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Update on Extracellular Matrix

The Multiple Roles of Arabinogalactan Proteins in Plant Development[1]

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Arabinogalactan proteins (AGPs) are: (a) structurally complex macromolecules composed of a polypeptide, large branched glycan chains, and lipid?; (b) markers of xylem development in Arabidopsis primary root?; (c) abundant extracellular matrix components in tobacco styles?; (d) active in tip growth of lily pollen tubes?; (e) able to promote somatic embryogenesis in carrot cell cultures?; (f) involved in programmed cell death in Arabidopsis cell cultures?; (g) present in Ancient Egyptian mummies and hieroglyphs?; (h) found in Mountain Dew[2]?; (i) active ingredients in Juzen-taiho-to, a Sino-Japanese herbal medicine?; (j) some of the above?; or (k) all of the above?

In plants, organ generation is continuous with the appearance of roots, stems, leaves, flowers, and fruits occurring during development from embryo to mature plant. Cell division and expansion are fundamental to this continuous organ generation. Control of these two processes has both temporal elements (when will division or expansion occur?) and spatial elements (what will be the orientation of the division plane, or what will be the direction of expansion?). These two processes, occurring at or near meristems, are joined by a third fundamental process, cell differentiation, to form organs during development.

What factors control these fundamental processes of cell division, expansion, and differentiation? Or, in simpler terms, consider a cell at or near an apical meristem. What determines whether this cell will divide, expand, and/or differentiate? Clonal analysis has shown that, at least for differentiation, cell position is a stronger determinant than cell lineage. Thus, the fate of a particular cell depends more upon the identity of its neighbors than upon the identity of its mother. In this context, markers of cell identity and mechanisms of cell-to-cell signaling immediately come to the fore, and this in turn directs attention to the cell surface as the likely site of initial events. Receptor-like protein kinases and many other cell surface molecules are currently being investigated for function in cell signaling and recognition. One such class of cell surface macromolecules, arabinogalactan proteins (AGPs), is considered here.

AGPS ARE UBIQUITOUS IN PLANTS AND HAVE COMPLEX STRUCTURES AMENABLE TO INFORMATIONAL/SIGNALING FUNCTIONS

AGPs are widely distributed in the plant kingdom, probably occurring in every cell of every plant from bryophytes to angiosperms. These proteoglycans are typically 60 to 300 kD, are often more than 90% carbohydrate, and have a core polypeptide that is usually rich in Hyp, Ala, Ser, and Thr. Approximately eight cDNAs encoding the core polypeptide of confirmed AGPs have been characterized, and approximately eight additional cDNAs, isolated by differential screening or other approaches, are also believed to encode AGPs (Sommer-Knudsen et al., 1997; Serpe and Nothnagel, 1999). Many apparent homologs are now being recognized in expressed sequence tag databases for Arabidopsis, rice, and other plants. All of these cDNAs encode polypeptides with a N-terminal signal sequence for entry into the secretory pathway and a domain rich in Pro/Hyp, Ala, Ser, and Thr (Sommer-Knudsen et al., 1997).

Divergence occurs beyond these two common domains, however, so several AGP types have been distinguished (Du et al., 1996). Figure 1 shows a "classical" AGP. According to cDNA sequences, classical AGPs contain a hydrophobic transmembrane domain at their C terminus. In the mature AGP, however, this hydrophobic domain is absent and replaced by a glycosylphosphatidylinositol (GPI) lipid anchor. The site of this processing is yet to be elucidated in plant cells but occurs in the endoplasmic reticulum in animal and yeast cells. Amino acid sequence motifs appropriate for such processing have been identified in all known and putative classical AGPs (Schultz et al., 1998). Some classical AGPs contain a short domain, rich in basic amino acids, that interrupts the Pro/Hyp, Ala, Ser, Thr-rich domain (Gao et al., 1999). "Non-classical" AGPs contain either a Cys-rich C-terminal domain or one or two Asn-rich domains that follow or enclose the Pro/Hyp, Ala, Ser, Thr-rich domain. None of the known non-classical AGPs contains a hydrophobic C-terminal domain or codes for GPI modification. Other macromolecules appear to be

