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Isolation and Chemical Characterization of Yariv Beta Glucosyl Reagent –Positive Macromolecules Released by *Gymnocolea inflata* When Leaf and Branch Development are Desuppressed

BY

NAHED SALAMA

A dissertation submitted to the Graduate Faculty in Biology, The City University of New York in partial fulfillment of the requirement for the degree of Doctor of Philosophy

2000

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Isolation and Chemical Characterization of Yariv Beta Glucosyl Reagent – Positive Macromolecules Released by *Gymnocolea inflata* When Leaf and Branch Development are Desuppressed

By
Nahed Salama

Advisor: Professor D. V. Basile

The presence of ammonium ion in the nutrient medium of axenically cultured plants of *Gymnocolea inflata* (Huds.) Dum. resulted in desuppressed cell proliferation in otherwise suppressed leaf and branch primordia. Antagonists of hydroxyproline-protein synthesis had produced these changes in the pattern of cell proliferation in relation to leaf and branch development earlier in four other species of leafy liverworts.

Since molecules released into the culture medium correlated with both ion- and antagonist-induced desuppression were stained by Yariv β-glucosyl reagent, a reagent diagnostic of arabinogalactan-proteins (AGPs), it was hypothesized that there was a causal relationship between the release of AGPs and desuppressed leaf and branch development. Because Yariv β-glucosyl reagent sometimes binds to macromolecules lacking an hydroxyproline-protein component, it was deemed necessary to chemically characterize the Yariv reagent-positive molecules as a test of this hypothesis.

Two monoclonal antibodies (McAbs) developed against Yariv reagent-positive molecules released by desuppressed *G. inflata* plants were used to affinity purify the molecules they specified away from other components of their culture medium. The affinity purified molecules were subjected to SDS-PAGE, amino acid, as well as carbohydrate composition and linkage analysis.

The results of all the analyses showed the Yariv reagent-positive molecules specified by the McAbs to be *bona fide* hydroxyproline-containing AGPs. The amino acid composition of the protein moieties of the AGPs released correlated with desuppressed development was distinctly different from those released or extractable from plants with normally suppressed leaf and branch development. This finding is interpreted to mean that it was the altered composition of the AGPs that resulted in their function being impaired.

By extrapolation, the results of this investigation support two related hypotheses. The first is that the Yariv β-glucosyl reagent-positive molecules released when suppressed leaf and branch development in *G. inflata* and other liverworts are desuppressed AGPs. The second is that certain AGPs play a pivotal role in determining where and when during the course of plant development cell proliferation is suppressed or desuppressed.

TO MY HUSBAND AND DAUGHTER

WITH MY GRATITUDE

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I am especially grateful to all of my professors, friends and colleagues who interrupted their busy lives to guide, advise and help me throughout my graduate experience.

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Personal debts can never be adequately acknowledged, my husband and my daughter gave me unstinting support in more ways than one, their detailed commentary and effort to make this manuscript are forever appreciated.

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Introduction

Some time ago, it was discovered that whenever ammonium ion was a component of any of several macronutrient salt formulations used to prepare media for the axenic culture of a leafy liverwort, *Gymnocolea inflata* (Huds.) Dum., a profound departure from their normal pattern of development was observed (Basile and Basile 1980). Specifically, plants that typically produce only two rows of leaves and rarely branch, developed three rows of leaves and branched much more frequently than when cultured in the absence of ammonium ion or when growing in their natural habitats. In addition, the stems, branches, and leaves were much more robust because of an obvious increase in the number of cells that composed them.

Because the changes in leaves' number and arrangement and branching types and frequency were consistent and characteristic effects produced by antagonists of hydroxyproline-containing proteins on morphogenesis of four other species of leafy liverworts (for review of pertinent literature, see Basile and Basile 1980), it was hypothesized that ammonium ions were somehow antagonizing normal hydroxyproline-protein synthesis and function in this plant. Nothing was found in the existing scientific literature that suggested that ammonium ion would or could interfere with hydroxyproline synthesis.

Nevertheless, evidence in support of the hypothesized effect was the finding that there was significantly less peptide-bound hydroxyproline in the acid hydrolyzed cell wall fractions of the plants cultured in ammonium-containing

media (Basile and Basile 1980). The nature of the hydroxyproline-protein being altered in correlation with changes in leaf and branch development was not known at the time. Subsequent studies, however, suggested that the hydroxyproline protein being altered might belong to a large class of hydroxyproline-containing glycoproteins and proteoglycans, the arabinogalactan-proteins (AGPs) (Basile et al. 1986; Basile and Basile 1987, 1990, 1993).

When extracts from *Gymnocolea inflata* plants cultured in the absence or presence of ammonium ion were compared by cesium chloride density gradient analysis and probed with Yariv β-glucosyl reagent, a reagent considered diagnostic for AGPs, there was statistically significant differences in the Electrophorotic and buoyant density range in the Yariv reagent-positive fractions, (Figure 1 & 2). Those from plants cultured in the absence of ammonium had a buoyant density range of from 1.50 to 1.66. Those cultured in the presence of ammonium had a range extending only to 1.62. Apparently there was either a loss or a release of the higher buoyant density Yariv reagent-positive molecules induced by the presence of ammonium in the medium. When the aqueous culture media in which the plants had been growing were similarly compared, the higher buoyant density fractions were found to be associated with ammonium-containing medium. In short, it appeared that ammonium caused some high buoyant density, Yariv β-glucosyl-positive molecules to be released into the culture medium by these plants correlated with the desupression of leaf and branch development.

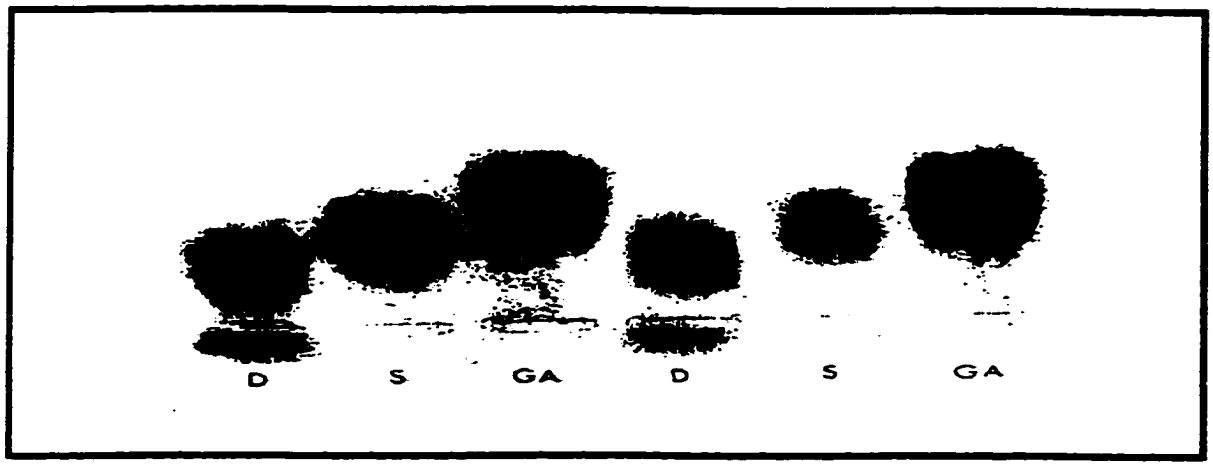


Figure 1:

AGPs from plants with desuppressed (D) and suppressed (S) leaf development and Gum Arabic (GA) as a standard, compared by glyoxyl gel electrophoresis stained with Yariv's β-glucosyl agent (from Basile & Basile, 1993).

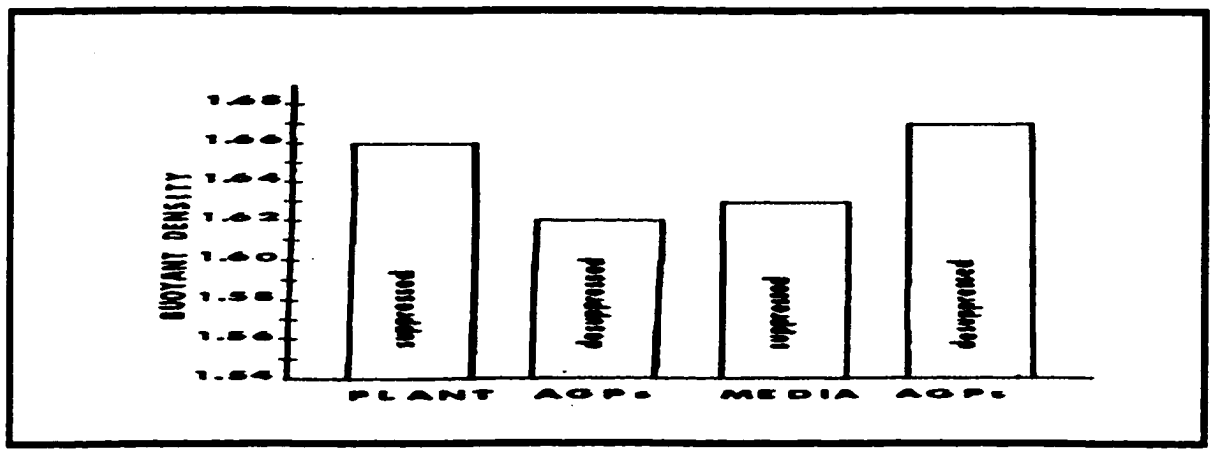


Figure 2:

A comparison of mean buoyant densities of AGPs extracted from suppressed and desuppressed plants, as well as from the media in which they were axenically cultured. An ANOVA based on six experiments indicated that the differences in mean buoyant density between suppressed and desuppressed plants, and between the media on which they were growing are statistically significant at 95% probability (from Basile and Basile, 1994).

Because Yariv β-glucosyl reagent is considered to be diagnostic for arabinogalactan- proteins (Clarke et al. 1979; Nothnagel 1997), it was tentatively concluded that ammonium ion was exerting its morph-genetic influence by virtue of altering the synthesis and thereby the function of one or more AGPs). By extrapolation, it was further hypothesized that one or more AGPs played a pivotal role in the correlative control system mediating the spatial and temporal control of growth suppression (Basile and Basile 1993) (Figure 3).

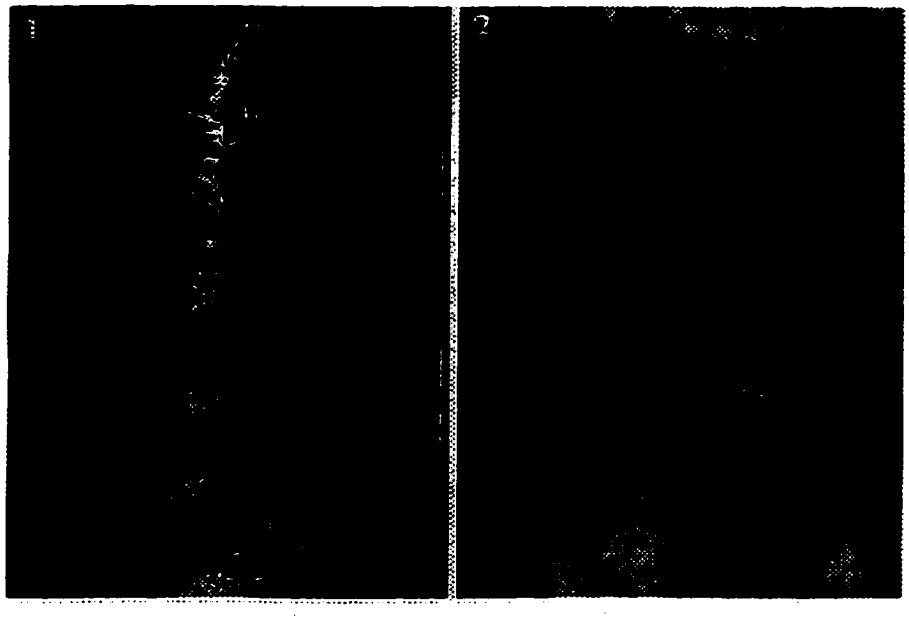


Figure 3, (1 and 2):
Scanning electron micrographs of *Gymnocolea inflata*, at the same magnification. (1) Habit of plant cultured on ammonium-free media. The small and well-spaced leaves arranged in two rows on an un-branched stem are characteristic of field grown plants. (2) Habit of plant after transfer from ammonium-free to ammonium-containing medium. Development with respect to the size and number (of rows) of leaves and the frequency of branching has been "desuppressed" (Basile et al., 1999).

Unfortunately, it is now known that Yariv β-glucosyl reagent can, sometimes, react with complex carbohydrates that are not AGPs (Serpe and Nothnagel, 1995) and also with proteoglycans that lacked measurable levels of hydroxyproline in its protein moiety (Baldwin et al. 1993; Hillestad et al. 1977; Mollard and Joseleau 1994). Since the bulk of the evidence linking the desuppression of leaf and branch development in leafy liverworts was obtained from experiments utilizing antagonists of hydroxyproline synthesis, it is essential to determine whether the Yariv reagent-positive molecules being released into the culture media were indeed hydroxyproline-containing AGPs.

In order to isolate, identify, and characterize the molecules that appear to be released from plants correlated with ammonium ion-induced changes in leaf and branch development, an attempt was made to develop monoclonal antibodies (McAbs) that were mono-specific for them.

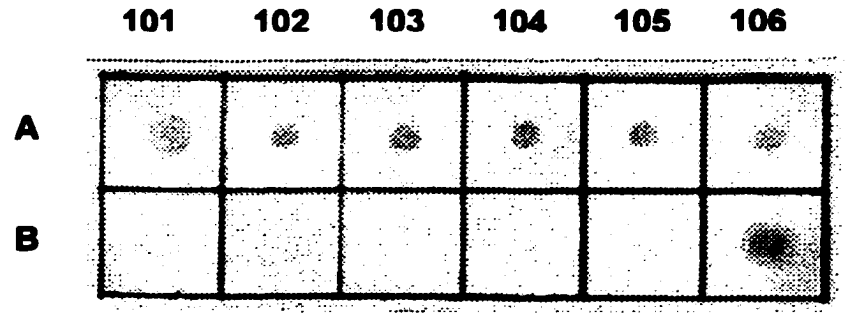


Figure 4:
Comparative dot blot analysis of pooled AGPs extracted from culture media that supported "desuppressed" leaf and branch development in row A, versus media promoting "suppressed" leaf and branch development in row B (Basile et al., 1999).

Five McAbs, were reported that bound Yariv β-glucosyl reagent-positive molecules and only appeared in the culture medium when the culture conditions were changed so as to induce increased cell proliferation in plants correlated with an experimentally induced increase in the size and number of leaves and branched produced (Figure 4) (Basile et al., 1999).

Two of these five McAbs were chosen to prepare affinity columns for the purpose of isolating and chemically characterizing the molecules they specify.

Reported here is the results of the chemical characterizations that show the molecules released into the medium and specified by these two McAbs, 101 and 103, are bona fide arabinogalactan-proteins.

LITERATURE READINGS

I. Introduction

Several recent discoveries have altered our view of the cell wall from that of a static structure to one in which the wall represents a virtual extension of the cytoplasm. Cell walls contain surface markers that foretell patterns of development and mark positions within the plant (Knox et al., 1989; knox, 1990; Pennell and Roberts, 1990). They contain components for signaling and communication by symplastic continuity through plasmodesmata (Robards and Lucas, 1990). Walls also maintain molecular continuity with the plasma membrane and cytoskeleton (Roberts, 1990). The firmer connections between the cell wall and plasma membrane also results from adaptation to osmotic stress (Zhu et al., 1993 a & b). Signals from the cell wall elicited by insect predation induce the production of defense molecules (Ryan, 1990), and shells of protein and lignin are formed in response to invading fungal and bacterial pathogens (Vance et al., 1980; Bowles, 1990).

Sections through a meristem show cells as an organized cluster of protoplasts partitioned by a matrix. For cells to develop their functional form and individuality, they must elongate and differentiate.

Coordinate expansion and differentiation of the individual cells is achieved by alteration of the structure of the developing wall, the mechanical determinant of cell form.

The plant cell wall is composed of many complex interacting molecules. The scope of this study will be primarily limited to the consideration of a particular class of cell surface macromolecules, the *arabinogalactan proteins (AGPs)* as well as other molecules that they may be related to AGPs. Various lines of evidence indicate that these proteoglycans are involved in several aspects of plant development.

II. Definition of AGPs

Arabinogalactan-proteins are proteoglycans (Fincher et al., 1983), or glycoproteins (IUB-IUPAC Joint Commission on Biochemical Nomenclature, JCBN, 1982 and 1987) that are widely distributed in the plant kingdom.

The term *"arabinogalactan"*, as explained by Nothnagel 1997, specifies certain structural characterization of the carbohydrate portion of AGPs.

The *"galactan"* portion of the name requires that the major carbohydrate portion(s) of the macromolecule must have a polysaccharide backbone or framework in which galactosyl residues are important components.

The *"arabino"* portion of the name requires that arabinosyl residues must also be important components, either as substituents on the galactan framework or as residues within the galactan framework itself.

However, further specification is required since several distinct polysaccharides that are properly named "*arabinogalactan*" have been found in plants and microorganisms (Clarke et al., 1979a&b; Daffe et al., 1993). AGPs

are also considered β-lectins by virtue of their ability to bind with β-D-glucosyl Yariv reagent in a selective manner (Showalter, A.M., 1993).

Although some variation occurs, AGPs typically contain high portions of carbohydrates and only 2-10%protein by weight (Clarke et al., 1979a; Showalter and Varner, 1989). The carbohydrate moiety consists of mainly arabinose and galactose with minor amounts of other sugars including uronic acids. Linkage analysis indicates a backbone of (1→3)-β-D-galactan, which is connected to core polypeptide. Side chains, of (1→6)-β-D-galactan, are attached at carbon atom 6 of some of the galactosyl residues in the backbone. In turn, these are substituted with arabino-furanose and other less abundant monosaccharides, often as terminal residues (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 are reported for several AGPs (Qi et al., 1991; Kieliszewski and Lamport, 1994).

The structure elucidation studies of extracted cell wall polymers AGPs (Carpita, 1989; Gane et al., 1994) have some times revealed the presence of 3-, 6- and 3,6- linked galactopyranosyl residues that are characteristic of AGPs (Figure 5). However, Nothnagel (1997) reported that these investigations did not establish whether these galactopyranosyl residues were linked to polypeptides thus establishing them as AGPs or to something else.

Aspinal (1973) classified plant arabinogalactans as either (type I) or (type II). Type I arabinogalactans have a linear (1→4)-β-D-galactan backbone with

arabinose oligosaccharide' side chains. Type II arabinogalactans have a highly branched framework consisting of a (1→3)-β-D-galactan backbone with (1→ 6)-β-D-galactan side chains attached at carbon atom 6 of some of the galactosyl residues in the backbone. Most of the arabinosyl residues in type II arabinogalactans are attached at carbon 3 of some of the galactosyl residues in the side chains (see Figure 5).

Controversy existed as whether AGPs was part of the cell wall or simply present as soluble molecules in the cell wall space (Fincher et al., 1983; Bacic et al., 1988). Biochemical studies (Serpe and Nothnagel, 1994, 1995) have shown that at least some AGPs are tightly bound to the cell wall.

Another class of the plant cell surface macromolecules, which is close structure wise to AGPs, is extensin. These macromolecules are cell wall proteins belonging to the larger class of hydroxyproline-rich glycoproteins (HRGPs). Although the protein portions of extensins and most AGPs are both rich in hydroxyproline (Hyp), these two macromolecules are usually clearly distinguishable by several other characteristics. Isoelectric points tend to be basic for extensins and acidic for AGPs. Although AGPs have large glycan side chains, extensins have many short carbohydrate side chains consisting of arabinose oligosaccharides (monosaccharides to tetra-saccharides) attached to Hyp, and galactose monosaccharides attached to Ser (Fincher et al., 1983; Showalter an Varner, 1989; Showalter, 1993).

