] show very low percentages of TUNEL positive nuclei (Table 2). Moreover, with increasing incubation time, both the (α-D-Gal)$_3$ Yariv reagent-treated cells and the untreated cells demonstrated reduced percentages of TUNEL-positive nuclei. This observation provides support to the idea of social control of PCD as promulgated by Barres *et al* (1992) and Raff (1992) in animal cell cultures and by McCabe *et al* (1997) in plant cell cultures, whereby neighboring cells grown at high density provide signal molecules or growth factors that suppress PCD.

Table 2. Average percentage of apoptotic (i.e., TUNEL-positive) cells in *Arabidopsis* suspension-cultured cells treated with Yariv reagent

Time (hr)	Untreated	50 μM (α-D-Gal)$_3$	50 μM (β-D-Gal)$_3$	80 μM (α-D-Gal)$_3$	80 μM (β-D-Gal)$_3$
24	–	5.98	30.38	7.30	47.51
48	–	2.29	34.66	3.47	80.14
72	0.87	2.44	45.43	4.52	100.00

Internucleosomal DNA fragmentation was detected in (β-D-Gal)$_3$ Yariv reagent-treated *Arabidopsis* cells, but not in (α-D-Gal)$_3$ Yariv reagent-treated cells or in untreated cells. Such fragmentation was both time and concentration dependent and was detectable after 48 hours of treatment with 80 μM (β-D-Gal)$_3$ Yariv reagent or after 72 h of 60 μM (β-D-Gal)$_3$ Yariv reagent (data not shown). Such time-dependent internucleosomal DNA fragmentation is characteristic of PCD in contrast to necrosis, which is characterized by rapid cell death and non-specific DNA degradation.

Microscopic examination of *Arabidopsis* cells treated with (β-D-Gal)$_3$ Yariv reagent showed characteristic structural changes in cell morphology not observed in (α-D-Gal)$_3$ Yariv reagent-treated cells or in untreated cells (data not shown). At the light-microscope level, cells treated with the 80 μM (β-D-Gal)$_3$ Yariv reagent displayed cytoplasmic shrinkage and condensation; this was also observed at the electron-microscope level in addition to chromatin condensation and nuclear membrane blebbing. Notably, other organelles (e.g., mitochondria) were intact. Such microscopic changes are characteristic of animal apoptosis (Wyllie 1980). Some of these changes are also recorded for plant PCD (McCabe *et al* 1997). Plant PCD research is beginning to receive significant attention but currently comprises few studies, particularly at the ultrastructural level.

On the basis of these three criteria, (β-D-Gal)$_3$ Yariv reagent induces *Arabidopsis* suspension cultured cells to undergo PCD. The precise mechanism whereby Yariv reagent induces this process is unknown but clearly implicates AGP involvement. One idea is that Yariv reagent serves to disrupt plasma membrane–cell wall interactions involving AGPs, which initiates a PCD signal transduction cascade. Such a scenario is hypothesized and supported in the case of an animal extracellular matrix–plasma membrane interaction (Frisch and Francis 1994). Another related idea is that AGPs must be present or assemble as multimeric complexes at the cell surface to relay signal trans-

duction information. Disruption of such complexes or their assembly may prevent growth signals from being relayed and thus trigger PCD.

3. CONCLUSIONS

1. LeAGP-1 is a novel classical AGP distinguished by its Lys-rich subdomain.
2. LeAGP-1 is the major AGP present in tomato cultured cells and media.
3. LeAGP-1 immunolocalizes in tomato to the cell walls of cultured cells, to the primary, and especially the secondary, cell walls of developing metaxylem elements of the stem and petiole, and to the cell walls and intercellular spaces present in stylar transmitting tissue.
4. Transgenic *LeAGP-1* antisense tomato plantlets were deficient in *LeAGP-1* mRNA and LeAGP-1 protein and showed severe overall growth inhibition and had difficulty rooting.
5. We speculate that LeAGP-1 is also associated with the plasma membrane via a GPI anchor where it could interact with cell wall components (including itself) and function as a cell adhesion molecule, possibly by regulating growth or PCD.
6. The ability of $(\beta\text{-D-Gal})_3$ Yariv reagent to trigger PCD implicates AGPs in this process.
7. It is worth noting that xylem is produced as a result of PCD and that AGPs, including LeAGP-1, are often expressed in such cells.

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