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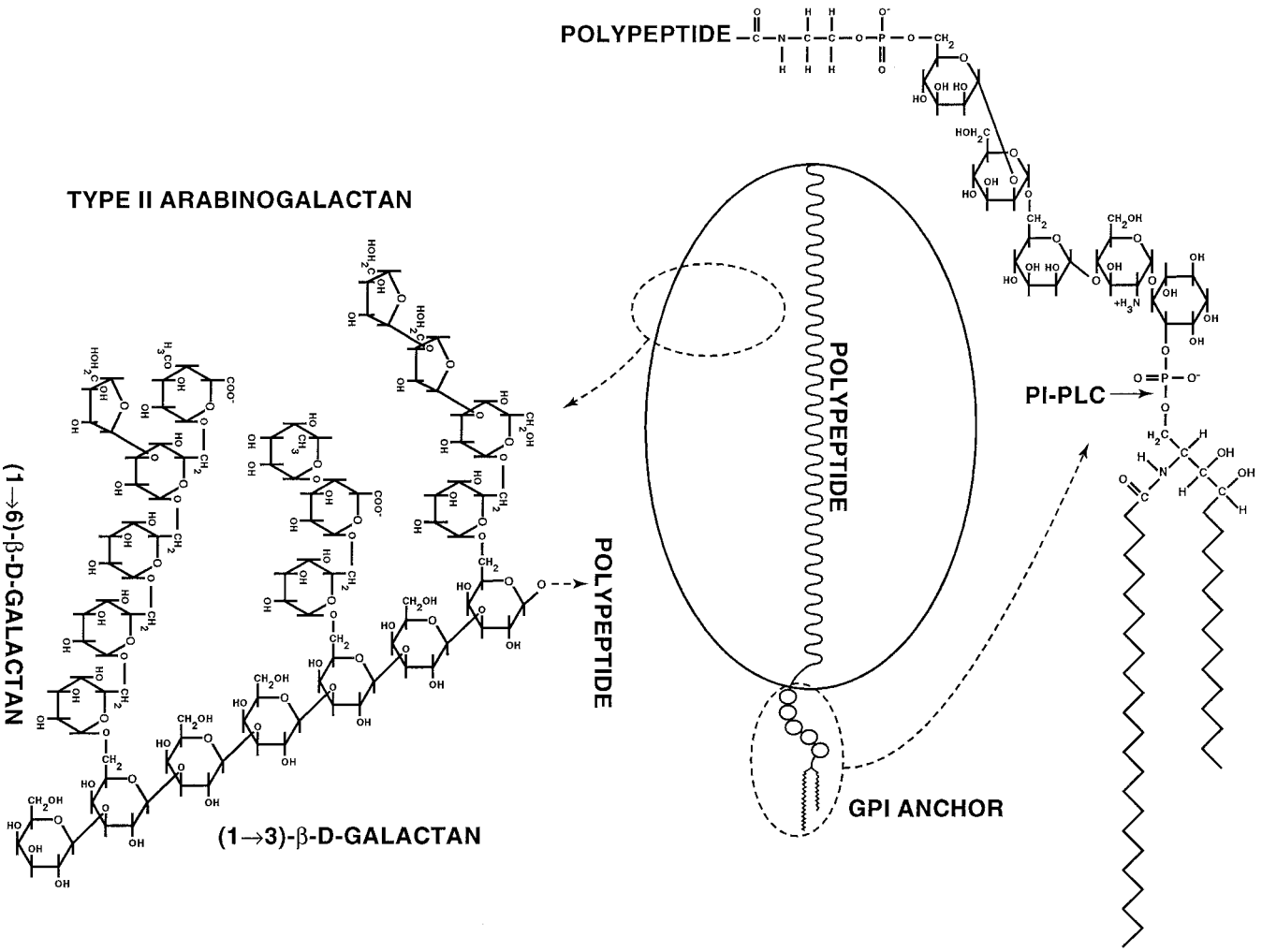