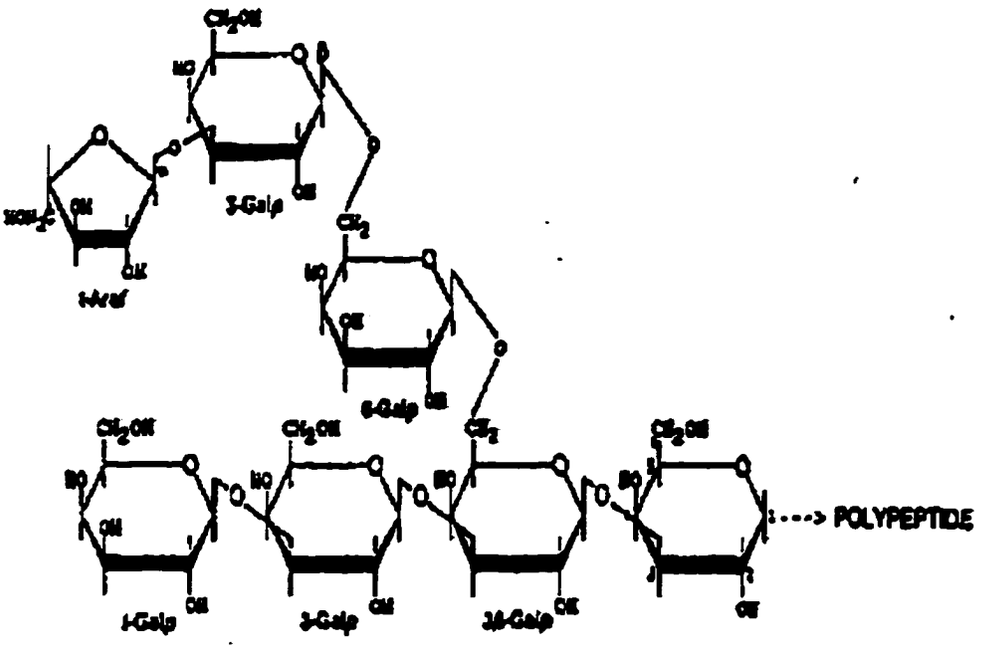


Figure 5: Hypothetical structure of a portion of the characteristic glycan of AGPs. The (1→3)-β-D-galactan backbone is connected to the core polypeptide. Side chains of (1→6))-β-D-galactan are attached at carbon atom 6 of some of the galactosyl residues in the backbone. Most of the α-L-arabinosyl residues are attached at carbon atom 3 of some of the galactosyl residues in the side chains. The sugar residues are provided with abbreviated labels that indicate the linkage arrangement. Linkage at carbon atom 1 is implied in this abbreviated labeling. Galactosyl residues in AGPs are typically present in a six-member ring, or pyranossyl, form, which is indicated by a *p* at the end of the abbreviated label. Arabinosyl residues in AGPs are often present in a five-member ring, or furanosyl form, which is indicated by an [*f*] at the end of the label . Other sugars, such as glucuronic acid and rhamnose, are also attached to the (1→6)-β-D-galactan side chains in many AGPs. Although illustrating some of the glycosyl linkages found in AGPs, this hypothetical structure is much smaller than the glycans of 30-150 sugar residues that are typically attached to a number of amino acid residues in the AGP core polypeptide (Nothnagel, E. 1997).

III. Probes of AGPs

Two types of powerful tools are used to unravel and typify AGPs: The polyclonal or monoclonal antibodies and the Yariv Phenylglycosides.

1. Antibodies:

Many groups of investigators, such as Anderson et al., 1984; Kikuchi et al., 1993; and Matsuoka et al., 1995, have produced antibodies against AGPs. Almost all these antibodies were, at least partly directed against the sugar moiety of the AGPs, specially arabinosyl and galactosyl residues. Furthermore, their binding could be inhibited by mono-and disaccharides (Anderson et al., 1984; Pennell et al., 1989) or by more complex oligo-and polysaccharides, such as gum arabic and AGP-like molecules from larch and wheat (Anderson et al., 1984; Knox et al., 1989; Pennell et al., 1989). Among antibody preparations, only five were reported to bind to protein rather than carbohydrates. Deglycosylated proteins were used as the immunogen in three of these cases (Gleeson et al., 1989; Lind et al., 1994; Sommer Knudsen et al., 1996), whereas the other two involved use of a polypeptide (John and Keller, 1995).

Identification of carbohydrate epitopes has been a challenging problem. This problem was avoided in a few cases by using a conjugated carbohydrate hapten of defined structure as the immunogen (Misaki et al., 1988; Northcote et al., 1989; Kikuchi et al., 1993). More often the immunogen was an AGP, a mixture of AGPs, or in some cases of monoclonal antibody production, membrane vesicle or whole protoplasts. Consequently, the epitopes of some

monoclonal antibodies remain unknown or have been characterized only to the extent of showing that carbohydrate is an important component of the epitope.

Monoclonal antibodies have been used to localize AGP epitopes in different tissues. The JIM4 antibody (Knox et al., 1989) was obtained after immunization with carrot protoplasts. The antibody recognized AGP epitopes from the medium of suspension-cultured carrot cells and gum arabic, as well as others, possibly non-AGP or AGP-like molecules. It was demonstrated that the epitope was located at a specific set of cells during the development of the carrot root apex. The expression of the antigen occurred well before pattern formation was visible, and was restricted to two small groups of cells in the future vascular bundle and was maintained through the root system. The epitope was also found in the vascular tissue of the cotyledons, exclusively on the outer side of plasma membrane. The same epitope has been localized to somatic embryos of carrots (Stacy et al., 1990). Here, the epitope was restricted to the protoderm of early somatic embryos and to the pro-vascular tissue of the root apex and the cotyledons. The JIM4 epitope therefore seemed to accompany pattern formation during the differentiation of the vascular tissue.

The JIM8 antibody was obtained after immunization with sugar beet protoplasts (Pennell et al., 1991) and was selected for its binding with gum arabic. The antibody bound to AGP epitopes from suspension-cultured carrot cells, but also to rhamnogalacturonans, which are non-AGP cell wall components, and to non-AGP gums. The epitope was localized in oilseed rape flowers. During flower development, the epitope was located on the sexual

organs and appeared progressively along several tissues up to the egg cell and the sperm cell. The epitope was present on specific cell types only during a short period, after which it disappeared. The JIM8 epitope was also expressed during the early stages of embryogenesis in the whole embryo, and at later stages only in the suspensor cells. The same epitope was located on a specific set of single cells in embryogenic cells suspensions of carrot (Pennell et al., 1992). These data suggested a role for the JIM8 epitope at or near the phases of the plant cycle where embryogenic cells are formed.

The monoclonal antibodies JIM 13 and 15 were raised against two AGP fractions isolated from the conditioned medium of an embryogenic carrot cell suspension and showed to be specific for AGPs (Knox, 1993). JIM 13 and 15 recognize complementary patterns in the developing carrot root. JIM 13 recognized the epidermal cells and the future xylem axis, but not the cortex. The JIM 15 epitope was complementary to this pattern and bound to the cortex, but not to the epidermis, with a small overlap with JIM 13 in the stele. The same epitope were found on cells in the developing xylem of maize, where they seemed to be markers of those cells which were committed to programmed cell death during xylem formation (Schindler et al., 1995).

Monoclonal antibodies have been used for detecting epitopes on the cell surface (Knox et al., 1989; Pennell et al., 1991; Schindler et al., 1995), or extra-cellular in cell walls or in the medium of suspension cultures (Kikuchi et al., 1993; Knox et al., 1991; Schindler et al., 1995), or extra-cellular in cell walls or in the medium of suspension cultures (Pennell et al., 1989; Knox et al., 1991; Li Y. et

al., 1992), or both simultaneously (Knox et al., 1991; Herman and Lamb, 1992; Pennell et al., 1992; Schindler et al., 1995). Occasionally epitopes have detected intracellular, predominantly in multi-vesicular bodies and/or associated with the vacuole (Herman and Lamb, 1992; Pennell et al., 1992; Schindler et al., 1995). With these antibodies not only the patterns of expression of membrane-bound AGP epitopes have been demonstrated, but also the intra- and extra-cellular presence of AGP epitopes. The significance of the finding that AGPs are found on both the cell membrane and in the cell wall is not clear, but it indicates that AGPs are very abundant in the surroundings of all cells.

The data shows that specific epitopes are present only on a limited number of cells or cell types during their differentiation and that some epitopes may show only transient expression. The outer side of cells contains sets of AGPs or AGP epitopes which can change of constitution during differentiation, meaning that cell lineage's will show subsequent appearance and disappearance of specific epitopes (Pennell et al., 1991; Knox J.P., 1993). The AGP epitopes may reflect a tissue pattern, which is determined by cell position. They also show extensive modulation of cell surface AGP epitopes during cell development (Knox J.P., 1990). The extensive control mechanism for the marking of cell position is an absolute necessity for the correct development of a multi-cellular organism.

This raises the question whether the different AGP epitopes are merely a result of differentiation or whether they can cause these events, or possibly both. Is differentiation accompanied by the formation of new AGP epitopes, resulting in

a change in the surface of the cells? or can newly formed AGP epitopes, after changing the surface direct the development of cells, possibly by an altered interaction with the neighboring cells? These intriguing questions of what came first, the AGP epitope or the differentiation, may be hard to answer. It must be clear, however, that only the detection of an epitope does not exclude molecules other than the AGP. How can we know that the localized epitopes are really belonging to AGPs? This may be irrelevant since the AGP epitopes may be located on AGPs, as well as related, but different molecules. If the epitope is essential for an activity, the rest of the molecule, whether AGP or not, may only be a vehicle for delivering the message. The observed modulation of epitopes might therefore be the result of extensive changes in AGPs, but also of totally different molecules, containing the same, modulated epitopes.

2. Yariv Phenylglycosides:

An important class of molecular probes investigating the AGPs, was discovered by Yariv et al., 1967. The general chemical name for this class of intensely re-colored probes (Figure 6) is 1,3,5-tri-(p-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene. Although the common names " Yariv antigens" and "Yariv reagents" have been widely used for these probes, the name Yariv phenylglycosides" seems preferable because it is structurally descriptive and avoids the implication of an antibody-like characteristic of AGPs.

The chemical synthesis of Yariv phenylglycoside, was originally reported by Yariv et al., 1962 and subsequently in somewhat more detail by Basile et al., 1989.

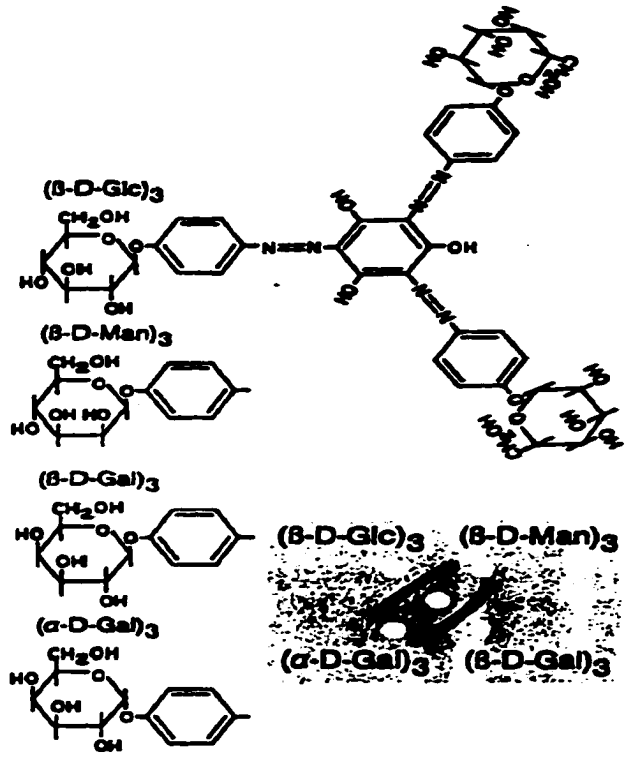


Figure 6:

Structures and AGP-precipitating activities of the most useful Yariv phenylglycosides. The chemical structure of $(\beta\text{-D-Glc})_3$, the Yariv phenylglycoside containing three β-D-glucoside arms, is shown at the top. Along the left side are shown the structures of the phenylglycoside arms that are present in the $(\beta\text{-D-Man})_3$, $(\beta\text{-D-Gal})_3$ and $(\alpha\text{-D-Gal})_3$ Yariv phenylglycosides . In the lower right corner the results of a double-diffusion precipitation assay are shown (Nothnagel and Lyon, 1986). Five wells were punched in a layer of agarose gel. An aliquot of rose CM-AGP was placed in the wells as labeled. As the AGP and Yariv phenylglycosides diffused toward each other, strong precipitation lines formed with the $(\beta\text{-D-Gal})_3$ and $(\beta\text{-D-Glc})_3$ Yariv phenylglycosides but not with the $(\alpha\text{-D-Gal})_3$ and $(\beta\text{-D-Man})_3$ Yariv phenylglycosides. Because of the subtle structural differences between these Yariv phenylglycosides, $(\alpha\text{-D-Gal})_3$ and/or $(\beta\text{-D-Man})_3$ are very useful as negative controls in experiments involving $(\beta\text{-D-Gal})_3$ and/or $(\beta\text{-D-Glc})_3$ (Nothnagel, E., 1997).

It has long been known that AGPs bind to Yariv antigens, synthetic molecules containing β-glycosides (Yariv et al., 1967; Clarke et al., 1978). The glycoside linkage in Yariv antigens has to be in the β-anomeric conjugation to react with AGPs. AGPs are sometimes also called β-lectins (Jermyn and Yeow, 1975; Clarke et al., 1978). Yariv phenylglycosides having sugars with cis-hydroxyl or other substitutions on C2 of the β-D-glycopyranosyl determinant do not bind to AGPs (Nothnagel and Lyon, 1986). For example, while $(\beta\text{-D-Glc})_3$, Yariv antigen can bind AGPs $(\beta\text{-D-Man})_3$ or $(\alpha\text{ -D-Gal})_3$, Yariv antigens do not react with AGPs. These latter Yariv antigens can serve as valuable controls to distinguish non-specific effects. Although the exact mechanism of the binding between Yariv antigens and AGPs is not understood, it is generally believed that the binding suggests the ability of AGPs to bind to certain β-linked glucans in the cell wall. Rohringer et al. (1989) reported that AGPs isolated form leek seeds were able to bind to components of the cell wall of wheat cells. It was speculated that the wheat cell wall components recognized by AGPs are β-linked bound sugars (Rohringer et al., 1989). Several β-linked polysaccharides are very abundant in plant cell walls. The most abundant one is cellulose 1,4-glucan. Other β-linked polysaccharides include callose, arabinogalactan, mannan and xylan (Aspinall, 1981). Structures similar to phenyl-β-glycosides might also be present in the cell wall polysaccharides and phenolics, so AGPs might be able to bind these cell wall components.

Solvent characteristics influence the interaction between AGPs and Yariv phenylglycosides. Perhaps of most practical importance is the effect of ionic

strength in aqueous solutions. Precipitation of the AGP-Yariv phenylglycoside complex does not occur in pure water but instead requires the presence of 1% (w/v) NaCl or some other solutes of equivalent or greater ionic strength (Jermyn an Yeow, 1975; Clarke et al., 1978; Gleeson an Jermyn, 1979; Komalavilas et al., 1991). This effect can be exploited as the basis of a powerful method for the purification of AGPs.

IV. Distribution of AGPs

Several investigations revealed that the taxonomic distribution of AGPs is very broad. AGPs are found in angiosperms, gymnosperms (Bobalek and Jonson 1983) and in various lower plants (Clarke et al., 1978) such as bryophytes (Basile and Basile, 1990).

The distribution of AGPs on the anatomical level is also broad. They are located in almost all tissues including leaves, stems, roots, floral parts and seeds (Fincher et al., 1993). At the sub-cellular level, cyto-chemical and immunological methods revealed the presence of AGPs in the cell wall (Clarke et al., 1975; Clarke et al., 1978; Samson et al., 1984; Basile and Basile, 1987), and on plasma membrane (Clarke et al., 1978; Komalavilas et al., 1991). Cytochemical and immunological studies on extracted cell wall polymers have also revealed the presence of 3-, 6-, and 3, 6-linked galactopyranosyl residues that are characteristic of type II arabinogalactans, see figure 7.

Although, the chemical analysis did not establish whether the arabinogalactan polysaccharides were linked to polypeptides or other cell wall polymers such as pectins (Pellerin et al., 1995).

Controversy existed as to whether AGPs were part of the cell wall or simply present as soluble molecules in the cell wall space (Fincher et al., 1983; Bacic et al., 1988). Recent biochemical studies (Serpe and Nothnagel, 1994 and 1995) have shown, however, that at least some AGPs are tightly bound to the cell wall. Other studies revealed that large amounts of AGPs are secreted and accumulated in special tissues, such as cell wall spaces. Hori and group, 1980, showed that AGPs accumulated as soluble molecules in the medium of suspension-cultured plant cells. In this regard Clarke et al., 1979, have reported that large amounts of secreted AGPs accumulate on the stigma of flowers. Furthermore, copious amounts of gum Arabic (known also as gum Acacia), a mixture containing AGPs are secreted upon wounding the bark of *Acacia senegal.*

AGPs also occur in some cytoplasmic organelles. Biochemical analyses have revealed the presence of AGPs or their precursor in Golgi apparatus (Kawasaki, 1987 a; 1987 b). Secretion of AGPs to the cell surface in the floral style has been suggested to involve AGP transport in multi-vesicular bodies that are produced by Golgi apparatus or perhaps by the endoplasmic reticulum (Sedgley and Clarke, 1986). Evidences show that there is a possibility of turnover pathway of AGPs. Since some partially degraded AGP-containing multi-

vesicular bodies were found in the vacuole, some multi-vesicular body-vacuole pathway was proposed for endocytosis and turnover of AGPs (Herman and Lamb, 1992).

V. Structure of AGPs

As stated by Nothnagel (1997), no AGP has been characterized to the extent that its complete chemical structure is known. In his view, the determination of a complete structure will not be accomplished for many more years because the partial characteristics determined for a variety of AGPs indicate that they are heterogeneous and among the most structurally complex macromolecules known. The AGPs that have structurally characterized to the greatest detail are generally those that can be obtained in relatively large quantities. In general, the carbohydrate moiety (usually more than 90%) is typically rich in arabinose and galactose, organized primarily as type II arabino-3,6-galactan (Aspinall, 1973). The protein component (usually less than 10%) is typically rich in Hyp/Pro, Ala, Ser. And Thr.

A. Polypeptide Components of AGPs:

Molecular mass of AGPs is typically 30 to 300KDa. The core polypeptide makes 2-10% of this mass, with the carbohydrates accounting for the dominant remaining portion. The most abundant amino acid residues in AGPs are typically Hyp, Ala, Ser, Thr, and Gly. However, variations of the typical AGPs structure exist, for example three Hyp-deficient AGPs have been reported (Hillestad et al., 1977; Baldwin et al., 1993; Mollard and Joseleau 1994). One His-rich AGP

(kieliszewski et al., 1992) and one Ala-poor AGP (Qi e al., 1991) have been found. Higher protein percent, 30-65%, also have been reported.

Polypeptide Sequence:

As adequate purification schemes of AGPs were developed, partial amino acid sequences for AGPs from carrots (Jermyn and Guthrie, 1985), ryegrass (Gleeson et al., 1989) rose (Komalavilas et al., 1991) and maize (Kieliszewski et al., 1992) gradually appeared in literature. The amino acid sequences were rich in Hyp, Pro, Ala, Ser, and Thr.

The most important recent advance in the investigation of AGPs has been the cloning of cDNAs encoding the core polypeptides of several AGPs (Chen et al., 1994). However, the nucleotide probes designed from peptide sequences of isolated AGPs provided the most certain approach for isolating cDNAs that encode AGPs. In the early attempts of cloning, the high redundancy of codons and high GC content led to difficulties in achieving countable results, however these problems were eventually overcome through the use of RNA probes and information regarding codon usage in plants (Chen et al., 1994). Other screening mechanisms have also resulted in isolation of cDNA that have been suggested to encode putative AGPs. Pea ENOD5 may have been the first of such example. This cDNA was isolated through screening for nodulin genes that were xpressed early in the pea-Rhizobium interaction (Scheres et al., 1990). Largely because the polypeptide predicted to be encoded by ENOD5 has an amino acid composition that resembled that of bean AGP (Van Holst et al., 1981). Scheres et al. in 1990 suggested that ENOD5 encoded an AGP. The ENOD5 sequence

contains repeated Pro-Ala that had been suggested to be a diagnostic characteristic of AGPs (Showalter and Varner, 1989). It has been noticed that the amino acid Hyp is also coded for as Pro. However, without the isolation and characterization of ENOD5, the suggestion of cDNA's encoded an AGP remain tentative.

Several authors (Showalter and Varner, 1989; Li and Showalter, 1996) have noted that short runs of Pro alternating with Ala or Ser have been found in several AGPs. An oligonucleotide probe corresponding to Pro-Ala-Pro-Ala-Pro was used with some success in screening for AGP genes (Li and showalter, 1996).

One of the highly occurring forms of sequences is Pro-X-Pro-Z-Pro, where the X and Z are any amino acids. Most often X and /or Z were found to be Ala, sometimes Ser or Thr, and rarely Val, Glu, or Gly. When both X and Z are Ala the sequence is palindromic, as is the longer sequence Hyp-Thr-Hyp-Val-Hyp-Thr-Hyp . Kieliszewski and Lamport (1994) have noted the presence of palindromic sequence in number of extra-cellular matrix proteins and have discussed the potential functional roles of such sequences in protein folding and self-assembling. Because the palindromic sequences found so far in AGPs are quiet short, it seems unlikely that their palindromic character would have a large effect on overall AGPs structure.

Early attempts to characterize AGP polypeptides, sometimes, indicated the presence of more than one N-terminal amino acid, an observation which raised the possibility that AGP molecule contained more than one polypeptide

chain (Fincher et al., 1983). The results of such comparisons so far have been consistent with the presence of a single polypeptide chain in each AGP.

The presence of several N-terminal amino acids in an AGP preparation is more likely due to the presence of several co-purifying AGPs in the preparation, rather than to several polypeptide chains in a single AGP (Chen et al., 1994, Du et al., 1994).

The sequence information available, so far, for AGPs has revealed few homologies at the level of amino acid sequence. Homology at the level of domain structure was commonly evident, at least for some subgroups of AGPs.

As additional cDNA encoding AGPs or putative AGPs became available, sequence homologies may become more evident. Currently, however, AGPs appear to represent a relatively diverse group at the level of amino acid sequence.

Biosynthesis of Polypeptide Component:

Comparative researches between cDNAs and their mature AGPs, which they encode for, indicate that the core polypeptides of AGPs are synthesized as they enter the rough endoplasmic reticulum, the first part of the usual secretory pathway of endoplasmic reticulum -to- Golgi apparatus -to- cell surface. During this process, a signal sequence of amino acids at the N-terminal is cleaved. The removal of this domain allows the entry of AGPs being synthesized into the endoplasmic reticulum (Chen et al., 1994; Mau et al., 1995).

Peptidyl-Proline Hydroxylation:

As in animal systems, Hyp in plant proteins is synthesized by post-transitional modification of peptidyl Pro (Pollard et al., 1981) and the process requires molecular oxygen (Lamport, D. T. A., 1963), Fe^{2+}, α-oxoglutarate, and an appropriate peptidyl Pro substrate (Sadava, et al., 1971; Tanaka, et al., 1981). Peptidyl-pro hydroxylase, the enzyme that catalyzes this conversion, has been detected by its activity in a number of plants. The activity of the enzyme can be blocked by 3, 4-dehydroproline (Cooper and Varner, 1983) and other inhibitors, some of which have been applied in plant cell cultures with the resulting effect of blocking culture growth (Schmidt et al., 1991; Serpe and Nothnagel, 1994). Most often examination of hydroxylase in vitro is difficult because defined substrates are not readily available.

B. Polypeptide-Carbohydrate Linkage:

Numerous studies of carbohydrate-polypeptide linkage have shown that Hyp, Ser, and Thr residues are all potential O-glycosylation sites in the AGPs. A serious limitation currently exists as to which Hyp/Pro, Ser, or Thr residues in a polypeptide are likely to be glycosylated. Although the sequences Asn-X-Ser and Asn-X-Thr code potential N-glycosylation sites, analogous sequence codes for O-glycosylation sites are yet to be established. In the case of Pro, a prerequisite problem is the identification of codes that govern hydroxylation to Hyp. Based largely on studies of extensins, some of these rules are emerging. Kieliszewski and Lamport (1994) suggested that (i) Lys-Pro, Tyr-Pro, and Phe-Pro are not hydroxylated, whereas Pro-Val is always hydroxylated; and (ii)

appearance of Hyp in blocks, such as Hyp_4, increases the likelihood of attachment of arabinose oligosaccharides (Kieliszewski et al., 1995) but may not be important with regard to attachment of large glycans as occur in AGPs. Matsuoka et al., 1995 suggested that hydroxylation of Pro is likely if this residue occurs in a reverse turn region of a polypeptide.

Although the presence of O-glycans is certainly predominant in AGPs, potential N-glycosylation sites were also present in some of the polypeptides (Wang et al., 1993; Sommer-knudsen et al., 1996).

C. Carbohydrate Component of AGPs:

Primary plant cell wall generally contain 5-10% protein (Darvill et al., 1980) as glycoprotein. Of the total glycoprotein, about 40-60% is carbohydrate with Gal and Ara as the major monosaccharides, and Xyl, Rha, Glc, and uronic acids as minor monosaccharides. The glycan moieties appear to have a common structure consisting of β-(1→3)-linked D-galactan backbones to which side chains of (1→6)-linked β-D-galactosyl residues are attached through 0 to 6 (Figure 7).

Some of the side chains are generally substituted with L-arabinosyl and uronosyl residues as well as minor amounts of glucosyl, rhamnosyl, xylosyl, or fucosyl residues depending on the source of AGP.

Working on the fine structure of the glycan moiety of an AGP isolated from mature radish, Tsumuraya et al. (1990) reported that this AGP is characterized by side chains consist of consecutive, at least up to 20, (1→6)-linked β-

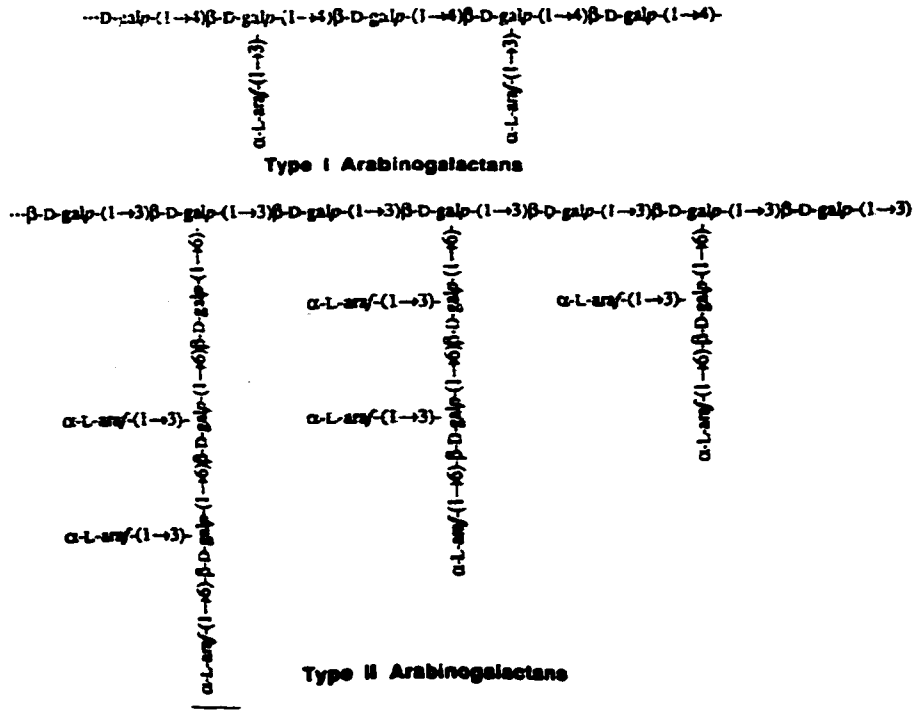


Figure 7:

Type I Arabinogalactans have a linear (1→4)-β-D-galactan backbone with arabinose oligosaccharide side chains. Type II Arabinogalactans have a highly branched framework consisting of a (1→3)-β-D-galactan backbone with (1→6)-β-D-galactan side chains attached at carbon atom 6 of some of galactosyl residues in the backbone (Aspinall, 1973).

galactosyl groups, and their acidic derivatives substituted with single 4-0-methyl-D- glucopyranosyluronic acid (4-Me-GlcpA) at non-reducing ends.

This structural feature of the AGP was revealed based on specific degradation of the proteoglycan with a novel exo-β - (1→3)-D-galactanase. Consecutive (1→6)-linked β-galactosyl residues have been reported to exist only

in AGPs but not in other plant carbohydrate polymers. Hence, it is anticipated that antibodies directed against sufficiently long consecutive probe for AGPs.

Various estimates have been made for the size of the glycans moieties attached to the core polypeptide of AGPs. Cultured tobacco cells produced several AGP fractions having average glycan chain size ranging from 46 to 140 sugar residues, with approximately 70% of the Hyp residues carrying such glycans (Kawasaki, 1987 a&b). Other estimates of average glycan chain size include, 30 sugar residues in the AGP component of gum arabic (Qi et al., 1991), 95 sugar residues in AGPs from the stigmas and styles of *N. alata* (Gane et al., 1995 a&b), and 150 sugar residues in a radish leaf AGP (Tsumuraya et al., 1984 a&b). Although results for many AGPs are consistent with a pure (1→ 3)-β-D-galactan backbone in these glycans, chemical degradation studies have shown that the backbone is interrupted in some AGPs. In an AGP form *L. multiflorum*, the backbone seems to consist of blocks of approximately 7 residues of 3-linked galactopyranose, separated by residues of 6-linked galactopyranose or 5-linked arabinofuranose (Bacic et al., 1987). A smaller block size of only 4 residues of 3-linked galactopyranose, separated by 6-linked galactopyranose, was found in an AGP from *Acacia robusta* (Churms and Stephen, 1984). This made the investigators suggest that the repeating-block structure in the glycan of some AGPs may have important functional consequences, however, this area of research is largely unexplored.

Although D-galactosyl and L-arabinosyl residues are, by definition, characteristic of all AGPs (see figure 7), wide variation exists with regard to the

other sugar residues that might be also present. Two of the most thoroughly characterized AGPs, one from *L. multiflorum* (Bacic et al., 1987) and another form *N. alata* (Gane et al., 1995 a&b), were found to have carbohydrate portions consisting almost solely of galactosyl and arabinosyl residues in approximately 2:1 molar ratio, with only trace amount of neutral sugars and no uronic acids present. More often however, AGPs have been reported to contain some amounts of other sugars that may include L- rhamnose, D-mannose, D-xylose, D-glucose, L. fucose, D-glucosamin, and the uronic acids D-glucuronic acid and D-galacturonic acid (Jermyn and Yeow, 1975; Clarke et al., 1979 a&b; Fincher et al., 1983; Tsumuraya et al., 1984 a&b and 1988).

Although structural studies of most AGPs have yielded results that they are consistent with model wherein the carbohydrate is present as large glycan (Fincher et al., 1983), smaller glycans have also been reported in some AGPs or AGP-like molecules. Analysis of an AGP from gum arabic revealed the presence of large glycan chains but also smaller Hyp-arabinosides, similar to those of extensins (Qi et al., 1991). Macromolecules that seem to contain both the large glycans of AGPs and the small arabinose oligosaccharides of extensins have also been found in *Z. mays* (Kieliszewski et al., 1992) and *N. alata* (Lind et al., 1994).

In general the structural analysis of carbohydrate glycans as described by Fincher, 1983 and Nothnagel, 1997 remains a difficult and likely important part of understanding of AGP function. More powerful tools for structural analysis are still needed to establish the overall as well as subtle structural features.

Suspension-cultured endosperm cells *of Lolium multiflorum* are considered a convenient system for studying AGP biosynthesis specifically, because they lack significant levels of cell wall glycoprotein, but secrete AGP into the medium. In these cells, a membrane-bound hydroxylase hydroxylates poly-L-in vitro (Cohen et al., 1983).

Biosynthesis of Polysaccharides:

The knowledge, in regards to the enzymes of polysaccharides synthesis, is still considered inadequate. However, activities of several glycosyl-transferases, the principle class of AGPs carbohydrates biosynthesis enzymes, have been investigated. A mixed membrane preparation from cultured endosperm cells of ryegrass was shown to incorporate radioactive galactosyl residues from UDP-Gal into various endogenous macromolecules, including the ones having the (1→6)-β-D-galactan linkages expected in AGPs (Mascara and Fincher, 1982). Further biochemical analysis with separated membrane fractions from ryegrass showed that the galactosyltransferase responsible for the synthesis of (1→6)-β-D-galactan was localized in the Golgi apparatus (Schibeci et al., 1984).

Because the (1→3)-β-D-galactan backbone presence in some AGPs (Churms and Stephen, 1984; Bacic et al., 1987) has been suggested, the synthesis of AGP glycan might involve a block assembly mechanism(Clarke et al., 1979 a&b; Fincher et al., 1983). Such a mechanism might be analogous to the synthesis of N-linked glycoproteins where oligosaccharides are synthesized as dolichol phosphate glycolipids and then transferred in one step from the lipid

to the polypeptide. Although direct evidence for block assembly of AGPs is lacking, it is noteworthy that Mascara and Fincher (1982) and Hayashi and Maclachlan (1984) in their researches, observed that a portion of glycosyl residues from radioactive UDP-Gal became incorporated into glycolipids.

D. Shapes of AGP as Visualized by Electron Microscopy:

Two models have been proposed for the macromolecular organization of AGPs. In the "wattle blossom" model by Fincher et al. (1983), the carbohydrate portion of AGPs exists as large glycans (approximately 100 residues) of globular shape that they are attached to the polypeptide. The polypeptide is located at the core of the AGP, which has an overall spheroidal shape. In the "twisted hairy rope" model by Qi et al. (1991), the carbohydrate portion of AGPs is present both as medium-sized glycans (approximately 30 residues) of extended conformation and as short linear oligosaccharides. These glycans and oligosaccharides are attached in repetitive blocks along the polypeptide. The overall shape of the AGP is elongated with short projections "hairs" along its length.

Imaging of AGPs, by transmission electron microscopy after rotary shadowing, has provided evidence that some AGPs may conform to one macromolecular model, whereas other AGPs may conform to the other model. Qi et al. (1991) found that an AGP from gum arabic appeared as a rod-like but flexible molecule of some thickness, a shape consistent with the twisted hairy rope model. Images of AGPs from carrot (Baldwin et al., 1993) and tobacco (Cheung et al., 1995), on the other hand, were ellipsoidal or spheroidal as predicted by the wattle blossom model. In both of these latter cases, the AGPs

exhibited a tendency to self-associate into aggregates. Cheung et al. (1995) suggested that this self-association was consistent with an adhesive property of AGPs.

VI. Functions of AGPs

Possible functions of AGPs can be inferred from their physical properties and their cellular localization. In a review by Fincher et al. (1983) all statements about functions were regarded as speculative.

AGPs have potential for two major types of interaction: macromolecules-to-macromolecules or macromolecules-to-small molecules ligand interaction.

If portion of the galactan framework adopt either a helical or triple helical conformation, with the substituents oriented to outside of the helix, several surfaces would be available for cooperation interaction; the regular areas of helix face, the helical grooves of the triple helix, and the substituents chains.

Macromolecules such as antisera and lectins (Gal-specific) bind to AGPs by specific interaction with a particular component of the AGP chains; although lectins specific for the other saccharide components (Ara, Rha) are not known, they may exist and could be involved in specific recognition reactions. Other macromolecules such as polysaccharides and proteins may interact nonspecifically with the AGPs causing changes in viscosity and gel-forming potential of AGP solution. Small ligands such as flavonol glycoside (Jermyn, M. A., 1978 a&b) and the artificial β-glucosyl antigens that may aggregate to a relatively large complex, also bind AGPs. It seems likely that the AGPs could

interact with other polysaccharides such as pectins in the middle lamella, and it is possible that they may also make contact with membrane-bound and wall-associated lectins (Jermyn and Yeow, 1975).

Furthermore, it is possible that the outer chains of AGPs are the functional regions of the molecules with the protein and β-galactan backbone serving only to present information inherent in the variety of terminal substituents. There is some evidence that terminal disaccharides of plant gums have taxonomic significance (Anderson et al., 1969), in addition, these groups may be implicated in the expression of identity of individual plant tissues or cell surface determinants such as the blood group and transplantation antigens of animal cells.

In a more recent investigation by Nothnagel (1997), the same facts about the functions of AGPs are confirmed. He states that the dominant hypothesis among current investigators is that AGPs function in plant development, perhaps as markers of cell identity or even as regulatory agents. This hypothesis is based primarily on observations that show expression and /or modification of some AGPs occurs in spatial or temporal patterns that correlate with certain aspects of plant development.

A. AGPs Mechanism of Action at the Cellular Level:

Various lines of evidence indicate that AGPs are involved in fundamental cellular processes, which can be considered the basics of plants' developmental and morphological activities. Some of the cellular processes, which may be

controlled by AGPs, are cell division, cell expansion, cell differentiation and, in some cases, cell death.

1. Cell Division:

A series of investigations of morphogenesis in leafy liverworts led to the hypothesis that AGPs are involved in the regulation of cell division in these lower plants (Basile, 1990; Basile and Basile, 1990 and 1993). This hypothesis was formulated as part of a larger hypothesis which holds that once cell proliferation is underway in a developing plant or plant part, then predictable form arises from the suppression of cell division at specific times and locations (Basile and Basile, 1993). This hypothesis was based in part on the results observed when developing gametophytes of liverworts were treated with hydroxyproline (Basile, 1979), 2,2'-dipyridyl (Basile, 1980) 3,4-dehydroproline (Basile et al., 1985), or other antagonisits (Basile, 1990) of the synthesis of hydroxyproline-containing proteins. In several species of liverworts, these treatments led to an altered pattern of morphogenesis that involved development of ventral leaves at locations where such leaves were not normally expressed. The hypothesis held that these new ventral leaves developed because of the lack of some unidentified hydroxyproline-containing protein which led to desuppression of cell division.

Subsequent investigations were focused on the identification of this hypothetical hydroxyproline-containing protein. Histochemical detection with $(\beta\text{-D-Glc})_3$ Yariv phenyl glycoside, see figure 7, showed that AGPs were common components in cell walls of liverworts (Basile and Basile, 1987).

Correlative evidence linking AGPs and suppression/desupression of cell division was obtained in experiments with *Gymnocolea inflata* (Basile and Basile, 1993). When axenically cultured on liquid medium, this leafy liverwort developed either a normal "suppressed" leaf pattern or a variant "desuppressed" leaf pattern, depending upon whether the nitrogen in the culture medium was supplied as nitrate alone or as ammonium with or without nitrate (Basile and Basile, 1980). The AGPs extracted from the plants or separated from the conditioned cultured medium were analyzed by centrifugation in cesium chloride gradients. The "supressed" plants retained a high buoyant density AGP fraction and released a low buoyant density AGP fraction into the medium. These results were interpreted as being consistent with the hypothesis that AGPs are involved in the regulation of cell division (Basile and Basile, 1993).

A different experimental approach led to an evidence of the involvement of AGPs in the proliferation of cells from higher plants. Serpe and Nothnagel (1994) used (β-*D*-Glc)$_3$ Yariv phenyl glycoside as an agent to perturb AGPs and their functions in living cells. When applied in the medium of suspension-cultured rose cells, (β-*D*-Glc)$_3$ caused inhibition of culture growth in a concentration-dependent manner, with complete inhibition occurring at 50 μM (β-*D*-Glc)$_3$. The (α-*D*-Man)$_3$ and (β-*D*-Gal)$_3$ Yariv phenylglycosides, which do not bind AGPs did not inhibit growth. After being held fully inhibited for 7 days in (β-*D*-Glc)$_3$ containing medium, the cells were transferred to normal medium and thereupon resumed growth with a time-course similar to that of control cells. Cell sized in control and (β-*D*-Glc)$_3$-treated cultures were similar, indicating that the

mechanism of growth inhibition involved suppression of cell division. Analysis of the treated cells showed that approximately 95% of the (β-*D*-Glc)$_3$ that was associated with the cells was bound to the cell wall AGPs.

2. Cell Expansion:

Reduced cell expansion in some systems has been found to be accompanied by reduced AGP abundance. Suspension-cultured tobacco cells adapted to medium containing specific amount of NaCl exhibited less cell enlargement than control cells growing in normal medium, an effect thought to be due to decreased cell wall extensibility in the salt-adapted cells (Iraki et al., 1989; Zhu et al., 1993 a&b). As detected with (β-D-Glc)$_3$ 0.16 mg of AGPs per milligram of total membrane protein was present in an enriched plasma membrane fraction from control cells grown in normal medium. In contrast, AGPs were barely detectable in the plasma membrane fraction from the salt-adapted cells. The amount of AGPs accumulating in the culture medium was also less with the salt-adapted cells than with the control cells. Based on these observations, Zhu and his group hypothesized that AGPs participate in cell expansion.

Stronger evidence of a role of AGPs in cell expansion in *Arabidopsis* was reported by Willats and Knox (1996), who used β–Glucosyl Yariv reagent (β–GluY), a synthetic phenyl glycoside that interacts specifically with AGPs in seedlings growing rooted in medium solidified with gellan gum. The presence of 30 μM of β–GluY in the medium did not affect seed germination but did reduce subsequent root growth to less than one-third of that in seedlings growing in

normal medium or in medium containing 30 μM α–Yariv phenyl glycoside (α–GalY) that does not bind AGPs. The effect of β–GluY was reversible in that root growth, which increased upon transfer of the seedlings from β–GluY–containing medium to the normal medium. At the level of light microscopy, the principal morphological effect of β–GluY occurred in the zone of root elongation where the epidermal cells became bulbous rather than elongated.

Willats and Knox (1996) also found that elongation of suspension-cultured carrot cells, normally inducible by dilution of the culture into medium without 2,4-dichlorophenoxyacetic acids, was blocked by 30 μM of β–GluY but not by α–GluY. Reversibility of the effect of β–GluY in this carrot cell system was not reported.

3. Cell Death:

Treatment with $(\beta\text{-D-Glc})_3$ proved to be very lethal to some cultures and reversibly inhibited cell division in other cultures.

Cell death has been observed as a response to AGPs perturbation in several cell cultures (Langan and Nothnagel, 1996). Although 50 μM of $(\beta\text{-D-Glc})_3$ reversibly inhibited (Serpe and Nothnagel, 1994), this $(\beta\text{-D-Glc})_3$ treatment quickly halted the growth of a recently initiated rose cell suspension culture and then gradually killed all the cells during the remainder of the 7-day treatment period. As the new cell line was passed into normal medium, however, the cells gradually improved their ability to survive the 7-day treatment with 50 μM of $(\beta\text{-D-Glc})_3$.

When Langan and Nothnagel (1996) applied 25μM of $(\beta\text{-D-Glc})_3$ to *Nicotiana edwardsonii* cultures , it killed most of the cells in 24 hours and all remaining cells within 5 days.

During a 7-day treatment period with 25μM of $(\beta\text{-D-Man})_3$, a Yariv phenyl glycoside that does not bind AGPs, see figure 7, the *Nicotiana* cells exhibited viability and growth characteristics that were indistinguishable from those of untreated controls.

The mechanism by which $(\beta\text{-D-Glc})_3$ rapidly killed these cell cultures have not yet been established. In particular, it is not clear whether $(\beta\text{-D-Glc})_3$ acted by simply damaging the cells, or whether it acted through a more complex mechanism .