Figure 1. Hypothetical model of a classical AGP carrying a GPI lipid anchor. The ellipse represents the 15- by 25-nm size of carrot AGPs. The wavy line represents the core polypeptide, which, for a 141-kD AGP containing 5.6% protein, has a calculated length of 24 nm, essentially the same as the length of the ellipse. The GPI anchor is similarly drawn to approximate scale and is based on the ethanolamine cap found at the truncated C terminus of pear and *N. alata* AGP core polypeptides (Youl et al., 1998) and on the ceramide lipid found in rose AGPs (Svetek et al., 1999). Biochemical evidence shows that Arabidopsis AGPs also contain GPI anchors (Sherrier et al., 1999). The structure of the oligosaccharide linker between the ethanolamine and lipid is as found in animals and microorganisms. The site of cleavage by phosphatidylinositol-specific phospholipase C (PI-PLC) is indicated. The type II arabinogalactan chains typically consist of 30 to 150 sugar residues and are attached at many Hyp, Ser, and/or Thr residues in the core polypeptide. The side chains shown on the (1→3)-β-D-galactan backbone are based on oligosaccharides characterized from various AGPs, but their placement as shown is hypothetical. No complete structure has been solved for any AGP. Reproduced with permission from Serpe and Nothnagel (1999).

chimeras of AGPs and extensins, containing both the large type II arabinogalactan polysaccharides of AGPs and the short Hyp-oligoarabinosides of extensins.

AGPs may be the most structurally complex macromolecules in nature. This complexity is most evident in the large, branched-glycan chains. Synthesis of a polypeptide is directed by a template (mRNA), but no such template is known for the synthesis of a polysaccharide. Instead, the required information is believed to reside in glycosyltransferase specificities: a particular glycosyltransferase is needed to add the first sugar to an amino acid in the core polypeptide and then additional particular glycosyltransferases are needed to form each type of sugar-sugar bond as the glycan chain grows. For the synthesis of highly complex chains (Fig. 1), many glycosyltransferases, and therefore many genes, are required. Such genome investment is unlikely to have survived evolution unless it enabled essential function. Localized on the plasmalemma, bound to the cell wall, or soluble in the cell wall space (Serpe and Nothnagel, 1999), AGPs are in positions where variations in their complex structures could serve to mark cell identity or to signal neighboring cells. When development requires or directs a change in cell surface signals, remodeling could be readily accomplished by phospho-

lipase cleavage of GPI anchors to shed AGPs from the plasmalemma.

HIGHLY REGULATED EXPRESSION OF AGP EPITOPES ACCOMPANIES DEVELOPMENT OF ROOT VASCULAR TISSUE

Numerous investigations have demonstrated that expression of AGPs is developmentally regulated in both space (different organs or tissues produce different AGPs) and time (one tissue or organ produces different AGPs at different developmental times). Regulated expression was first demonstrated at the level of the mature AGP by electrophoretic and biochemical techniques. More recently, hybridization techniques have demonstrated regulated expression of mRNAs encoding AGP core polypeptides. Most demonstrations of regulated expression, however, have involved the use of monoclonal antibodies directed against carbohydrate epitopes.

An excellent example is the developmentally regulated expression of plasmalemma- and cell wall-associated AGPs in root tips. The JIM13 monoclonal antibody, which binds an AGP epitope competitive with β–D-Glc*p*UA-(1→3)-α-D-Gal*p*UA-(1→2)-L-Rha trisaccharide, labels particular cells during pattern formation in vascular tissue. The labeling pattern is somewhat species specific, however, since JIM13 labels cells associated with xylem in carrot, radish, pea, and Arabidopsis and cells associated with phloem in onion and maize, and exhibits lesser variations in the details of labeling within these groups of dicotyledons and monocotyledons (Casero et al., 1998; Šamaj et al., 1998).

Dolan et al. (1995) very graphically described the pattern for Arabidopsis, in which the JIM13 AGP epitope appears in a single cell (the metaxylem initial) in a cross-section cut just above the quiescent center and then spreads nonclonally to neighboring cell files farther up the root (Fig. 2). These observations are consistent with an important link between development and cell position. An interesting modification of JIM13 expression occurs in Arabidopsis mutated in *scarecrow*, a gene that encodes a putative transcription factor. The normal asymmetric division of the cortex and endodermal initial (Fig. 2) is disrupted in this mutant, resulting in a primary root in which the cortex (which normally lacks JIM13 epitope) and the endodermis (which normally expresses JIM13 epitope) are replaced by a single cell layer between the epidermis and pericycle. This mutant cell layer stains with JIM13, which, together with other evidence, indicates the presence of a combination of characteristics that are normally expressed either only in the cortex or only in the endodermis (Di Laurenzio et al., 1996).

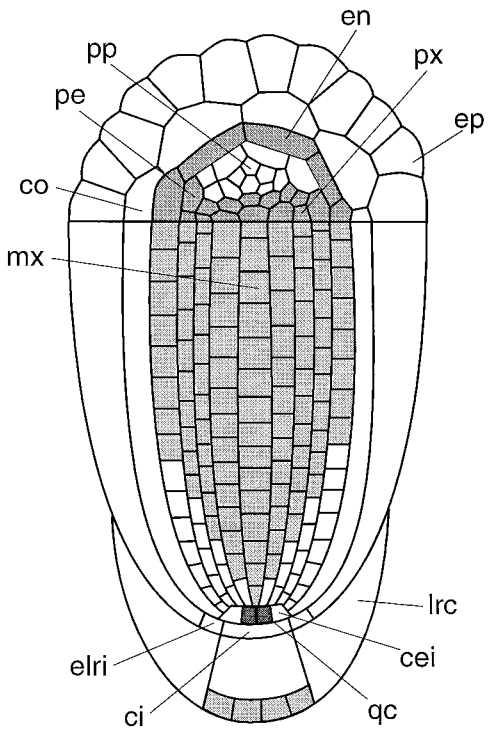

Figure 2. Pattern of AGP expression (shades of gray) detected by JIM13 antibody in Arabidopsis root tip. The JIM13 epitope is not expressed in the quiescent center (qc) but is expressed in a single cell, the metaxylem initial, just above the quiescent center. Moving upward from the metaxylem initial, expression continues in the metaxylem (mx) and spreads outward until protoxylem elements (px), pericycle cells at the xylem pole (pe), and endodermis (en) become labeled. The most distal of the columella cells in the root cap are also labeled by JIM13. Other cells present but not labeled by JIM13 are the cortex (co), protophloem element (pp), epidermis (ep), lateral root cap (lrc), cortex and endodermal initial (cei), columella initials (ci), and epidermis and lateral root cap initial (elri). Reproduced with permission from Dolan et al. (1995).

SEVERAL AGPS ARE EXPRESSED IN PISTILS AND FUNCTION IN FERTILIZATION

Resulting from the most sustained effort in AGP research, five AGPs and AGP-like molecules from the style and stigma of *Nicotiana alata* have been characterized, and cDNAs encoding their core polypeptides have been cloned (Sommer-Knudsen et al., 1997). The extent and specificity of expression in the pistil vary considerably among these five molecules. Transcripts of *AGPNa1* are expressed in the style but also in other organs. Expression of *AGPNa2* is high in cell cultures and very low, but detectable, in styles. Expression of *AGPNa3* occurs exclusively in pistils and most abundantly in the stigma (Fig. 3A). Although classified as Pro rich, the proteins encoded by *NaPRP4* and *NaPRP5* have several features in common with AGPs and are highly expressed in the transmitting tract of the style.

The core polypeptides of the transmitting tissue-specific AGPs TTS-1 and TTS-2 of *Nicotiana tabacum* are very similar to each other, and TTS-1 is 96.9% identical to *Na*PRP4. The mRNA and protein levels of TTS are maximal from near anthesis until several days after pollination (Cheung and Wu, 1999). Transcripts of *TTS-2* are very abundant in transmitting tract tissue (Fig. 3), and immunocytochemistry with antibodies directed against the TTS-1 core polypeptide confirm an abundance of the protein in the extracellular matrix of the transmitting tissue (Cheung et al., 1993).