In studies of development of vasculature, Schindler et al. (1995) and Dolan et al. (1995) noted that certain AGP epitopes seemed to identify cells committed to program cell death. As suggested by Nothnagel (1997), if AGPs are involved in programmed cell death, then perturbation of AGPs with $(\beta\text{-D-Glc})_3$ may have activated such a program in cell culture.

4. Cell Position:

As described previously, the antigenic property of AGPs has led to the generation of monoclonal antibodies. In one case an antibody, JIM4, has been shown to be specific for AGPs and not react with other cell surface rabinosylated and hydroxyproline-containing glycoproteins such as the extensins and lectins of the *Solanaceae* (Pennell et al, 1989).

Knox et al. (1989) described the specific, and restricted distribution of a set of plasma membrane antigens in seedling of *Daucus carota L.* The antibody JIM4 recognized an epitope common to a set of glycoproteins of the plasma membrane of a carrot cell line and an arabinogalactan protein secreted by the cell line. They demonstrated that in the carrot seedling the most abundant expression of the membrane-associated epitope occurs in the vascular tissues. Examination of the root apical meristem of carrot seedling indicated that the expression of the epitope is a very early event in the continuing development of the root meristem. The surface epitope is expressed within one or two cells of the dome of apical initials, before the pattern of future vascular tissue can be discerned and well before its actual differentiation. These findings could be used as early determinants of plant morphology and cell differentiation.

B. AGPs Involvement in Plant Development:

The presence of AGPs in the plant during development has been studied by number of authors. The dominant hypothesis among current investigators is that AGPs functions in plant development act perhaps as markers of cell identity or even as regulatory agents.

□ **Reproductive Phase of Development:**

AGPs have a role in **style functions**, the first hydroxyproline-rich macro-molecules in styles to be structurally characterized were the arabinogalactan proteins (AGPs) which are found in the extracellular secretions of the styles and stigmas of all angiosperms species surveyed (Hoggart and Clarke, 1984). They may be involved in the adhesion of pollen to the stigma surface, may provide a

nutrient supply to growing pollen tubes, may play a role in control of water balance, or could act as anti-microbial or anti-fungal agents (Labarca and Loewus, 1973; Fincher et al., 1983).

In regard to the presence of AGPs in the style, the exact function of AGPs or indeed of any of the other hydroxyproline-rich glycoprotein in styles has not been established. They may be presented as structural components or have a role in pollen tube growth either as adhesive or nutrients, or they may act simply by forming a matrix that can support the germination and growth of pollen tubes (Clarke and Gleeson, 1981). Their location in the extracellular matrix also suggests that, like other transmitting tract with specific gene products, they might be involved in defense against pathogens attack (Bradley et al., 1992; Gasser and Robinson-Beers, 1993; Lind et al., 1994; Showalter, 1993).

AGPs of stigmas and styles of *Nicotiana alata* are developmentally regulated. The amount of AGPs in these organs increases during floral development from green bud to mature flower, as shown both by direct quantification of buffer-soluble AGPs using the β-glucosyl Yariv reagent (Gell et al., 1986) and by quantitative immuno-cytochemistry using a monoclonal antibody directed to terminal α-L arabinofuranosyl residues (Sedgley and Clarke 1986).

In a recent investigation by Sommer-Knudsen et al. (1996) a basic galactose-rich style glycoprotein (GaRSGP) encoded by a previously characterized style-specific cDNA (NaPPP4) has been isolated from *Nicotiana alata* and structurally characterized. The glycoprotein is composed of

approximately 25% (w/w) protein and 75% carbohydrates. The glycoprotein is rich in the amino acids lysine, proline and hydroxyproline, and the mono-sacharides in galactose and arabinose. A carbohydrate epitope(s) is formed on both GaRSGP and another style-specific glycoprotein but not on glycoproteins from other tissues. This finding provides further evidence for the existence of a style-specific carbohydrates epitope(s), which may play a role in style functions.

AGPs and **ovary development**: The AGPs from the ovary of *Nicotiana alata* were also found to be developmentally regulated, as the different charge classes of AGPs altered during floral development. The AGPs from the mature ovary had charge characteristics that were distinct from those previously reported for the stigma and style. However, the concentration of AGPs (0.6 μg/ml fresh weight) in the ovary did not change during development, or in response to either compatible or incompatible pollination. The AGPs of the ovary are mainly associated with the epidermis of the placenta (Gane et al., 1995 a&b).

- **Somatic Embryogenesis:**

The addition of AGPs, isolated from seeds, to an old carrot cell line which had lost its embryonic potential, restored the ability to produce somatic embryos (Kreuger, M. et al., 1995). In a similar experiment, the addition of AGPs to an embryonic cell line resulted in an increase in embryonic potential. In *Picea abies* the addition of seed AGPs permanently altered the state of development of a cell line. Embryo previously unable to mature were now able to do so, and this ability was maintained after the cell lines had been transferred to medium without AGPs (Egertsdotter, U. et al., 1995).

- **Pattern Formation in Roots and Coleoptiles:**

A spatial and temporal expression of arabinogalactan proteins (AGPs) in the root development was investigated with monoclonal antibodies specific for AGPs. Association of the JIM13 epitope with xylem development in roots of *Arabidopsis thaliana* (Dolan et al., 1995), Just above the cell of the quiescent center, the central metaxylem initial cell was found to contain the JIM 13 epitope. Labeling with the JIM13 epitope was also observed in the columella cells closest to the tip of the root cap and in cells already sloughed off from the cap. During secondary thickening later in root development, detection of the JIM 13 epitope gradually decreased as lignification of the vessel elements proceeded (Dolan and Roberts, 1995). Epitopes of another monoclonal antibody JIM14, on the other hand, were present in abundance in the cell wall of mature sieve tube elements of the phloem and at much lower levels on the plasma membrane of most cells in the root.

Orderly expression of AGPs relative to differentiation of tracheids and other cells also been investigated in the subapical zone of maximum elongation in maize coleoptiles (Schindler, T., et al., 1995). As expressed per coleoptile length and as detected by either $(\beta\text{-D-Glc})_3$ or MAC207 antibody, the total detergent/low salt-extracted AGP content of the coleoptile was highest in 3-day-old seedling and then gradually decreased by about half during the next 4 days.

C. <u>AGP Mechanism of Action at the Molecular Level:</u>

The experimental evidence regarding the action of AGPs on a molecular level is insufficient, as viewed by Nothnagel, 1997. Questions were raised

considering the molecular action of AGPs, such as: Do AGPs interact with, or bind to, any particular molecules at the plant cell surface?

If interactive molecules can be found and identified, then light might be shed on the function of the AGPs themselves. No available evidence conclusively shows that AGPs interact with specific molecules at the cell surface. Various preliminary observations, however, suggest that such interactions might occur, for example, that AGPs may interact with pectins in the cell wall.

Sequential extraction of isolated cell walls has sometimes yielded pectin fractions that contain the 3, 6-linked galactopyranosyl residues that are characteristic of AGPs (Carpita, 1989). However, it is possible that pectins and AGPs do not interact together but simply happened to get purified under similar conditions.

VII. AGPs Possible Mode of Action:

In an analysis of the possible mode of action of AGPs by Kreuger and van Holst, 1996, the presence of AGPs on the outer side of the plasma membranes is interpreted as indication of the role played by AGPs in the interface of the membrane, and the extracellular matrix. Certain AGPs epitopes may be actually involved in the deposition and crystallization of macromolecules, thereby directing planes of growth, development and cell shape (Kieliszewski M.J. et al., 1992).

Cell position as well as cell lineage determines cell fate (Irish V.F., 1991; Sussex I.M., 1989) and this is, at least partly, determined by the cell wall. In

multi cellular organisms, all cells will depend on and influence each other. Cells that have entered a new cell layer by division will differentiate according to their new position or new cell layer rather than their origin. When isolated protoplasts of embryos of *Fucus spiralis* were placed in contact with the cell wall of another type, their fate were switched (Berger, F. et al., 1994). This sudden change in development clearly needs cell-cell communication to trigger these events, and cell walls appear to have the ability to alter the cell's fate.

Another possible manner for AGPs to function is as substrate for the production of a oligosaccharin by an enzyme.

The small oligosaccharin can then exert its function as a signal molecule, and the remainder of the AGPs can be quickly recycled, which would be in accordance with the observed high turnover (Kreuger, M. and van Holst, GJ. 1996). Indeed the processing AGPs, thereby possibly releasing biologically active compounds, has been reported (Kjellbom, P. et al., 1994).

IIX. Conclusion

There are many indications that AGPs have a role in cell identity and/or cell-to-cell signalling. A role in developmental processes is supported by a vast amount of circumstantial evidence and some direct evidence. The structure of AGPs has a possibility of creating many different types of molecules, which all can have a different function. The fact that each tissue, and possibly each cell type, has its own set of AGPs, indicates a relation with cell identity. The localization on the plasma membrane and at the cell wall is in line with the AGPs

involvement in developmental processes. The high turnover of AGPs will enable the cells to react quickly to a changed environment, by rapid removal and synthesis of new types of AGPs. Some direct evidence of involvement in differentiation is given by the fact that addition of nanomolar quantities of specific AGPs to cell cultures, change the developmental fate of the cells. This activity of AGPs seems to be related to the presence of specific epitopes. It may very well be that only specific AGP epitopes are essential for the activity, irrespectively of whether they are present on AGPs or on other molecules. The occurrence of a receptor for AGPs or AGP-derived molecules, however, awaits discovery.

Materials and Methods

I. Plant Material and Culture Conditions:

1. Plant Material:

The plant used in this study as a source of antigen is axenically cultured gametophyes of *Gymnocolea inflata* (Huds.) Dum, (Hepaticae) collected from Mt. Desert Island, Main, USA. They were derived from the same stock cultures used in earlier experiments by Dr. D. Basile and Dr. M. Basile since 1972.

2. Culture Media and Culture Condition:

Two types of media are used to culture the plant:

(i) Aqueous Knop media, lacks ammonium ions [appendix I]

(ii) Aqueous knudson, contains ammonium ions [appendix II]

The macro- or micro-nutrients, that were used to formulate knop and knudson media were taken from early work of D. Basile, 1978.

Cultures were incubated on lighted shelves in an air-conditioned chamber with maintained temperature of $18 \pm 2^{\circ}$ C and received continuous illumination between 700 and 1000 lux from cool white fluorescent bulbs. The *Gymnocolea inflata* were cultured on knop medium for twelve weeks then some were isolated and freeze dried using liquid Nitrogen, others were induced to proliferate by transferring to knudson media for another twelve weeks (Basile and Basile, 1980).

II. Extraction of Crude AGPs from Knudson Plant and Media:

AGPs were isolated from dried plant [appendix III], as well as media [appendix IV], through a series of precipitations using variable centrifugal speeds, and ethanol (80%) for dialysis. The concentrations of AGPs were measured using radial diffusion assay with Yariv β-glucosyl reagent [appendix V].

III. Isolation of Molecules Containing the Epitope(s) for JIM 101 and JIM 103:

Of the immunological methods attempted for the isolation of the antigens specific for JIM 101 and JIM 103 monoclonal antibodies, immuno-affinity purification using cyanogen bromide-activated sepharose 4B, proved to be successful over a variety of other methods such as the Acti-Disk ultra high capacity glutaraldehyde (GTA). The Acti-Disk method allows a small amount of the antibody to come in contact with the antigen (presumably AGPs) and provides insufficient time for this contact

Immuno-Affinity Purification Technique:

To undertake this technique for the isolation of Yariv's positive active molecules of JIM 101 or 103 from knudson media or knop plant, certain procedures were needed prior to using monoclonal antibodies.

1. Isotyping of Antibodies:

Serotec's anti-rat monoclonal isotyping reagents were used to characterize the class and subclass of the rat monoclonal antibodies JIM 101 and JIM 103 of

concentrations ranging from 5 to 20 μg/ml. The principle of this test system is based on red cell agglutination. A positive agglutination result is produced when highly specific antibody (which is coupled onto sheep red blood cells) recognizes and binds to the particular isotype to which it is directed (Serotec's anti-rat isotyping kit).

2. Affinity purification of the Monoclonal Antibodies:

The antibody purification reagents are mixtures of polymers formulated to concentrate and purify gamma globulins, IgG or IgM through size exclusion action. The reagents entrap the antibody and partition out other proteins and lipids (E-Z Sep, Pharmacia). Final suspension of the antibody is in 0.1M Trizma Base (adjusted to 9.0 → 9.5 pH) or Phosphate Buffer Saline (PBS) pH = 7.2.

3. Measurement of Monoclonal Antibody Concentration:

The colorimetric Bicinchonicic Acid for Protein Assay (BCA - Pierce Company) was used to determine the concentration of the immunoglobulins JIM 101 and JIM 103. The protein combines with Cu^{+} which is detected with a selective reagent for Cu^{+}, namely BCA. The product of the reaction is a chromophore, which could be detected using a spectrophotometer. To determine the protein concentration, the spectrophotometric absorption readings were compared to a protein standard curve of known concentration.

CNBr Activated Sepharose 4B Beads Technique:

CNBr-activated Sepharose 4B (Pharmacia Biotech) is a pre-activated gel for immobilization of ligand containing primary amines. The standard procedure is as follows:

The required amount of gel was weighed (one gram of CNBr powder makes 3-5 ml gel). Additives were washed away with 200 ml of 1 mM HCl per gram of freeze-dried powder. Ligand, JIM 101 or JIM 103 monoclonal antibody, each separately was dissolved in coupling buffer, 0.1 M $NaHCO_3$ (pH= 8.3) containing 0.5 M NaCl. The mixture, containing gel and Ligand, was covalently bound, as specified by Pharmacia Biotech, by mixed end-over-end for 1 hour at room temperature or overnight at 4° C. Excess ligand was washed away with 5 gel volumes of coupling buffer. The swollen gel contained approximately 2 mg of antibodies per ml. Any remaining active groups were blocked with Glycine buffer (pH= 8). The resulting complex was mixed for 2 hours then washed with at least three cycles of alternating pH and 5 gel volumes of each buffer. Each washing cycle consisted of a first wash with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl buffer (pH 8) containing 0.5 M NaCl (Pharmacia Biotech).

The column was equilibrated in Phosphate Buffer Saline (PBS) with 0.1% sodium azide. Lyophilized and ethanol precipitated AGP molecules from knudson culture medium or knop plant (10 mg), were dissolved in 10 ml of PBS (pH 7.2) with NaN_3 and mixed with 20 ml of CNBr sepharose 4B column, coated with about 40 mg of JIM 101 or JIM 103. The mixture was then left to react overnight at 4° C (Baldwin et al., 1993).

Unbound material was removed by washing with 10 column volumes of PBS with sodium azide. The bound AGP was subsequently eluted with 0.1M NaCl, 0.15M glycine buffer (pH 2.4), then collected in 800 µl, aliquot into 1.5 ml

"eppendorf" tubes containing 200 µl of 1 M Tris buffer(Baldwin et a., 1993). Eluted molecules were tested for the presence of JIM 101 or JIM 103 reactive AGP by an immuno-dot blot assay [appendix VI].

When the immunoaffinity purification of the AGP molecules was performed for the reason of carbohydrate analysis, about 50 mg of AGPs (antigen) were alkaline hydrolyzed prior to passing through the column [Appendix VII].

IV. Characterization of the Affinity- Purified Molecules:

In the process of characterization of the affinity purified molecules, the following questions may be answered:

- Are the isolated molecules arabinogalactan proteins?
- What are the apparent molecular weights of these glycoproteins?
- How many molecules (peptide chains) are present in the core protein of the affinity purified molecules and in the crude AGP used for the column?
- What are the apparent molecular weights of the isolated peptide chains molecules?
- What is the percent of the protein in the eluted molecules?
- Does the glycoprotein show some special features in terms of the constituent amino acids?
- Does the two isolated carbohydrates show any special features different from the classic type in terms of carbohydrate composition and linkage?

The following procedures were designed to assist in answering the previously raised questions:

A. Yariv Reagent Dot Blot:

A Dot Blot Assay with Yariv's reagent was used to test if the isolated molecules are AGPs. Isolated samples (1-3 μg) were dissolved in sterile distilled water, then dotted onto nitrocellulose, air dried and blocked with 3% dried skimmed nonfat milk powder dissolved in PBS (pH 7.4) for 1 hour then washed for 5 minutes in PBS, and immersed for 15 minutes in β-glucosyl Yariv reagent. The amount of concentration of Yariv reagent used was 0.15 mg cm^{-3} in 1% NaCl. The nitrocellulose was washed in PBS (pH 7.4), to decrease background staining before observations were made (Baldwin et al., 1993). Positive results would indicate that the uncoupled molecules are AGPs.

B. Estimation of Molecular Weights of Affinity Purified and Crude AGPs Using SDS-PAGE:

SDS-PAGE has a high resolving power. Proteins migrate according to their sizes making estimations of molecular weights possible. The latter effect is a consequence of uniform binding of SDS to various proteins, resulting in a constant charge to mass ratio. A complete description of buffers and gel preparation techniques are taken from BIO-RAD SDS-PAGE manual, combined with special staining methods; Silver Stain (sensitive at μg of protein concentration) and Coomassie Brilliant Blue (sensitive at mg of protein concen.). BIO-RAD running buffer Tris/Glycine/SDS has pH = 8.83, also high range molecular weight BIO-RAD Kaleidoscope pre-stained markers were used.

Due to the interference from the carbohydrate chains, the binding of SDS to glycoprotein is lower than proteins. This leads to slower migration, and results in molecular weight estimations, which are too high. The migration of glycoprotein,

relative to that of deglycosylated standards, varies with the percentage of acrylamide in the gel. Generally, at higher acrylamide concentrations, the relative migration is faster and the estimated molecular weight is lower.

The concentrations worked with the affinity purified molecules were 7.5 and 10%. Glycoprotein bands are frequently broad in shape and usually appear as "smears" rather than bands.

Mini Protean II Electrophoresis Cell made by BIO-RAD, California, USA was the employed apparatus. The voltage used was 100 V for about 1 to 11/2 hours running time or until the highest marker molecular weight was adequately moved into the gel.

C. Characterization of The Protein Moiety of AGPs:

Two preliminary tests were done on the immunopurified molecules to ensure the presence of amino acids, in general, and hydroxyproline, in specific. Hydroxyproline is an amino acid known to be present in this type of glycoprotein. These tests are: (i) Thin Layer Chromatography (TLC), and (ii) Colorimetric Test. These were followed by an amino acid composition analysis in which High-Performance Liquid Chromatography (HPLC) with reversed-phase chromatography was employed.

The performed protein composition analysis, also called molar ratio, can give information, sometimes, about the properties of a glycoprotein (Fukuds and Kobala, 1993):

a) A high content of Ser, Thr and Hyp may indicate a substantial amount of O-linked carbohydrates in the glycoprotein.

b) A high content of Cys may indicate extensive disulfide bonding and visa versa.

c) A high content of Asn, Ser, and Thr may indicate N-linked glycans.

Nevertheless, to get reliable amino acid compositions analysis, the proteins has to be purified then analyzed using HPLC.

V. Protein Analysis:

In order to obtain compositional analysis data of proteins, preliminary procedures were performed to separate off hydrolyzed proteins from un-hydrolyzed proteins and carbohydrates. Also, the isolated samples had to be freed of salt and detergent.

a) Hydrolysis of Proteins and Peptides:

A solution of the affinity purified molecules was mixed with 200-500 fold excess 6 N hydrochloric acid (distilled two or three times) in a thick glass tube designated for evacuation and sealing. Then immersing the tube in liquid Nitrogen or solid CO_2 to freeze the liquid. The atmospheric air then replaced by pure nitrogen gas, and the tube was evacuated to approximately 1 mm., and subsequently sealed. Hydrolysis was carried out for 18-72 hrs. at temperature of $110^{\circ}C \pm 1^{\circ}C$. After hydrolysis, the hydrochloric acid was removed by slow evaporation in a vacuum desiccator containing NaOH (Brenner et al., 1969).

b) Separation of Amino Acids from Proteins and Polysaccharides:

Acid hydrolyzed glycoproteins contained peptides, proteins and carbohydrates in addition to amino acids. One of the successful methods of separation of amino acids is Gel Filtration. This method was used to separate high molecular weight carbohydrates and peptide chains from low molecular weight amino acids in Gel Filtration with Sephadex-25, a cross-linked, insoluble, but highly swelling dextran. Large molecules were excluded but smaller molecules or inorganic salts diffused without hindrance into the gel. When an aqueous solution of a mixture of such substances was passed through a sephadex column, the high molecular weight portion (carbohydrates and large peptide chains) was eluted in the first fractions. However, smaller molecules were retained for some time and then eluted with more water or diluted salt solution (Brenner et al., 1969).

c) Separation of Salts and Soluble Carbohydrates from Amino Acids:

Based on the method described by Louise Slade (personal Contact with Basile, D.), this process is an ion exchange process. The method has proven successful in removing both salts and soluble carbohydrates.

Dowex-50 resin (1g) was placed in a beaker with excess 3N NaOH to cover the resin, stirred with a magnetic stirrer for 20 min. It was then washed on a Buchner filter with de-ionized water until neutral (de-ionized distilled water had to be used) or reaching the pH of water if not 7. Resin was treated similarly with 3N HCl in a beaker, but poured into the column

without washing. Sample was added to column after adjusting it to pH2. The column was then washed with de-ionized water until neutral. Amino acids were eluted at once using 3N NH_4OH.