The abundance of these AGPs in the pistil, the binding of TTS proteins to pollen tubes, the uptake of *Na*PRP5 by

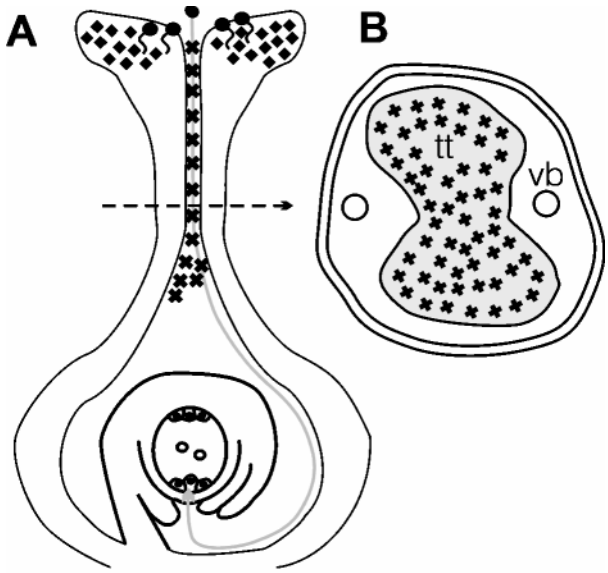

Figure 3. Tobacco pistil in longitudinal (A) and cross-section (B). Stigma-specific *AGPNa3* transcripts (♦) and transmitting tract-specific *TTS-2* transcripts (**x**) were visualized by in situ hybridization with a ^{32}P-labeled *AGPNa3* cDNA probe (Du et al., 1996) and a ^{35}S-labeled antisense RNA probe (Cheung et al., 1993), respectively. The gray line in A indicates the pollen tube growth path to the embryo sac. tt, Transmitting tract; vb, vascular bundle.

pollen tubes, and other observations have led to the hypotheses that pistil AGPs function in recognition, adhesion, pollen tube nutrition, pollen tube guidance, and other similar roles (Sommer-Knudsen et al., 1997; Cheung and Wu, 1999). Transgenic tobacco plants expressing antisense *TTS* mRNA displayed significantly reduced levels of both *TTS* transcripts and proteins and—in consequence—reduced growth of pollen tubes and reduced fertility. Plants transformed to constitutively express *TTS* accumulated the polypeptide in all vegetative and floral tissues, but glycosylation of these polypeptides was abnormally low everywhere except in the style. Most of these transgenic plants appeared normal. Plants transformed to constitutively express *Agamous*, a gene regulating gynoecial development, had abnormal sepals that expressed glycosylated TTS proteins and supported pollen germination and growth. These observations were interpreted as evidence that *Agamous* regulates the expression of both the TTS polypeptide and the glycosyltransferases that glycosylate it (Cheung and Wu, 1999).

PERTURBATION OF AGPS BLOCKS TIP GROWTH BUT NOT EXOCYTOSIS IN POLLEN TUBES OF SOME SPECIES

Fertilization in angiosperms relies on directed tip growth of pollen tubes through the style to reach the embryo sac. Immunolocalization shows that the cell wall behind the tip of *N. tabacum* (Li et al., 1995), lily (*Lilium longiflorum* L.; Jauh and Lord, 1996), and *N. alata* (Ferguson et al., 1999) pollen tubes contains AGPs, often appearing in circumferential bands that may correlate with the pulsatory growth sometimes exhibited by pollen tubes. The presence of AGPs at the tip of pollen tubes appears to be species dependent, however, since monoclonal antibodies detect AGPs at the tip of pollen tubes of lily but neither *N. tabacum* nor *N. alata*.