Hydroxyproline Analysis

In this method, hydroxyproline amino acid is first oxidized then the product is reacted with P-dimethylaminobenzaldehyde to form a chromophore that can be detected using a spectrophotometer (Stegemann, H., 1958).

Hydrolyzate containing amino acids were affinity purified by passage through a Dowex-50 column then tested for the presence of hydroxyproline (amino acid known to be present in almost all AGP).

Material:

Stock Buffer pH6:

- 50 g citric acid. H_2O.
- 12 ml glacial acetic acid.
- 120 g sodium acetate. $3H_2O$.
- 34 g NaOH
- bring it up to 1000 ml (added to it 10 ml toluene).

Working Buffer pH 6 (stable for several weeks):

- 500 ml stock.
- 100 ml distilled water.
- 150 ml n-propanol

Chloramine-T (stable for several weeks in dark bottle at 4 °C):

- 1.41 g chloramine-T
- 10 ml distilled water.

- 10 ml working buffer.

Aldehyde/Perchloric Solution (make fresh every day):

The following amounts were mixed, mixture was enough for testing 25 samples:

- 3.75 g P-Dimethylaminbenzaldehyde.
- 15 ml n-Propanol.
- 6.5 ml Perchloric acid.

Method:

1. Use 0.2 ml sample or standards (concentration 0.2-6 μg /ml).
2. Add 0.1 ml Aldehyde/Perchloric Solution. Heat to 60° C for 15 minutes using water bath, then cool to room temperature.
3. Read at 560 nm [100 μl sample of 5 μg/ml stock should read 0.600].

HPLC-Amino Acid Composition Analysis

Amino acid analysis is carried out on a Pico-Tag Amino Acid Analysis System (Cohen et al., 1989.) Duplicate samples were hydrolyzed in 12N HCl/proprionic acid at 150°C for about 25 min. Appropriate blanks, controls and standards were hydrolyzed in the same vessel as a batch hydrolysis. The amino acids were derivatized with phenylisothiocyanate (PITC) and analyzed by Reversed Phase HPLC (Heinrikson and Meredith, 1984; Bidlingmeyer et al., 1984). The resulting phenylthiocarbamyl amino acid derivatives were separated on a 4.6 x 300 mm Nova Pack C 18 column employing a modified Pico-Tag buffer system (Cornell University, Ithaca, NY).

Deglycosylation of AGPs Using Anhydrous Hydrogen Fluoride

Deglycosylation of JIM 101 and JIM 103 affinity purified AGPs and Knudson media were carried out in a custom made anhydrous hydrogen fluoride (HF) apparatus (figure 8) (apparatus design was provided by Shimizu, M., at Peninsula Labs to Basile, D).

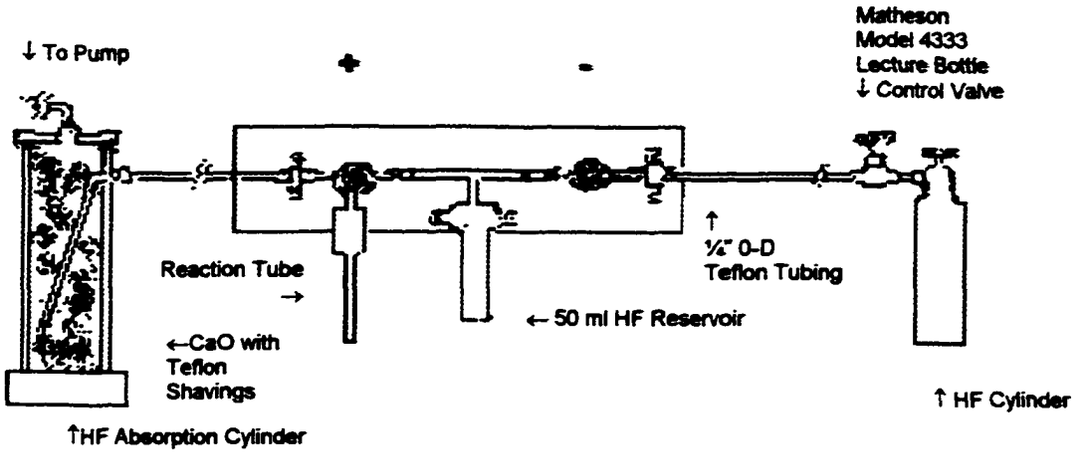


Figure 8:
Custom HF Reaction Apparatus used for the Deglycosylation of protein.

Pressure was partially removed and under suction anhydrous gaseous HF was released from its tank allowing a portion of the gas to condense into a reservoir tube using dry ice or liquid nitrogen. The gas was then evaporated using warm water then, in an evacuated reaction tube, the gas is mixed with AGP lyophilized sample with a ratio 1:5 respectively (Baldwin et. al., 1993) while cooling with dry ice or nitrogen. The mixture was then allowed to react for 1-2 hours while slightly cooling in an ice water bath. Cautiously, the extra HF was removed from the reaction tube using a suction pump. To ensure that all HF were evaporated and had been removed, evacuation was continued for an

additional hour. The reaction tube was then placed in desiccators containing KOH pellets to remove any residual HF in the tube. The deglycosylated protein was then dissolved in appropriate buffer for further process.

To separate carbohydrates from deglycosylated proteins, a size exclusion gel is used (Bio Gel P-2). The gel was swelled for 3 hours using Phosphate Buffer Saline (PBS) then the protein mixture was passed through a column containing the gel. Protein was eluted using excess PBS. Finally, protein was dialyzed then lyophilized (Schagger and VonJagow. 1987)).

Estimation of Molecular Weights of deglycosylated AGPs (Core Proteins)

The samples of deglycosylated AGP were re-suspended directly in 1X SDS-PAGE sample buffer with a ratio of 2 μg protein to 1 μl buffer. A sample of AGP (3 mg) was assumed to provide about 55 μg of protein after deglycosylation (Baldwin, T., School of Biological Sciences, University of Sains, Malaysia- personal contact). Samples were boiled and then stored at –20° C. Core proteins were run in BIO-RAD ready-made Tricine SDS-PAGE gradient gel with concentration ranging from 4 to 20% with BIO-RAD Tricine buffer. Standards used were wide range BIO –RAD, and a single standard with the value of about 2.5K to cover all probabilities of molecular weights.

Mini Protean Gel (BIO-RAD, CA) run at 70 V while the sample was in the stacking gel, then increased to 100 V while moving through the separating gel, with a total time of about 60 min. The gel was stained with BIO-RAD Silver Stain (BIO-RAD, CA).

VI. Carbohydrates Analysis:

The oligosaccharide containing the epitopes for JIM 101 and JIM 103 composition and linkage analysis were performed at CCRC Laboratory, University of Georgia, Athens, Georgia, using GC-MS.

Glycosyl Composition Analysis

The dried samples containing 20 μg of internal standard myo-inositol in a teflon-lined screw-capped test tube (100 μg) were hydrolyzed, overnight, with 500 μl of 1.0 M methanolic- HCL for 16 hours at 80°C. The next day the excess methanolic-HCL was evaporated at about 40°C with a stream of Nitrogen gas.

Methanol (250μL) was added to each sample and dried down again. The released methylglycosides were N-acetylated for 15 min at 45°C, using a mixture of acetylating reagent [methanol (200 μl), pyridine (20μl) and acetic anhydride (20μl)]. The acetylated samples were dried down at 80°C for 20min, under a stream of nitrogen and tri-methyl-sialylated (TMS) with 200μl of Tri-Sil reagent. The samples were cooled down, and the reagent was evaporated with a stream of nitrogen until becomes dry. The derivative samples were dissolved in 100μl of hexane and resolved on a 30m DB-1 column (J&W Scientific, Folsom, CA) using Hewlett-Packard 5985 GC-MS system. An initial temperature of 160°C were used, then raised to 200°C at 2° per minute, samples for identification were also runing along the side (York et al., 1986).

Carbohydrate Linkage Analysis

The samples were methylated using the NaOH/MeI method (Ciucanu and Kerek, 1984). The methylated material was then isolated by extraction into methylene chloride, then dried down and subjected to sequential hydrolysis (2M, TFA at 120°C), followed by reduction ($NaBD_4$) and finally acetylation (acetic anhydride and pyridine). The obtained sample of the partially methylated alditol acetates was analyzed using a fused silica (30m Sp2330 column) in a Hewlett-Packard 5985 GC-MS system with myo-inositol as an internal standard. The following temperature conditions were applied: 2 minutes at an initial temperature of 80°C, increased to 170°C at 30°C per minute, then to 240°C at 40° per minute and held for 5 minutes at 240°C (York et al, 1986).

RESULTS AND DISCUSSION

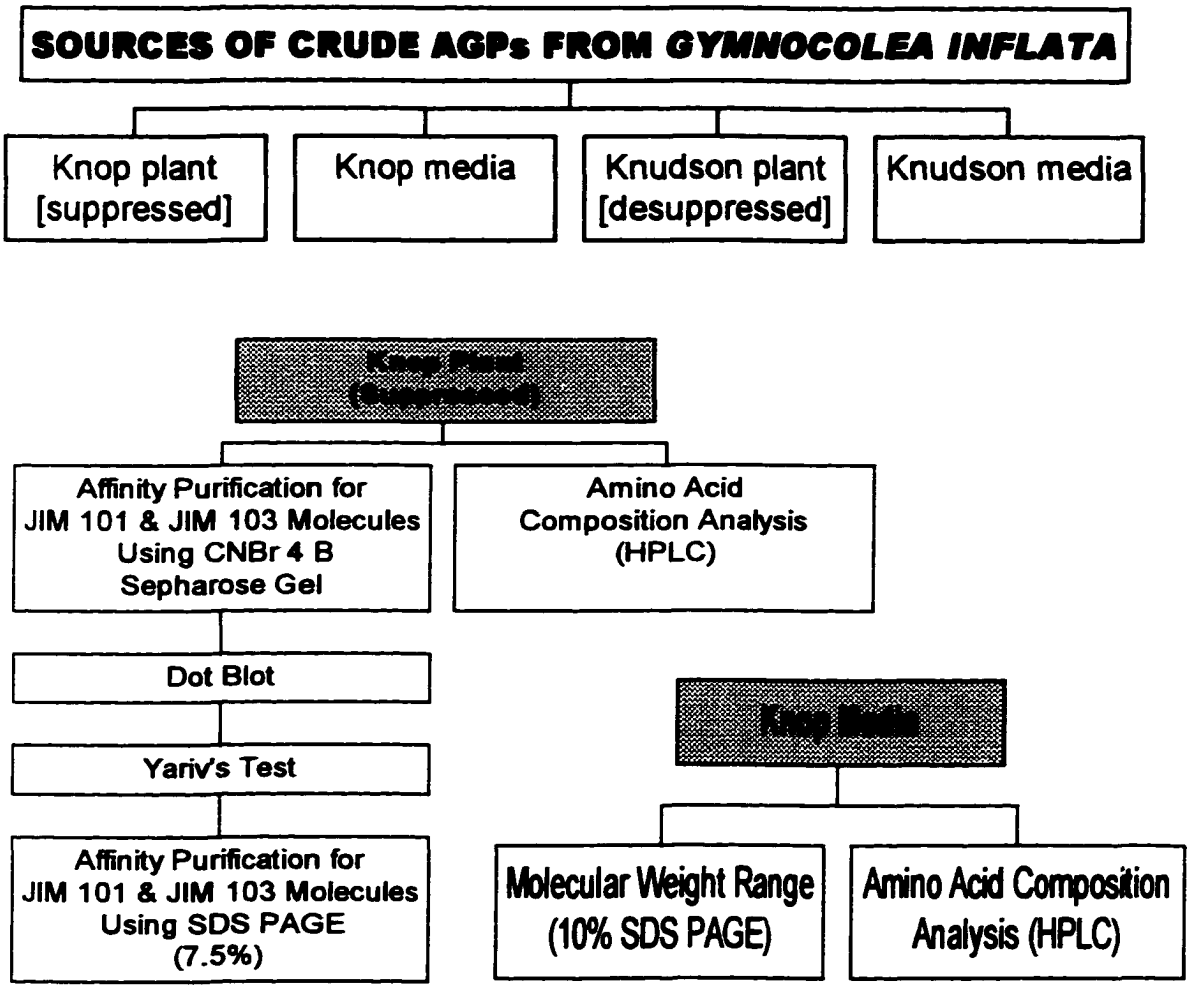
I: Research Strategies

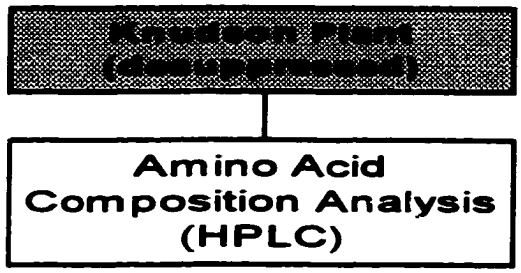
In the process of characterization of the isolated molecules, the following questions were answered:

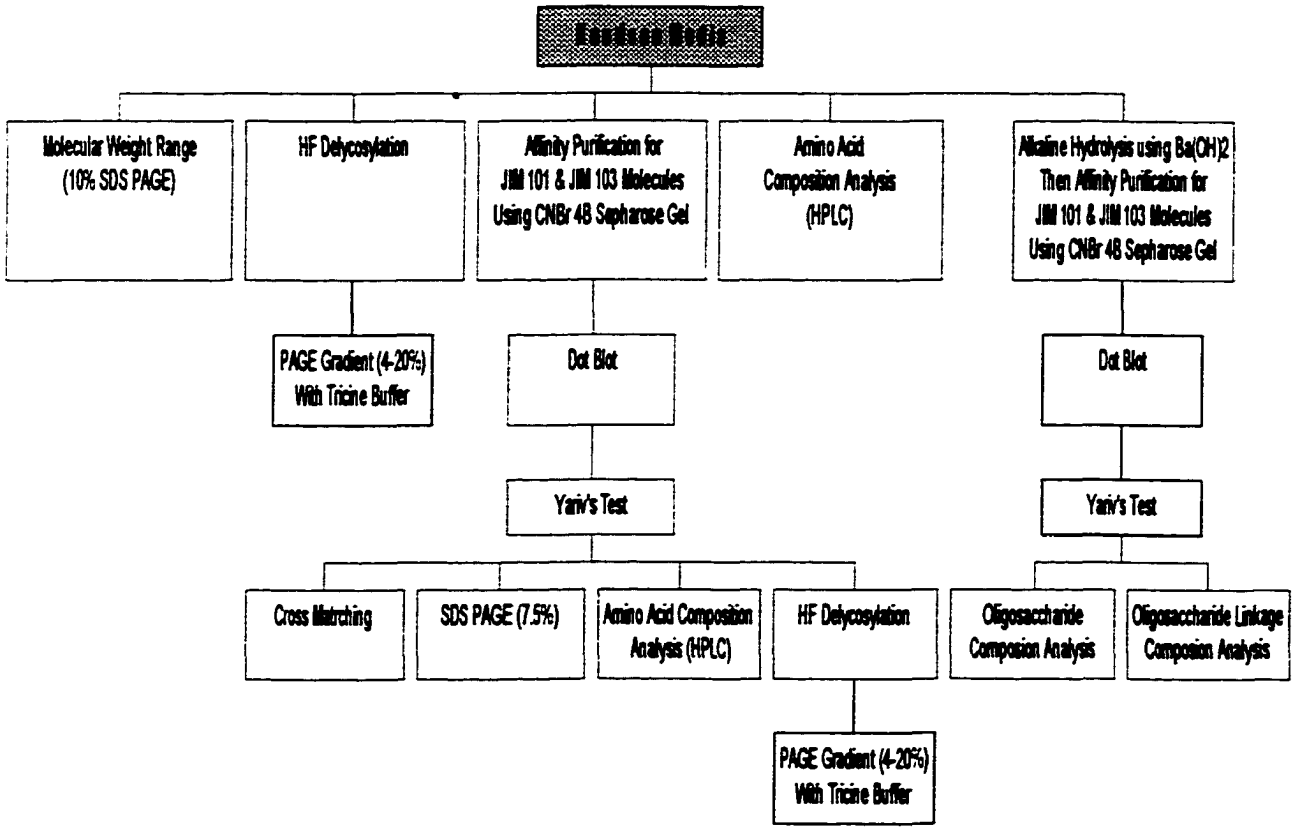
- What are the molecular weight ranges of crude AGPs? For example, that of knudson media and knop media. Do these molecular weights fit into the classic molecular weight ranges of the other native AGPs? (SDS PAGE).
- Is the core protein of the crude AGPs, formed of one or more protein backbones? (HF deglycosylation then Tricine SDS PAGE techniques).
- What is (are) the molecular weight(s) of the backbone of the core protein molecule(s) of the native AGPs? (Same techniques as above).
- How do the proteins of the 4 tested sources of AGPs similar to or different from one another? And from those other AGP known as the "classic" types? (HPLC amino acid analysis for the protein moiety of crude AGP isolated from knop media, knudson media, knop plant and knudson plant)
- Are the molecules, isolated by CNBr-activated Sepharose 4B columns and coated with JIM 101 or JIM 103 reactive to their own antibodies? (Immuno-blotting)
- Are the molecules isolated by JIM 101 and JIM 103 AGPs reactive to Yariv' reagent?

- What are the molecular weights of whole AGPs (glycosylated) containing the epitopes of JIM 101 and JIM 103 AGPs? (One time when the source of AGP is knop plant and another knudson media). What are the similarities and differences between the two situations? (SDS-PAGE)
- Is there cross-reaction between AGPs immunoaffinity purified by different JIM Monoclonal Antibodies? If there is a reaction, how is this reaction explained in light of the molecular weights of the whole molecules? (Immunoblotting)
- What are the molecular weights of the core proteins (deglycosylated molecules) of the affinity purified molecules containing the epitopes of JIM 101 or JIM 103? (Tricine-SDS-PAGE)
- What is the ratio between the molecular weights of the whole molecules of JIM 101 and JIM 103 and those of their core protein using knudson media as the source of antigen?
- Do the affinity-purified molecules contain HYP? (Thin layer Chromatography and chloramine-T to test for the presence of HYP in the affinity purified molecules, an amino acid known to be present in AGP) (This fact is used as a preliminary result on the way to getting a full amino acid analysis).
- Using the HPLC amino acid analysis for the protein moiety of JIM 101 and JIM 103 affinity purified molecules, what are the differences and similarities between JIM 101 and JIM 103 protein moieties and that of crude AGP of Gymnocolea inflata?

- What is the actual percent of the protein moiety compared to the total weight of affinity purified molecules using BIORAD protein assay?
- How are the carbohydrate compositions as well as the linkage analysis of JIM 101 and JIM 103, different or similar to the classic type of AGP? and, How these two epitopes are different or similar to one another?
- Draw a general conclusion about the protein moiety and molecular weight of the crude AGP of Gymnocolea inflata, and also the protein, carbohydrate and molecular weight of the eluted AGP molecules?







II. Selective Study of the Native AGPs :

The four native AGPs extracted from knop media, knudson media, knop plant and knudson plant of Gymnocolea inflata were extensively studied in a variety of ways. The studies performed on one or more of these native AGPs are: **1)** Molecular weight ranges of the whole molecule from knop and knudson media; **2)** Number and molecular weights of the polypeptide backbones of the AGPs from media of proliferating plant; **3)** Finally, the amino acid analysis study for all the four sources of native AGPs

1. Comparison of Molecular Masses of AGPs Secreted by suppressed vs. desuppressed plants:

In an attempt to determine the relative numbers of molecular masses of the "AGPs" being secreted by suppressed and desuppressed plants, extracts from the media of both were subjected to SDS-PAGE analysis. An example of the results is given in Figure 9. This procedure did not resolve the sample into discrete bands of glycoproteins. Each sample produced a more or less continuous "smear" corresponding to a range of molecular weight. Apparently this pattern is typical of many AGPs subjected to this type of analysis (Baldwin et al., 1993; Lind, et al., 1994; Serpe and Nothnagel, 1995).

What the SDS PAGE analysis did reveal was a difference in the ranges in their molecular weights. The Yariv-positive material released by the desuppressed plants had molecular weights from 30 Kda to well in

excess of 200 KDa while those released by the suppressed plants did not exceed 200 Kda (Figure 9). These results are in close agreement with an earlier comparative CsCl analysis that showed that desuppressed plants of *G. inflata* released Yariv-positive molecules with higher buoyant densities than did the suppressed plants (Basile and Basile 1993).