Perturbation experiments with Yariv phenylglycosides have shown that AGPs are involved in tip growth of lily pollen tubes. Yariv phenylglycosides form a class of synthetic, chromophoric molecules of the general structure 1,3,5-tri-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene. The β-glucosyl Yariv phenylglycoside (β-D-Glc)$_3$ and β-galactosyl Yariv phenylglycoside (β-D-Gal)$_3$ bind and precipitate AGPs, but α-galactosyl Yariv phenylglycoside (α-D-Gal)$_3$ and β-mannosyl Yariv phenylglycoside (β-D-Man)$_3$ do not and thus serve as negative controls. Long used as tools in the purification and detection of AGPs, Yariv phenylglycosides have recently become facile tools for probing AGP functions (Nothnagel, 1997). When applied to lily pollen tubes growing in vitro, (β-D-Glc)$_3$ causes growth to decelerate and stop within 5 to 10 min. If (β-D-Glc)$_3$ is removed from the medium within 1 to 2 h, growth resumes by formation of a new growing tip along the flanks of the original tip. When injected into the hollow style of lily, (β-D-Glc)$_3$ reduces the number of pollen tubes growing into the style and also reduces fertilization (Jauh and Lord, 1996). In vitro growths of pollen tubes of *Zea mays* and *Annona cherimoya* are similarly inhibited by (β-D-Glc)$_3$, but those of Arabidopsis, *Aquilegia exima*, and *N. tabacum* are unaffected (Roy et al., 1998).

The mechanism of pollen tube tip growth involves massive exocytosis of pectins and other polymers needed for the continuous assembly of new cell wall on the advancing tip. Within 5 min after the application of (β-D-Glc)$_3$, callose deposition is evident at the tip, and within 1 h, abundant matrix material accumulates between the plasmalemma and the pectin wall at the tip. Secretory vesicles that are abundantly present at the tip of control pollen tubes (Fig. 4A) are also present in (β-D-Glc)$_3$-treated tubes (Fig. 4B) (Jauh and Lord, 1996; Roy et al., 1998). Cytoplasmic [Ca^{2+}] in control pollen tubes exhibits a tip-focused gradient such that [Ca^{2+}] ≅ 500 nM immediately behind the tip but declines to less than 300 nM just 20 μm behind the tip. Within 1 to 2 min after adding (β-D-Glc)$_3$ to the medium, cytoplasmic [Ca^{2+}] begins to rise, exhibiting a tip-focused gradient of lesser slope, until [Ca^{2+}] ≅ 1 μM throughout the apical 75 μm (Roy et al., 1999). These effects of (β-D-Glc)$_3$ are unique compared with those of caffeine and other inhibitors that abruptly block pollen growth but always do so with an accompanying cessation of exocytosis and dissipation of the intracellular [Ca^{2+}] gradient. Roy et al. (1998, 1999) interpreted these observations as indicating that perturbation of AGPs interferes with wall assembly at the tip, thereby blocking growth, and also increases net Ca^{2+} influx at the tip, thereby elevating [Ca^{2+}] and maintaining exocytosis.

ACTION OF AGPS DURING SOMATIC EMBRYOGENESIS

The function of AGPs in somatic embryogenesis was first suggested by immunocytochemical studies with JIM4 and

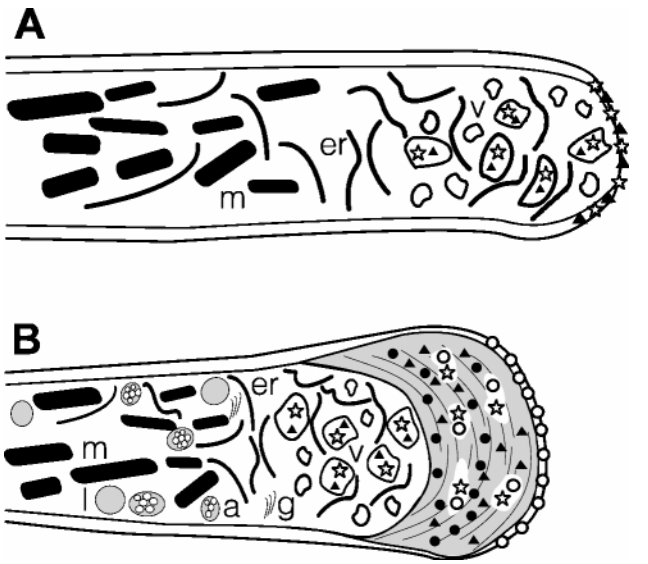

Figure 4. Tip region of lily pollen tube grown in vitro in control conditions (A) and after treatment with $(\beta$-D-Glc$)_3$ (B). Growth of the tube occurs at the tip via exocytosis of polymers (esterified pectins [▲], AGPs [☆], and others not shown) that assemble to a thin, pectin-rich wall at the tip. Growth is inhibited by $(\beta$-D-Glc$)_3$, but exocytosis continues and deposits wall components in the area (shaded) between the plasmalemma and the cell wall. The accumulated wall material is heterogeneous, with fibrillar regions that are rich in pectins (esterified [▲] or low-esterified [●]) and electron-translucent areas that are rich in callose (○) and AGPs (☆). The cytoplasm near the tip of the control and $(\beta$-D-Glc$)_3$-treated pollen tubes contains abundant secretory vesicles (v) and endoplasmic reticulum (er) with mitochondria (m) farther back, but lipid droplets (l), amyloplasts (a), and Golgi bodies (g) have moved forward in the treated pollen tube (Roy et al., 1998). Pectins, AGPs, and callose also occur in the wall back from the tip but are not marked with symbols.

JIM8 antibodies, which recognize AGP epitopes. In both carrot and maize cultures, the JIM4 epitope is particularly abundant in the outer cells and extracellular layers of compact clumps of embryogenic callus, but non-embryogenic cells are completely devoid of this epitope (Kreuger and van Holst, 1996; Šamaj et al., 1999). Applied to carrot cell cultures, JIM8 labels a subpopulation of cells in the path to somatic embryogenesis, and macromolecules produced by these cells are considered to provide a signal essential for progression to embryo formation (McCabe et al., 1997).

Other investigations of AGP function in somatic embryogenesis have involved manipulation of the amount or types of AGPs in the culture medium. These two factors have been modified by precipitating AGP molecules with either $(\beta$-D-Glc$)_3$ or anti-AGP antibodies (Thompson and Knox, 1998; Butowt et al., 1999), or by adding exogenous AGPs isolated from plant tissues of high embryogenic capacity. The latter approach, in particular, has been reported to enhance the efficiency of somatic embryogenesis in carrot, cyclamen, and Norway spruce (Kreuger and van Holst, 1996).

The mechanism of action of AGPs in somatic embryogenesis remains uncertain, although some convergence with other factors that influence somatic embryogenesis has been recently achieved. Extracellular endochitinases are secreted into the medium of embryogenic cell cultures and have been shown to play an important role in somatic embryo development of both gymnosperms and angiosperms. A search for plant substrates for this endochitinase led to the isolation and identification of AGPs containing GlcNAc. The addition of AGPs isolated from immature carrot seeds increased the number of somatic embryos formed in carrot protoplast cultures, and preincubation of the seed AGPs with endochitinase further increased the frequency of embryo formation. This observation suggests that oligosaccharides released from AGPs by the action of endochitinase act as signal molecules stimulating the development of embryos (van Hengel, 1998).

PERTURBATION OF AGPS ALTERS THE PROGRESS OF CELL DIVISION, EXPANSION, AND DEATH IN UNDIFFERENTIATED CELL CULTURES

The functions of AGPs in undifferentiated protoplasts and cultured cells of several species have been investigated by using monoclonal antibodies or Yariv phenylglycosides to bind, aggregate, and presumably inactivate AGPs. Addition of JIM13 antibody to the medium of sugar beet protoplasts inhibited proliferation but did not affect short-term viability (Butowt et al., 1999). With all species thus far reported, the growth of cell cultures is halted by the application of $(\beta$-D-Glc$)_3$ or $(\beta$-D-Gal$)_3$, which bind AGPs, but is unaffected by application of $(\alpha$-D-Gal$)_3$ or $(\beta$-D-Man$)_3$, which do not bind AGPs. Under moderate treatment conditions, the inhibition of growth is reversible upon washing $(\beta$-D-Glc$)_3$ or $(\beta$-D-Gal$)_3$ from the medium. The mechanism of growth stoppage involves inhibition of cell division in some cell cultures (Nothnagel, 1997; Thompson and Knox, 1998) but inhibition of cell expansion in others (Willats and Knox, 1996). Inhibition of cell expansion is also evident in Arabidopsis seedlings, in which $(\beta$-D-Glc$)_3$ causes a bulging of root epidermal cells (Willats and Knox, 1996) that mimics the phenotype of *reb1-1*, an Arabidopsis mutant that expresses a reduced level of root AGPs (Ding and Zhu, 1997). With some particularly sensitive cell lines or at somewhat higher treatment concentrations, $(\beta$-D-Glc$)_3$ or $(\beta$-D-Gal$)_3$ kills cells within 2 to 3 d (Nothnagel, 1997; Gao and Showalter, 1999).