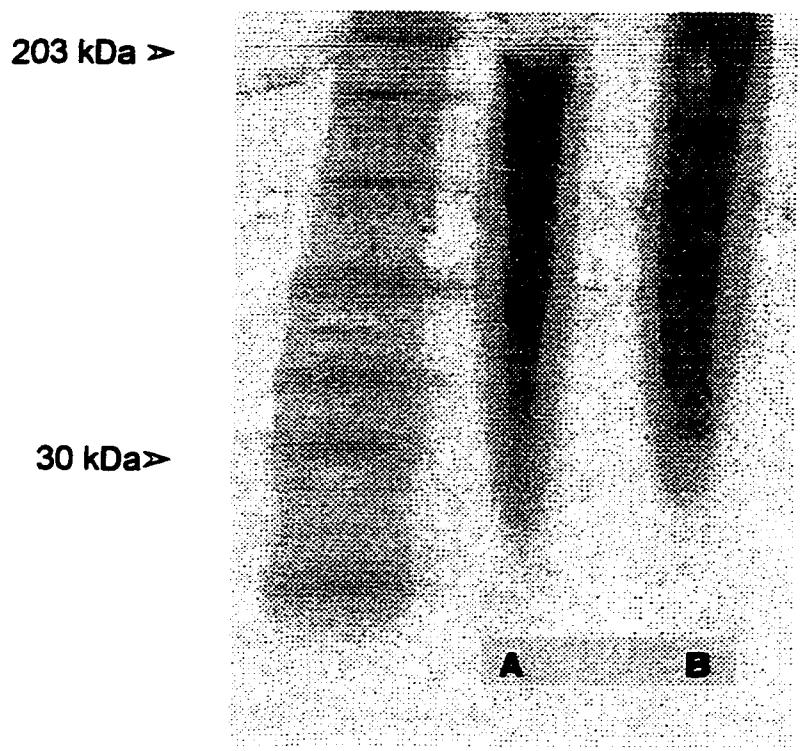


Figure 9:

Silver stained 10% SDS-PAGE run with culture media of suppressed (knop media) and desuppressed (knudson media) of Gymnocolea inflata, lanes A and B, respectively. Both give wide long "smeary" bands with molecular weight ends at about 30 kDa. However, it appears the knop media smear is missing the 203 kDa AGP molecule(s).

Although it has been already ascertained that the samples subjected to CsCl and SDSPAGE analysis yielded a variety of amino acids including hydroxyproline upon acid hydrolysis (Basile et al. 1999), it was not known how many proteins/polypeptides were present in the samples. These two methods of analysis failed to resolve the crude exacts into discreet bands of glycoproteins/proteoglycans, presumably due to their large complex carbohydrate components (Sommer-Knudson et al. 1996).

2. Deglycosylation of Crude AGP Obtained from Knudson Media (of desuppressed plant) and the Release of its Core Protein:

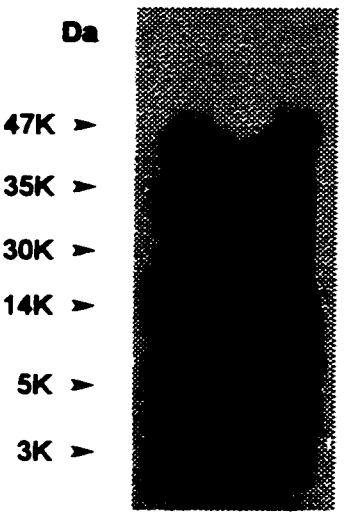
To determine how many proteins/polypeptides were present in the Yariv-positive molecules released into the medium by desuppressed plants, the samples were deglycosylated and the polypeptides thus released then subjected to SDS-PAGE analysis using Tricine-SDS PAGE of a gradient 4-20% and stained with Silver stain (Bio- Rad).

An example of the results of this analysis is given in Figure 10. The deglycosylated samples could be resolved into six discrete bands with molecular weights corresponding to approximately 3, 5, 14, 30, 35, and 47 KDa. In the extensive literature review by Nothnagel (1997), the range in molecular mass of AGPs is typically 30 – 300 KDa and the core polypeptide contribute to 1 to 10 % of this mass. Therefore, the results of the SDS-PAGE analyses of the crude and deglycosylated fractions

derived from the media of desuppressed plants are compatible with the hypothesis that they contain backbone proteins of AGPs.

Figure 10:

Tricine SDS-PAGE of gradient gel (4-20%), stained with silver stain. The well was loaded with 10 μg of deglycosylated knudson media (media of proliferating plant). The gel shows 6 tight bands of core protein of molecular mass of 3k, 5k, 14k, 30k, 35K and 47kDa.



3. Comparative Amino Acid Analysis of Yariv Reagent-Positive Fractions Extracted From Plants and Culture Media:

Yariv-positive fractions from suppressed and desuppressed plants and the media in which they had been cultured are acid hydrolyzed and analyzed for their amino acid content. The HPLC Reversed –Phase technique was used in accomplishing this task.

The native sources of AGP used in this analysis are knop plant (table 1& figure 11), knudson plant (table 2 & figure 12), knop media (table 3 & figure 13) and knudson media (table 4 & figure 14).

Table 1:

Amino acid analysis of protein moiety of ***Knop plant*** molecules.

Pool I, II and III are combinations of different eluants collected on different dates. [Res. = Residue of amino acid recovered]

Residue	Pool I		Pool II		Pool III	
	Pmol	% of Res.	Pmol	% of Res.	Pmol	% of Res.
ASX [ASP+ASN]	312.8	2.7	294.9	2.4	440.8	2.9
GLX [GLU+GLN]	974.8	8.1	983.2	8.0	1127.0	7.4
HYP	377.3	3.8	467.0	3.9	563.5	3.7
SER	1022.2	8.8	1130.7	9.2	1309.8	8.6
GLY	1976.3	15.9	1978.7	16.1	2284.5	15.0
HIS	171.6	1.5	135.2	1.1	213.2	1.4
ARG	802.5	6.8	884.9	7.2	1020.4	6.7
THR	749.1	6.4	749.7	6.1	99.0	6.5
ALA	1351.7	11.6	1388.8	11.3	1736.2	11.4
PRO	654.3	5.6	688.2	5.6	959.5	6.3
TYR	220.4	1.6	159.8	1.3	228.5	1.5
VAL	727.6	5.9	823.4	6.7	883.3	5.8
MET	123.3	1.1	122.9	1.0	198.0	1.3
CYS	39.4	0.3	61.5	0.5	30.5	0.2
ILE	460.7	4.1	553.1	4.5	609.2	4.0
LEU	902.1	7.5	860.3	7.0	1264.1	8.3
PHE	389.6	3.1	307.3	2.5	502.6	3.3
LYS	639.6	5.5	688.2	5.6	868.1	5.7

Figure 11:

Chromatograph of pool I amino acid analysis of ***Knop plant*** molecules. Run time is 26 minutes, injection volume is 50 :l and UV absorbency is at 254 nm.

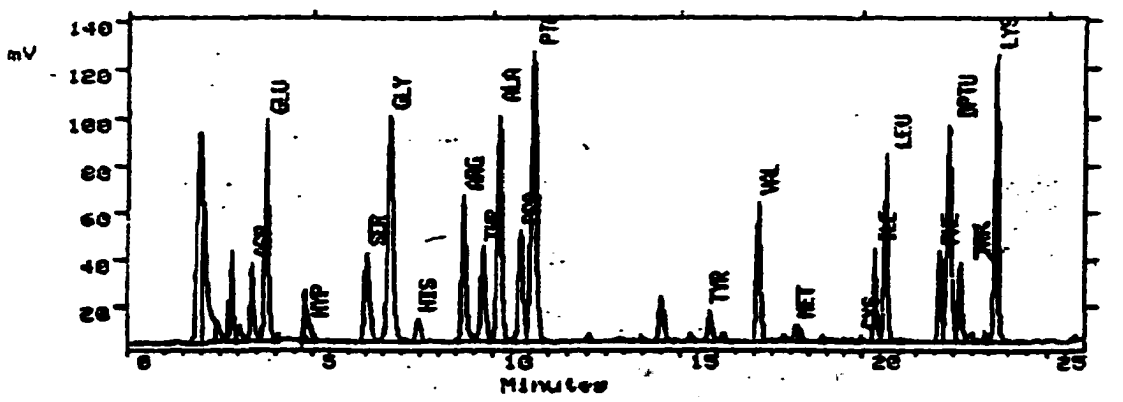


Table 2:

Amino acid analysis of the protein moiety of ***Knudson plant*** molecules. Pool I, II and III are combinations of different eluants collected on different dates. [Res. = Residue of amino acid recovered]

Residue	Pool I		Pool II		Pool III	
	pmol	% of Res.	pmol	% of Res.	Pmol	% of Res.
ASX [ASP+ASN]	314.4	2.8	195.6	3.1	178.0	2.6
GLX [GLU+GLN]	471.7	4.1	318.8	5.1	322.8	4.6
HYP	314.4	2.8	76.2	2.5	72.8	2.3
SER	1123.0	10.0	606.4	9.8	681.3	9.5
GLY	1516.1	13.5	941.2	14.8	1027.0	14.4
HIS	224.6	2.0	112.1	1.8	132.4	1.8
ARG	763.6	6.8	412.0	6.5	499.1	7.2
THR	1021.9	9.1	561.7	9.0	619.0	8.8
ALA	1493.6	13.3	887.4	13.5	976.8	13.7
PRO	595.2	5.3	361.6	5.6	396.4	5.6
TYR	157.2	1.4	62.2	1.0	98.6	1.4
VAL	696.3	6.2	404.2	6.5	448.6	6.5
MET	134.8	1.2	42.4	0.7	48.4	0.8
CYS	56.2	0.5	16.8	0.3	16.9	0.4
ILE	370.6	3.5	205.6	3.3	250.7	3.5
LEU	819.8	7.3	453.9	6.8	503.7	7.1
PHE	314.4	2.8	189.5	3.0	210.9	3.0
LYS	842.3	7.5	421.8	6.7	495.8	6.9

Figure 12:

Chromatograph of pool I amino acid analysis of ***Knudson plant*** molecules. Run time is 26 minutes, injection volume is 50 :l and UV absorbency is at 254 nm.

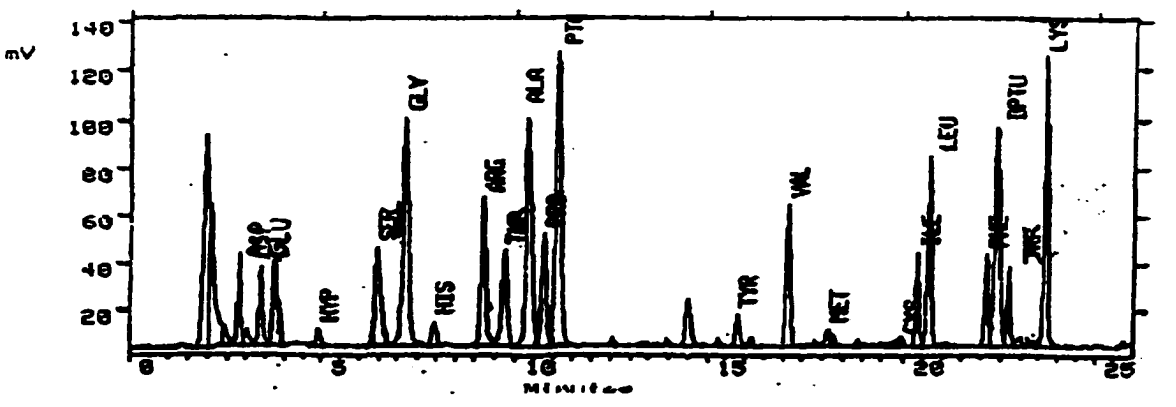


Table 3:

Amino acid analysis of protein moiety of ***Knop media*** AGPs molecules. Pool I, II and III are combinations of different eluants collected on different dates. [Res. = Residue of amino acid recovered]

Residue	Pool I		Pool II		Pool III	
	pmol	% of Res.	pmol	% of Res.	Pmol	% of Res.
ASX [ASP+ASN]	846.0	10.5	918.6	10.5	879.8	10.8
GLX [GLU+GLN]	926.7	11.5	1032.4	11.8	945.1	11.6
HYP	185.3	2.3	210.0	2.4	203.7	2.5
SER	741.4	9.2	927.4	10.6	798.4	9.8
GLY	1031.5	12.8	1268.6	14.5	1099.8	13.5
HIS	128.9	1.6	148.7	1.7	130.4	1.6
ARG	217.6	2.7	201.2	2.3	187.4	2.3
THR	556.0	6.9	577.4	6.6	578.4	7.1
ALA	805.9	10.9	909.9	10.4	863.6	10.6
PRO	475.5	5.9	472.4	5.4	472.5	5.8
TYR	104.8	1.3	113.7	1.0	97.8	1.2
VAL	475.3	5.9	472.4	5.4	439.9	5.4
MET	48.4	0.6	87.5	1.0	57.0	0.7
CYS	72.5	0.9	52.5	0.6	48.9	0.5
ILE	233.7	2.9	245.0	2.8	244.4	3.0
LEU	572.2	7.1	594.9	6.8	529.6	6.5
PHE	298.2	3.7	297.5	3.4	317.7	3.9
LYS	265.9	3.3	245.0	2.8	260.7	3.2

Figure 13:
Chromatograph of pool I amino acid analysis of ***Knop media*** molecules. Run time is 26 minutes, injection volume is 50 :l and UV absorbency is at 254 nm.

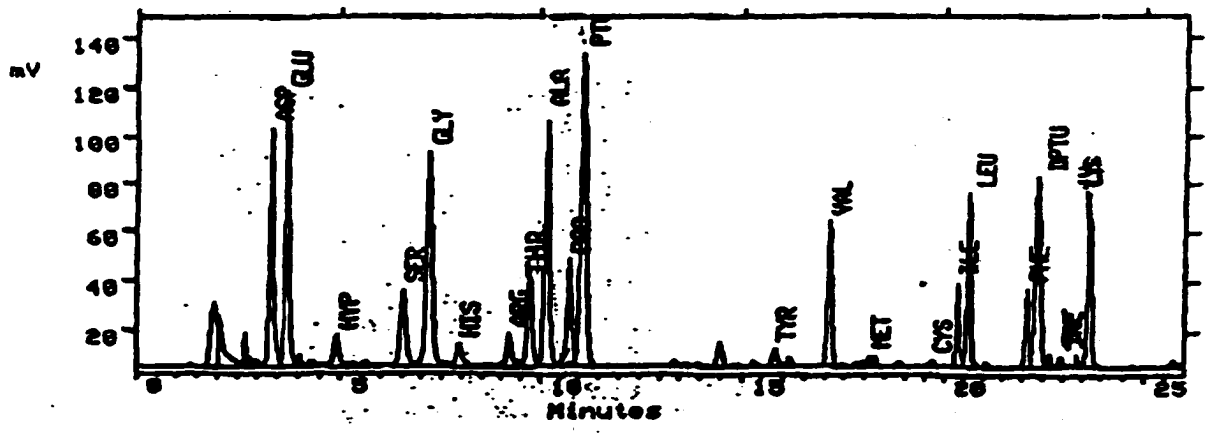


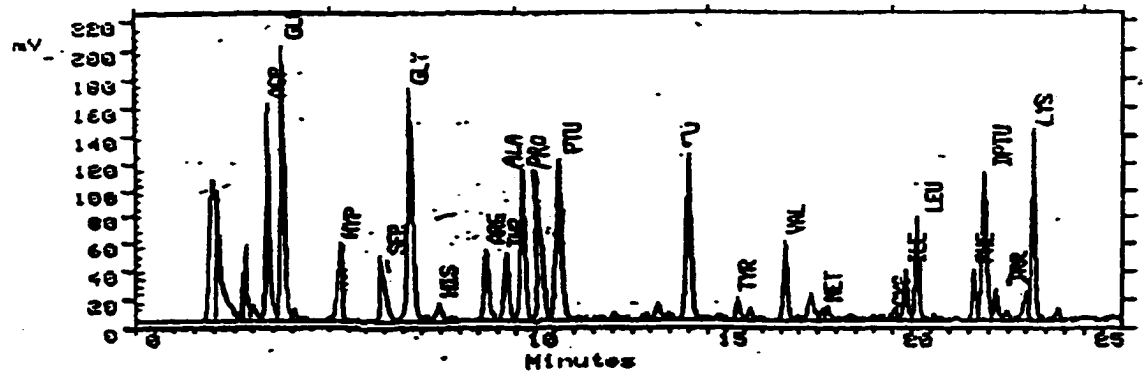
Table 4:

Amino acid analysis of protein moiety of ***Knudson media*** AGPs molecules. Pool I, II and III are combinations of different eluants collected on different dates. [Res. = Residue of amino acid recovered]

Residue	Pool I		Pool II		Pool III	
	pmol	% of Res.	pmol	% of Res.	Pmol	% of Res.
ASX [ASP+ASN]	977.7	8.2	1046.3	8.4	899.8	8.4
GLX [GLU+GLN]	1696.4	14.2	1913	15.3	1681.7	14.2
HYP	644.6	6.3	525.3	4.2	610.3	5.7
SER	81.5	6.8	845.5	7.6	781.0	7.3
GLY	2335.3	17.5	2163.5	17.3	1981.7	15.3
HIS	125.1	1.0	124.8	1.0	128.2	1.2
ARG	508.3	4.3	547.8	4.4	5142.2	4.8
THR	691.7	5.8	716.2	4.7	696.2	5.8
ALA	1129.4	9.5	1178.6	9.4	1167.6	9.5
PRO	712.5	8.1	950.5	8.5	921.2	8.2
TYR	106.5	0.9	137.9	1.1	96.5	0.9
VAL	486.7	4.2	509.5	4.1	514.3	4.5
MET	48.2	0.4	58.4	0.5	47.4	0.5
CYS	48.3	0.4	50.4	0.4	41.4	0.4
ILE	275.2	2.3	281.4	2.3	289.2	2.4
LEU	526.7	4.4	534.9	4.3	535.6	4.5
PHE	229.3	1.9	233.0	1.9	235.6	2.2
LYS	556.4	4.7	579.7	4.6	543.4	4.2

Figure 14:

Chromatograph of pool I amino acid analysis of *Knudson media* molecules. Run time is 26 minutes, injection volume is 50 :l and UV absorbency is at 254 nm.



? = Contaminant, probably Tris buffer

Table 5:
Amino Acid Composition Analysis in **mol % of crude AGPs** taken from: Knop media, Knudson media, Knop plant and Knudson plant of Gymnocolea inflata.

Amino Acids	Knop Media	Knudson Media	Suppressed Plant (Knop)	Desuppressed Plant (Knudson)
ASX	10.6	8.3	2.7	2.8
GLX	11.6	14.5	7.8	4.6
HYP	2.4	5.3	3.8	2.5
SER	9.9	7.2	8.9	9.8
GLY	13.6	16.6	15.7	14.2
HIS	1.6	1.1	1.3	1.9
ARG	2.4	4.5	6.9	6.8
THR	6.9	5.4	6.3	9.0
ALA	10.6	9.5	11.4	13.5
PRO	5.7	8.3	5.8	5.5
TYR	1.2	1.0	1.4	1.3
VAL	5.6	4.3	6.1	6.4
MET	0.8	0.5	1.1	0.9
CYS	0.7	0.4	0.3	0.4
ILE	2.9	2.3	4.2	3.4
LEU	6.8	4.4	7.6	7.1
PHE	3.7	2.0	3.0	2.9
LYS	3.1	4.5	5.6	7.0

The results of this comparative amino acid analysis are presented in Tables 1 through 5 and Figures 11 through 18. These data show that all the Yariv-positive fractions obtained by the methods used for their extraction and isolation contain polypeptides with twenty common protein amino acids including hydroxyproline as regular components. Although AGPs were originally considered to be "hydroxyproline-rich" (Fincher et al. 1983), it is now known that the amino acid composition of the polypeptide components of AGPs are highly variable (Nothnagel 1997) and some compounds referred to as AGPs are

hydroxyproline-deficient (Hillestad et al., 1977; Baldwin et al., 1993; Mollard and Joseleau, 1994). These data strongly support the hypothesis that the Yariv-positive material, being investigated, contains hydroxyproline-containing AGPs. Therefore, the Yariv-positive material being investigated here will be henceforth referred to as "AGPs".

There are number of differences in the composition of the separate fractions to indicate that they contain distinctly different proteins or polypeptides.

The most interesting result of this analysis with regard to hydroxyproline is its relative concentrations not its absolute. Because ammonium ion has had the same morphogenetic effect as antagonists of hydroxyproline synthesis, it was expected that the AGPs released by the desuppressed plants would be more deficient in hydroxyproline than that released by suppressed plants. (i.e., Plants cultured in Knudson's media vs. Knop's media). Contrary to what was expected, hydroxyproline is in considerably higher concentration, almost doubled, in the samples obtained from the media in which desuppressed plants were cultured. Obviously, another explanation as to "how ammonium ion is acting?" is required. It is apparently not via the impaired synthesis of hydroxyproline.