Gao and Showalter (1999) found that suspension-cultured Arabidopsis cells die within 3 d of application of $(\beta$-D-Gal$)_3$, and this death occurs not by simple necrosis but by induction of programmed cell death. The hypothesis that certain AGPs mark cells destined for programmed cell death had been previously suggested on the basis of immunocytochemical detection of highly regulated AGP expression during xylem development, a process concluding with programmed cell death (Dolan et al., 1995; Gao et al., 1999). Current models suggest that an integrated control system regulates both the cell cycle and programmed cell death, and the results observed upon perturbation of AGPs in plant cell cultures suggest that AGPs may play a role in this integrated control system. Gao and Showalter (1999) suggested that $(\beta$-D-Gal$)_3$ disrupts plasmalemma-cell wall connections and thereby activates a signal transduction

pathway that directs the cell away from cell cycle progression and toward programmed cell death.

PRACTICAL APPLICATIONS OF AGPS

Although scientists have not been particularly successful in identifying the precise functions of AGPs in plants, greater success has been achieved in finding uses for AGPs harvested from plants. The most important AGPs in this regard are those comprising the principal mass of gum arabic, an exudate collected from *Acacia senegal* trees (http://www.redbay.com/plthomas/arabic/). The Ancient Egyptians used gum arabic as an adhesive when wrapping mummies and in mineral paints when making hieroglyphs. Although gum arabic has also been used as an adhesive in modern times, the most important applications are in the food, pharmaceutical, cosmetic, and lithography industries. Gum arabic has the unique combination of being an excellent emulsifier while having low viscosity even at high concentrations. These properties make it very useful as a flavor encapsulator, an agent to prevent Suc crystallization in confections, and especially as a stabilizer of emulsions, including the citrus oil emulsion concentrates used in soft drinks such as Mountain Dew (Serpe and Nothnagel, 1999).

Other AGPs or AGP-related polysaccharides in certain medicinal herbs have been shown to have beneficial medical effects such as the activation of the complement system. Bioassays after fragmentation of these plant molecules commonly reveal that the immune-modulating activity resides in type II arabinogalactan chains (Fig. 1). An example is Juzen-taiho-to, a traditional Sino-Japanese herbal medicine which, upon ingestion, interacts with intestinal Peyer's patches where T lymphocytes are activated to produce growth factors that increase proliferation of bone marrow cells. This immune-modulating activity has been traced to macromolecules from rhizomes of *Atractylodes lancea* DC, a component of Juzen-taiho-to. One of the active macromolecules interacts strongly with $(\beta\text{-D-Glc})_3$, indicating that it is an AGP, and enzymic degradation of its type II arabinogalactan chains leads to loss of its pharmacological activity (Yu et al., 1998).

OUTLOOK

Analysis of AGP functions through the generation of transgenic plants is currently under way in several laboratories and will certainly dominate AGP research in the immediate future. Experiments involving antisense or gene knockout approaches to generate plants with low AGP levels are likely to yield informative results. Meaningful interpretation of transgenic experiments involving elevated expression of a native or exogenous AGP core polypeptide will require diligent analysis of the glycosylation state of the expressed product.

NOTE ADDED IN PROOF

The complete structure of the GPI anchor on pear AGPs has been solved (D. Oxley, A. Basic [1999] Proc Natl Acad Sci USA **96:** in press) and is as shown in Figure 1 with a partial β-D-Gal-(1→4) substitution on the Man adjacent to the GlcN.

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