The only clue based on these data derives from the observation that the Knudson's media "AGPs" are richer in the basic amino acids arginine, lysine and possibly glutamine than AGPs from Knop's media. The protein' backbone of AGPs are more typically neutral to acidic. Some AGPs secreted from plants cultured on ammonium-containing medium are apparently basic since they migrate toward the negative pole (cathode) during electrophoresis through a gel

Figure 15:

Column chart showing comparative studies between the amino acid compositions of Knop media AGP (suppressed) and Knudson media AGP (desuppressed). The degree of variance between the two sources, statistically (the F TEST), from all the data available = 0.68348

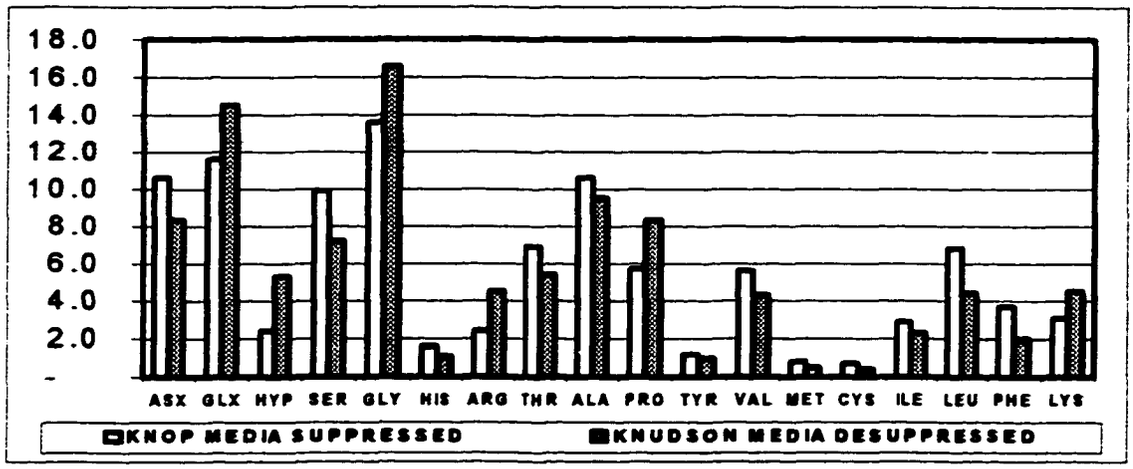


Figure 16:

Column chart showing comparative studies between the amino acid compositions of Knop plant and Knudson plant AGPs. The degree of variance between the two sources, statistically (the F TEST), from all the data available = 0.87539

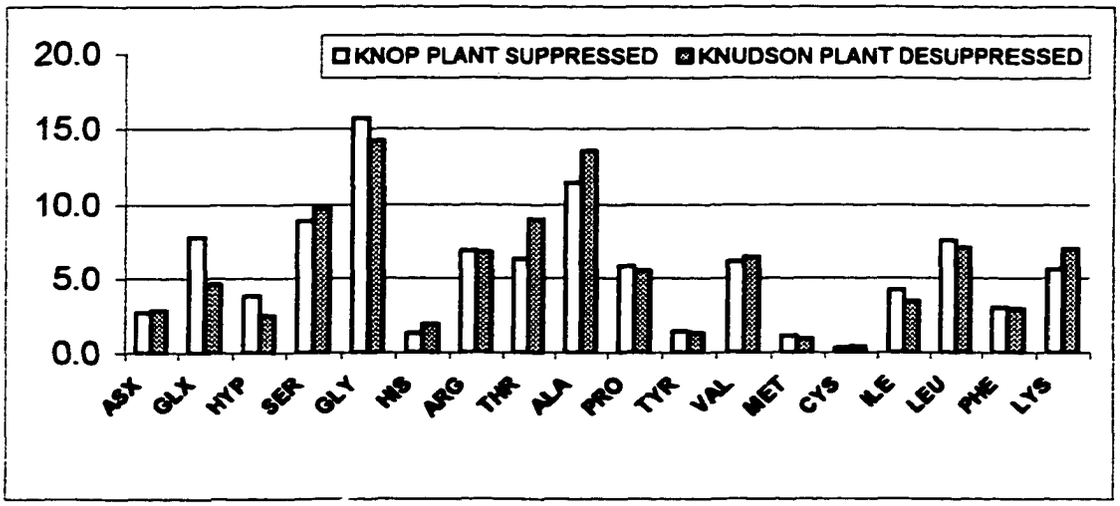


Figure 17:

Column chart showing comparative studies between the amino acid compositions of Knop plant and Knop media AGPs. The degree of variance between the two sources, statistically (the F TEST), from all the data available = 0.83156

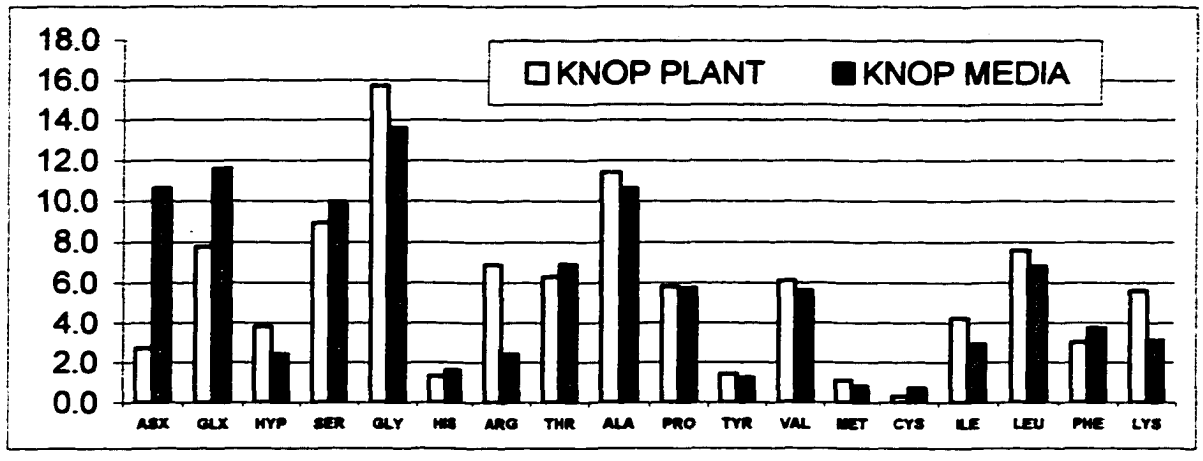
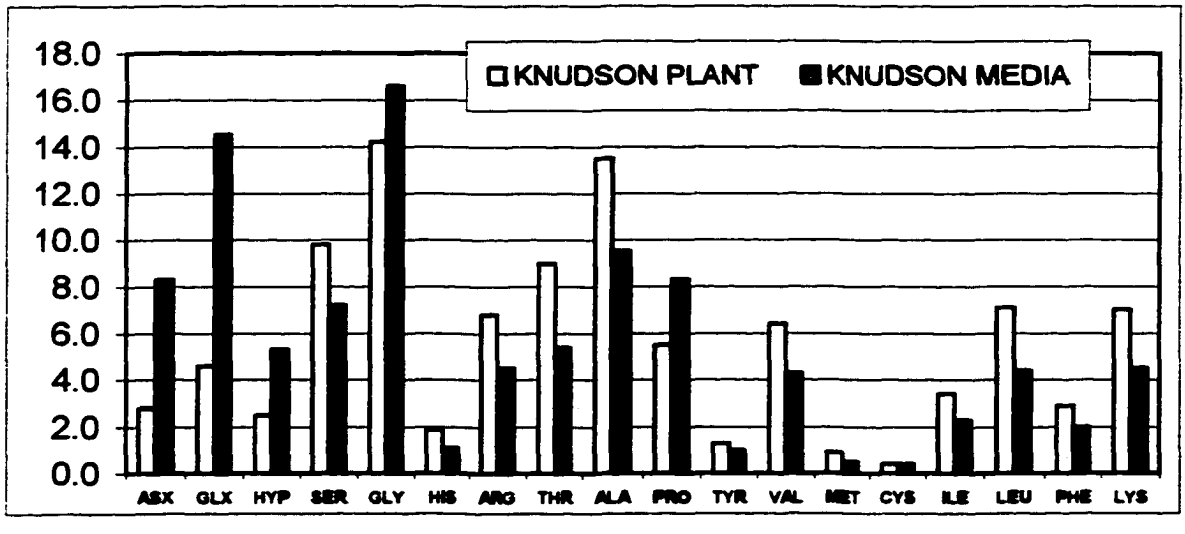


Figure 18:

Column chart showing comparative studies between the amino acid compositions of Knudson plant and Knudson media AGPs. The degree of variance between the two sources, statistically (the F TEST), from all the data available = 0.64302



buffered at pH 8.6 (see Figure 1). Perhaps the capacity for certain AGPs to locally suppress cell proliferation related to organ initiation and development is, in part, related to their net charge and/or the distribution of plus and minus charged components. It will be necessary to unequivocally identify and chemically characterize the hypothetical morphoregulatory AGPs to test this hypothesis.

Relatively, the most abundant amino acid was GLY, a hydrophilic, uncharged with polar side chain. Its molar ratio ranges from 13.6 to 16.6%. Having GLY as the abundant residue was also observed in other investigations such as Norman et al., 1990 and Baldwin, 1993.

In the contrary, ALA was slightly decreased. When the molar ratios of proteins from plants were compared to those from media of Gymnocolea inflata, a considerable drop of ALA was noticed. The ALA of the plant bound AGP was higher than that of the released, regardless of their morphogenic status, whether suppressed or desuppressed. Coincidentally, this result is in agreement with the analysis done by He Du and group, 1994, in which the ratio of ALA residue of bound AGP of N alata style was decreased from 15.9 to 6.2% once became unbound. ALA is a hydrophobic amino acid, this property may indicate the presence of an intrinsic membrane glycoprotein with multiple trans-membrane domains (Fukuda and Kobata, 1993).

With regard to SER residue, the results showing its consistency in all tested native AGPs. Incidentally, SER's are hydrophilic residues with non-

charged polar group and potential O-glycosylation sites in AGPs (Fukuda and Kobata, 1993).

The proportions of PRO, THR, and LEU were low. The presence of high ratios of PRO and THR residues would usually indicate the presence of substantial amounts of O-linked carbohydrates. It was noticed, however, that the molar ratio of PRO residue in media of proliferating plant was the highest of the four AGP sources. The reverse is true for the THR, the highest molar ratio was in AGPs extracted from the proliferating plant it self. LEU percent makes a noticeable drop in value in knudson media (4.4%) whereas the other three types of AGPs ranged between 6.8 to 7.6%.

At this point three things can be concluded. The first is that the putative "AGPs" being released into the culture medium have amino acid compositions that fall within the range acceptable for AGPs. The second is that they contain hydroxyproline. The third is that the "AGPs" released by desuppressed plants are significantly different from those released by suppressed plants in their relative concentrations of hydroxyproline and basic amino acids.

III. Isolation of Yariv β-Glucosyl Reagent-Positive Molecules Specific for JIM 101 and JIM 103 Monoclonal Antibodies

The initial reason for concluding that JIM 101 to JIM 106 are specific for epitopes of AGPs was because they bound the Yariv's β-glucosyl reagent-positive macromolecules we used as an antigen in the dot-blot screening procedure, see figure 4. Yariv's β-glucosyl reagent is generally considered to be diagnostic for AGPs (Nothnagel, 1997). However, because at least one AGP

was found to be "hydroxyproline-deficient" (Baldwin et al., 1993), it was deemed necessary to ascertain that the antigen we used to immuno-select the McAbs containing the "hydroxyproline-containing polypeptide" moiety.

All the results obtained thus far in this investigation support the original hypothesis that ammonium ion brings about desuppressed leaf and branch development by causing certain AGPs to be secreted into the culture media rather than being bound to the surfaces of the cells in which cell proliferation would otherwise be suppressed.

On the other hand, thus far, no specific molecule has been identified as an AGP and correlated with desuppressed leaf and branch development. To do this, two McAbs that bound Yariv-positive material that only occurred in the culture media of desuppressed plants were used to immuno-purified the molecules they specify away from all other media components.

Figures 19 A and B, and Figure 20 A and B show immuno-blotting when crude extracts from media in which desuppressed plants had been cultured were passed through affinity columns prepared with JIM 101and JIM 103, respectively, as primary antibodies and probed with their respective McAbs.

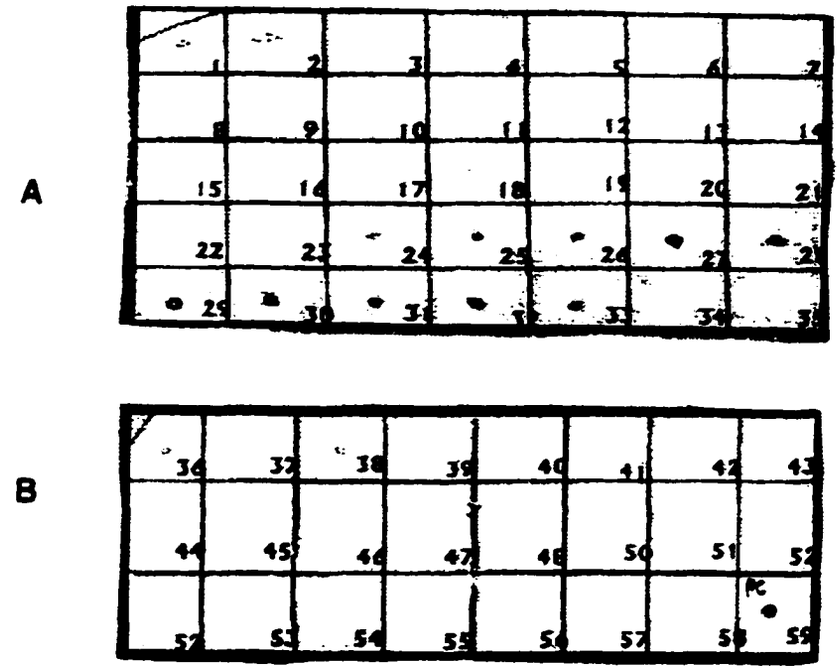


Figure 19:

Immunoblots of JIM 101 eluted AGP released into the media of desuppressed *gymnocolea inflata*. Nitrocellulose papers A and B were dotted with fractions collected during the purification. Blot A and B were probed using JIM 101 as the primary antibody with an alkaline phosophatase-conjugated secondary antibody. Square 58 is a negative control dotted into with PBS , in place of the antigen. Square 59 represents the positive control and contains crude AGP. Fractions 15 to 57 were collected after the addition of elution buffer. Fractions 24 to 33 are shown to contain most of the JIM 101 reactive material.

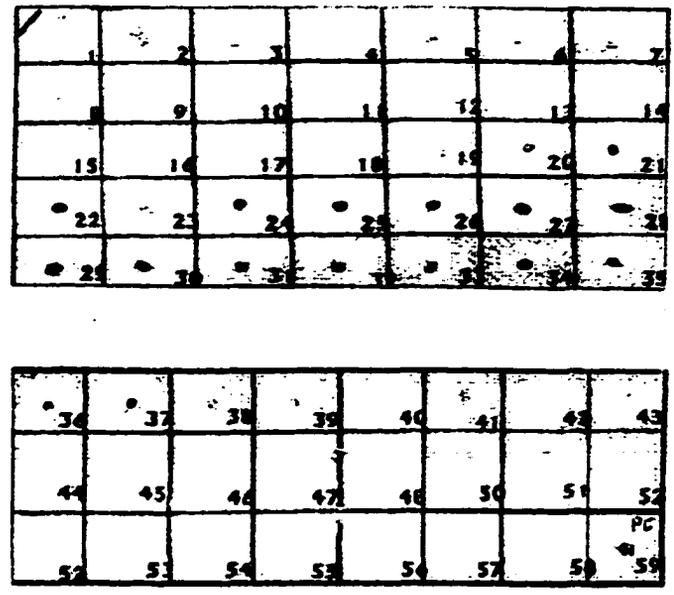


Figure 20:
Immunoblots of Jim 103 eluted AGP released into the media of desuppressed *Gymnocolea inflata*. Nitrocellulose A and B were dotted with fractions collected during purification. Blot A and B were probed using JIM 103 as the primary antibody with an alkaline phosphatase-conjugated secondary antibody. Square 59 (positive control) into which crude AGP was blotted. Square 58 (negative control) was dotted with PBS instead of antigen. Fractions 15 to 57 were collected after the addition of the elution buffer. Fractions 19 to 37 are shown to contain JIM 103 reactive material.

IV. Selective Study of The Affinity Purified Molecules

Preliminary to affinity purifying and chemically characterizing the molecules that bind to JIMs 101 and 103, some baseline data was obtained with regard the Yariv β-glucosyl reagent-positive macromolecules extracted from the suppressed (normal) and desuppressed (phenovariant) plants and the media in which they had been growing. Specifically, it was deemed necessary to ascertain

that the Yariv-positive extracts were not pure carbohydrate but actually contained polypeptide components characteristic of AGPs.

These studies answered important questions such as: Are the isolated molecules AGPs? Are they reactive to one another's monoclonal antibody?, How many polypeptide chains in the backbone of each of the isolated molecules?

1. Reactivity of the Immuno-affinity Purified Molecules to β-glucosyl Yariv's Reagent:

Detection and quantification of AGP were performed on nitrocellulose paper using β-glucosyl Yariv reagent, as described by Van Holst and Clark (1986). When the positive reacting elutes from these affinity columns were pooled, concentrated, spotted on nitrocellulose and probed by Yariv β-glucosyl reagent (Figure 21). Preliminary evidence was obtained that the two JIMs were selective for AGPs.

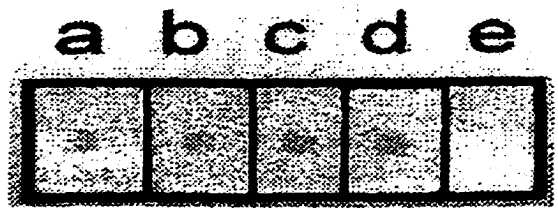


Figure 21:

Beta-Glucosyl Yariv's dot blot of immuno-affinity purified AGPs probed with Yariv's reagent. Sections a and b contain 101 and 103 eluted AGPs molecules, respectively. Sections c and d contain crude AGPs from knudson and knop media, respectively. Section e, negative control, only contains PBS.

2. SDS-PAGE and Measuring the Molecular Weights of Immunoaffinity Purified Molecules:

Extracts from suppressed plants and from the medium in which desuppressed plants had been cultured were purified using JIM 101 and JIM 103 affinity columns. The resulting samples were compared by means of SDS-PAGE (Figure 22).

The JIM 101 affinity purified AGPs extracted from the suppressed plants formed three bands of approximately 49, 97 and 203 Kda (lane B). These molecular weights suggest the possibility that the three bands represent the monomer, dimer and tetramer of the same AGP. However, the bands may, alternatively, constitute three different AGPs.

The JIM 101 affinity purified AGPs secreted into the culture media formed only two bands of 97 and 203 Kda (lane A). These two bands correspond to the two higher molecular weight fractions from the plant extract. Here too, the approximate molecular weights of the two bands suggests that the 203 Kda fraction may be composed of two 97 Kda fractions. Sommer-Knudson et al. In 1996 pointed out that the extreme "stickiness" of some AGPs presents a problem in keeping AGPs from being associated with other glycoproteins including other AGPs even under highly dissociating conditions.

JIM 103 affinity purified AGPs extracted from suppressed plants and the medium in which desuppressed plants had been growing, each formed only a single band of approximately the same molecular weight, 97 kDa (lane C and D).

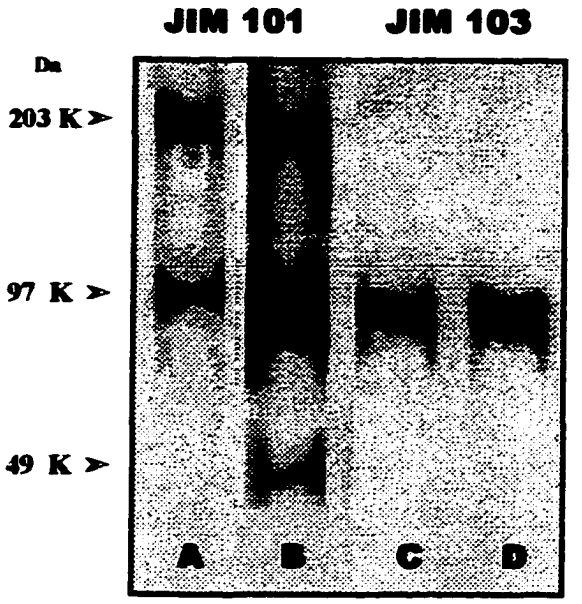


Figure 22:

Silver stain 7.5% SDS-PAGE run with immuno-affinity purified AGPs of JIM 101 in lanes A & B, and of JIM 103 in lanes C& D. The JIM 101 affinity purified extracted from suppressed plant (lane B) was forming three bands at molecular weights of 49, 97 and 203 KDa, and the one extracted from the media was forming two bands at molecular weights of 97 and 203 KDa. Whereas JIM 103 affinity purified AGPs extracted from desuppressed plants and the media in which they had been growing (lanes C and D), each was forming single band with a molecular weight at 97 KDa.

3. Cross-reactions among the Morphoregulatory Molecules:

The finding that both McAbs used to prepare affinity columns bind AGPs retained by suppressed plants and AGPs secreted into the culture medium when they are desuppressed raises the question as to whether the two McAbs are specifying the same AGP. A quick check was made by performing Immuno-blotting assay using JIM 101 eluted molecules with JIM 103 monoclonal antibody and JIM 103 eluted molecules with 101 monoclonal antibody to determine if the two McAbs cross-reacted.

The results of this assay (Figure 23) show that there is some cross-reactivity. However, the reaction of each McAbs to the antigen it specifies is much stronger. Apparently the antigenic sites are similar but not the same.

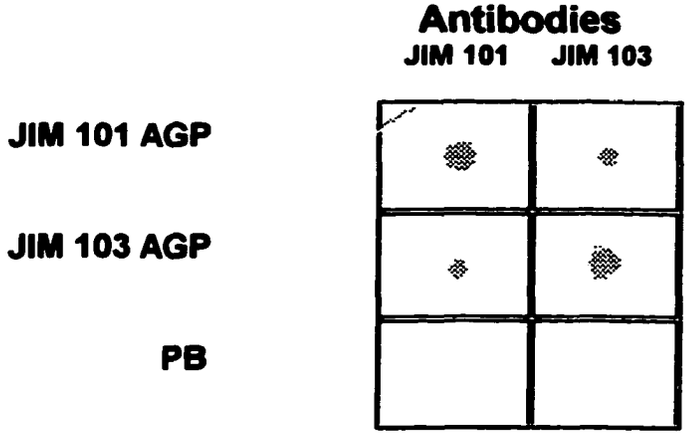


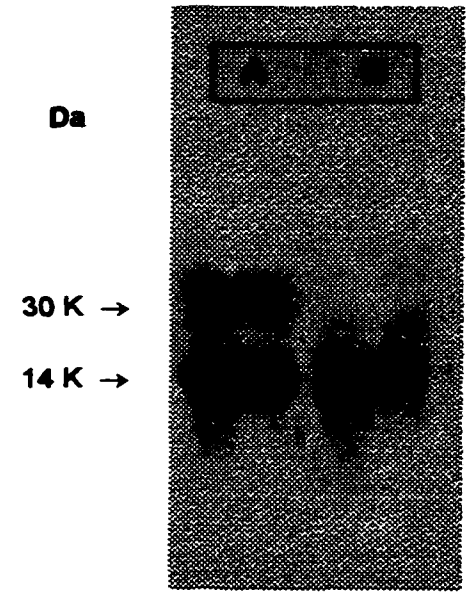
Figure 23:
Immuno-blotting of JIM 101 and JIM 103 eluted AGPs molecules against each others' monoclonal antibodies in addition to their own. This blotting shows a positive cross-reaction. The reaction of elutes with their monoclonal antibodies were much stronger.

4. Deglycosylation of The Affinity Purified Molecules and the Reveal of their Core Protein:

To find out whether the multiple bands produced by JIM 101 affinity purified AGPs are reflecting the two or the four monomers "sticking" to one another, the affinity purified samples were deglycosylated using anhydrous HF. The resulting protein backbones subjected to SDS-PAGE.

Consistent with the results of the SDS-PAGE analysis of the intact AGPs, the deglycosylated JIM 101 samples formed two bands (Figure 24, lane A), corresponding to a molecular weight of 14 and 30 KDa. The deglycosylated JIM 103 samples were forming one band corresponding to 14 KDa (Figure 24, lane B). Since the higher molecular weight band produced by the JIM 101 purified sample is approximately twice that of the

Figure 24:
Tricine SDS-PAGE of gradient gel, 4-20%, stained with silver stain. Deglycosylated AGPs of JIM 101 and JIM 103 affinity purified molecules in lanes A and B respectively. JIM 101 eluted molecules core protein shows two bands of molecular weights at 14 and 30 K Da, where as JIM 103 core protein shows one band of molecular weight at 14 K Da.



lower band, it is still possible that the 30 KDa band represents a complex formed by two of the 14 KDa proteins. There is no evidence, however, that the "stickiness" of certain AGPs is a property of their protein backbones.

These results are somewhat agreeable with previous finding by Gleeson et al., 1989, who observed that the secreted classical Hydroxyproline-rich AGP isolated from *Lolium multiflorum* culture media has decreased in molecular mass upon deglycosylation from 200 to 35 KDa. Baldwin and his group also reported the same observation in 1993. They noticed that the molecular mass of native glycosylated sample of carrot AGP runs as a smear of molecular mass ranging from 70 to 100 KDa, as opposed to its deglycosylated protein which runs as a single tight band of approximately 30 KDa.

Because of the fact that AGPs molecules are highly glycosylated molecules, this makes them travel on gels slower than their counterpart molecules with less or not glycosylated. Consequently, the molecular weight appears higher than their actual value.

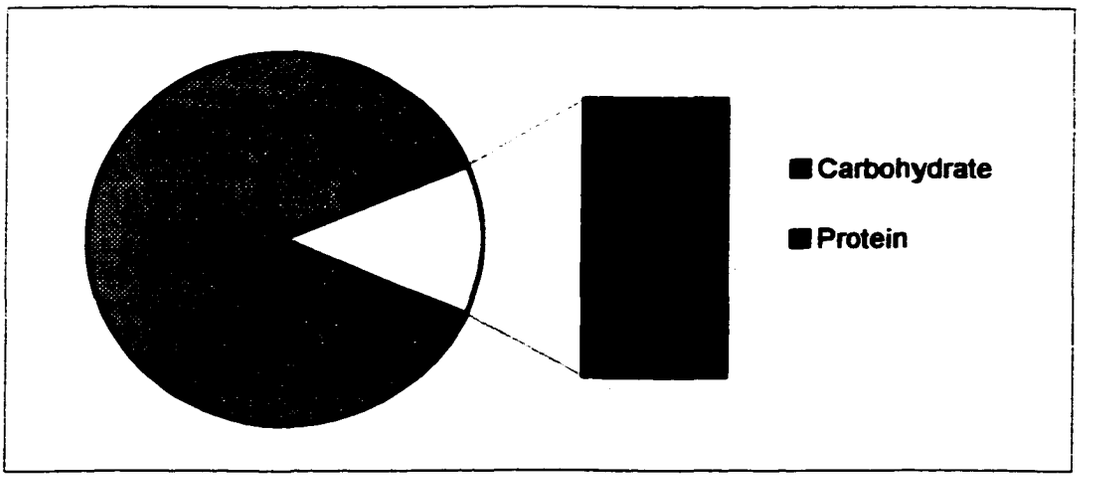
Two facts that could cause the protein core of AGPs to have a percent higher than 10, these facts are (1) the retention of some sugar linkages by the protein molecules of the deglycosylated AGP, and/or (2) the slow movement on the gel by the highly glycosylated molecules.

5. Determination of the Actual Core protein percent in the Deglycosylated AGP Molecules:

Based on the SDS-PAGE analysis of the affinity selected AGPs, the protein component of JIM 101 AGPs purified from the culture medium account for about 14.4% of the 97 KDa fraction and about 14.8% of the 203 KDa fraction. The protein component of JIM 103 AGPs purified from the culture medium also account for about 14.4% of the 97 KDa fractions.

Although the protein content of AGPs "typically" accounts for 2% to 10% of the total weight (Figure 25), appreciably higher percentages have been reported (Fincher et al., 1983).

It is possible, if not probable, that the deglycosylation of the AGPs was not totally completed and a small amount of carbohydrate is contributing to the observed weights.



(Figure 25)

A rigorous determination as to whether or not the14.4 % and the 14.8 % represent the approximate or actual percentage of the core proteins would not be a factor in the categorization of the affinity selected molecules as AGPs.

More interesting is the finding that both JIM 101 and JIM 103 selected AGPs that have the same molecular weights of their intact (i.e. 97 KDa) and deglycosylated (i.e. 14 KDa) molecules as determined by SDS-PAGE. This coupled with the finding that both JIMs cross-reaction with their affinity purified products suggests that these McAbs may be recognizing a common epitope and possibly a common AGP.

V. Chemical Characterization of Yariv β-Glucosyl Reagent Positive Molecules

This section is an extensive study on the protein and carbohydrate moieties of the immunoaffinity purified molecules.

1. Amino Acid Composition Analysis of The Immuno-affinity purified JIM 101 and JIM 103:

An HPLC analysis (Reversed-Phase technique) of the amino acid composition was performed on the acid hydrolysates of JIM 101 and JIM 103 purified AGPs. The results of these analyses were given in Tables 6 through 8 and Figures 26 through 28.

A comparison of the mean values of the mol % of the amino acids composing each of the samples does not provide evidence to support the SDS-PAGE-based evidence that JIMs 101 and 103 are specifying the

Table 6:

Amino acid analysis of protein moiety of ***JIM 101*** immuno-affinity purified AGPs molecules. Pool I, II and III are combinations of different eluants collected on different dates. [Res. = Residue of amino acid recovered]

Residue	Pool I		Pool II		Pool III	
	p mol	% of Res.	p mol	% of Res.	p mol	% of Res.
ASX [ASP+ASN]	830.3	7.2	842.6	7.1	815.5	7.2
GLU	934.1	8.1	1008.8	8.5	1019.4	9
HYP	657.3	5.7	676.5	5.8	645.6	5.7
SER	1153.2	10	1186.8	10.1	1132.62	10
GLY	1533.7	13.3	1578.5	13.3	1506.4	13.3
HIS	356.2	2.3	273	2.4	283.2	2.5
ARG	426.7	3.7	439.1	3.6	407.7	3.6
THR	680.4	5.9	700.2	5.4	611.6	5.4
ALA	853.3	7.4	878.2	7.6	1087.3	7.6
PRO	1060.9	9.2	1091.9	9.1	1053.3	9.3
TYR	219.1	1.9	225.5	2	203.9	1.8
VAL	565.1	4.9	581.5	4.8	521	4.6
MET	92.3	0.8	94.9	0.8	79.3	0.7
CYS	69.2	0.6	71.2	0.5	56.6	0.5
ILE	761.1	6.6	783.3	6.9	770.2	6.8
LEU	657.3	5.7	676.5	5.6	634.3	5.6
PHE	288.3	2.5	296.7	2.4	260.3	2.3
LYS	495.9	4.3	510.3	4.2	464.4	4.1

Figure 26:

Chromatograph of pool I amino acid analysis of *JIM 101* immuno affinity purified molecules. Run time is 26 minutes, injection volume is 50 :l and UV absorbency is at 254 nm.

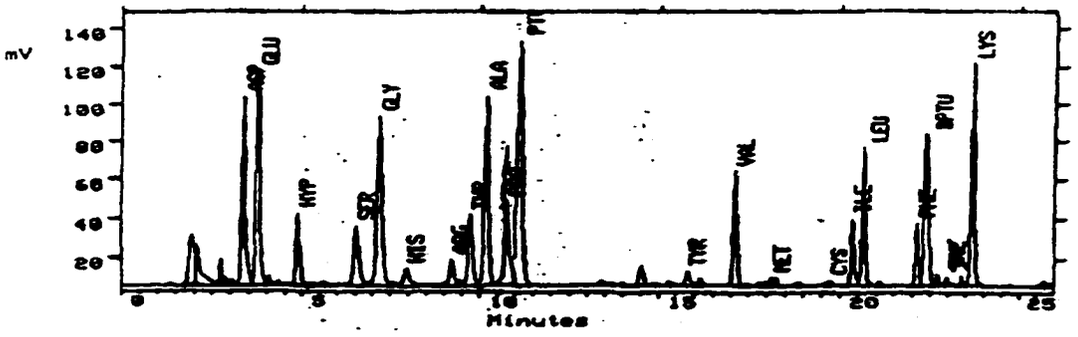
Table 7:

Amino acid analysis of protein moiety of ***JIM 103*** immuno-affinity purified AGPs molecules. Pool I, II and III are combinations of different eluants collected on different dates. [Res. = Residue of amino acid recovered]

Residue	Pool I		Pool II		Pool III	
	p mol	% of Res.	p mol	% of Res.	p mol	% of Res.
ASX [ASP+ASN]	756.5	10.0	789.7	10.5	831.2	9.5
GLX [GLU+GLN]	846.0	11.2	862.3	11.5	1023.6	11.7
HYP	504.4	6.7	467.4	6.2	498.7	5.7
SER	651.0	8.6	701.1	9.4	743.7	8.5
GLY	1220.2	15.2	1168.5	15.6	1285.1	14.7
HIS	130.2	1.7	104.8	1.4	131.2	1.5
ARG	134.2	1.8	145.1	1.9	140.0	1.6
THR	480.0	6.0	499.8	5.6	542.4	6.2
ALA	789.1	9.8	781.7	9.7	831.2	9.5
PRO	691.5	8.5	725.3	9.1	805.0	9.2
TYR	105.8	1.6	88.6	1.1	122.5	1.4
VAL	464.0	5.7	488.1	6.0	568.7	6.5
MET	65.0	0.9	64.5	0.9	78.7	0.9
CYS	65.0	0.9	63.2	0.8	70.0	0.8
ILE	154.6	2.1	129.0	1.7	140.0	1.6
LEU	506.7	6.2	378.8	5.1	454.9	5.2
PHE	179.0	2.4	145.1	1.9	201.2	2.3
LYS	309.1	3.9	362.6	4.8	279.9	3.2

Figure 27:

Chromatograph of pool I amino acid analysis of *JIM 103* immuno affinity purified AGPs molecules. Run time is 26 minutes, injection volume is 50 :l and UV absorbency is at 254 nm.



same AGP. Although the mol percent of many of the amino acids comprising their core proteins are very similar, there are few so distinctly different that it seems clear that the two McAbs specify different AGPs.

Most important to the main hypothesis being tested in this study is the finding that the JIM 101-purified and JIM 103-purified AGPs have amino acid compositions that are distinctly different from those from the suppressed plants and/or the medium in which they had been growing.

These data provide the best evidence obtained thus far to support the hypothesis that ammonium ion exerts its influence on cell proliferation *vis a vis* leaf and branch development in *G. inflata* by virtue of modifying the structure (composition) and therefore function of certain AGPs that function to help locally suppress cell proliferation (Basile and Basile 1993; Basile et al. 1999). Conversely, these data do not support the hypothesis that ammonium ion is acting as an antagonist of hydroxyproline protein synthesis; at least not in the manner of 3-4 dehydropoline, 2, 2-dipyridyl, or Lycorene (Basile and Basile 1980). These three compounds antagonize normal hydroxyproline-protein synthesis by impeding the conversion of peptidyl-proline to hydroxyproline (Basile 1990; Basile and Basile 1990 and literature cited therein). On the contrary, the presence of ammonium ion appears to have caused a significant increase in the mol percent of hydroxyproline present in the core proteins of JIM 101- AND 103-specified AGPs.

Table 8:

Amino Acid Composition Analysis in mol % of crude and immunoaffinity purified JIM 101 and JIM 103 AGPs of Gymnocolea inflata.

Amino Acids	JIM 101 Affinity Purified	JIM 103 Affinity Purified	Knop Media	Knudson Media	Suppressed Plant (Knop)	Desuppressed Plant (Knudson)
ASX	7.2	10.0	10.6	8.3	2.7	2.8
GLX	8.5	11.1	11.6	14.5	7.8	4.6
HYP	5.7	6.2	2.4	5.3	3.8	2.5
SER	10.0	8.7	9.9	7.2	8.9	9.8
GLY	13.3	14.8	13.6	16.6	15.7	14.2
HIS	2.4	1.5	1.6	1.1	1.3	1.9
ARG	3.6	1.7	2.4	4.5	6.9	6.8
THR	5.6	5.9	6.9	5.4	6.3	9.0
ALA	7.5	9.5	10.6	9.5	11.4	13.5
PRO	9.2	8.9	5.7	8.3	5.8	5.5
TYR	1.9	1.2	1.2	1.0	1.4	1.3
VAL	4.8	6.1	5.6	4.3	6.1	6.4
MET	0.8	0.9	0.8	0.5	1.1	0.9
CYS	0.5	0.8	0.7	0.4	0.3	0.4
ILE	6.8	1.7	2.9	2.3	4.2	3.4
LEU	5.6	5.4	6.8	4.4	7.6	7.1
PHE	2.4	2.2	3.7	2.0	3.0	2.9
LYS	4.2	3.7	3.1	4.5	5.6	7.0

Although no mechanism comes to mind to suggest how this is being done, it appears from the data in Table 8 that ammonium ion is altering normal hydroxyproline protein and/or AGP synthesis by promoting the incorporation of both proline and hydroxyproline into the core proteins of certain AGPs. Both of these amino acids are in considerably higher concentration in the AGPs secreted into the culture medium by desuppressed plants than in the AGPs either secreted or retained by the suppressed plants. It should be noted that the hypothesis

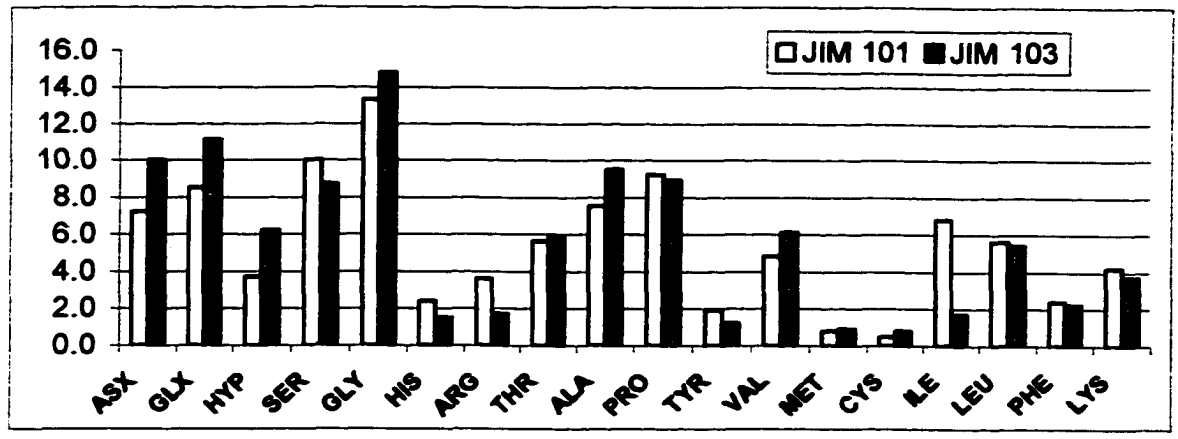


Figure 28:

Column chart showing comparative studies between the amino acid compositions of the immunoaffinity purified JIM 101 and JIM 103 molecules. This statistical comparative study of both amino acids is showing a degree of variance equal to 0.41648. This variance is the formula result of a famous statistical analysis known as "F TEST" to study the degree of equality between the two different "molecules". The value of this test is another proof that the amino acids of both JIM 101 and JIM 103 are somewhat significantly different.

does not require that prolyl hydroxylation is impaired, but only that normal hydroxyproline-protein synthesis is altered.

A change in the normal number and /or distribution of hydroxyproline residues has the same end result. The number or distribution of potential O-glycosylation sites present on the hydroxyproline will be altered, thereby altering the normal structure-function relationship. This interpretation is supported by the findings that the addition of hydroxyproline to the culture media of liverworts (Basile and Basile 1990), a moss (Mignone and Basile in press) and a flowering plant (Rauh and Basile in press) has the same developmental effect. In each case exogenous hydroxyproline acted to locally "desuppress" cell proliferation in

organ primordia, the development of which would otherwise be suppressed. It has been established that when exogenous hydroxyproline acts to alter the normal pattern development, it does so by virtue of being directly incorporated into hydroxyproline-containing proteins, thereby increasing rather than decreasing the number and distribution of O-glycosylation sites (Basile et al. 1987).

2. Comparative Analysis of JIM 101- and JIM 103-Binding Carbohydrate Epitopes:

It seems unlikely from the results of the comparative amino acid analysis that that the finding that both McAbs were selective for AGPs with the same apparent molecular weight (i.e. 97 KDa) was based on their affinity for the core proteins. Moreover, in those cases in which epitope analysis has been carried out for a McAb that specified an AGP, the epitope recognized by the McAb has been against a carbohydrate moiety (Nothnagel 1997).

To find out if JIMs 101 and 103 were binding the same carbohydrate epitope, the peptide bonds were cleaved by means alkaline hydrolysis and the epitopes affinity selected using the same JIM 101 and JIM 103 affinity columns used for affinity purifying the native AGPs. The results of comparative analyses of acid hydrolysates of the oligosaccharides obtained this way are presented in Tables 9 and 10 and Figures 29 through 31.

It may be seen by referring to Table 9 that arabinose and galactose are, by far, the most abundant monosaccharides present, further supporting the conclusion that both McAbs specify "arabinogalactan-proteins". On the other

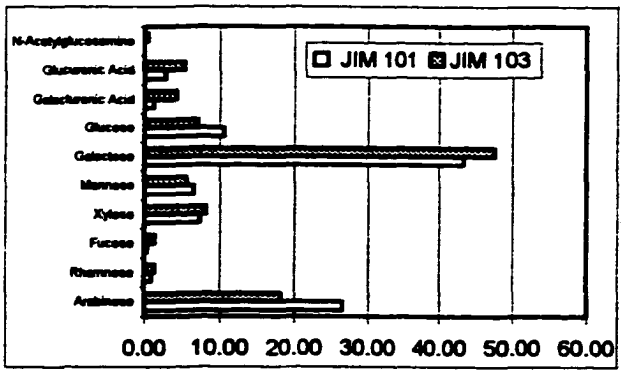
Table 9:
Sugar Compositions Analysis of the Oligosaccharides Containing the Epitopes for JIM 101 and JIM 103 Monoclonal Antibodies.

SUGAR	JIM 101	JIM 103
Arabinose	26.50	18.20
Rhamnose	0.95	1.40
Fucose	0.40	1.45
Xylose	7.40	8.20
Mannose	6.50	5.70
Galactose	42.50	47.70
Glucose	10.60	7.15
Galacturonic Acid	1.25	4.30
Glucuronic Acid	2.85	5.45
N-Acetylglucosamine	0.00	0.45

hand, it is difficult to deduce from these data why the two McAbs seem to be specifying the same AGPs based on comparative SDS-PAGE analysis. Some of the differences in monosaccharides composition are small enough to be attributed to experimental error. The differences in uronic acid content and the absence of detectable N-acetyglucosamine in the JIM101-specified epitope make it clear that the two McAbs are binding to two different oligosaccharides. Samples of the sugar composition chromatographs are showing for both: JIM 101 (Figure 30) and JIM103 (Figure 31).

Figure 29:

Bar graph showing comparative studies of the carbohydrate compositions in both JIM 101 and JIM 103 molecules.



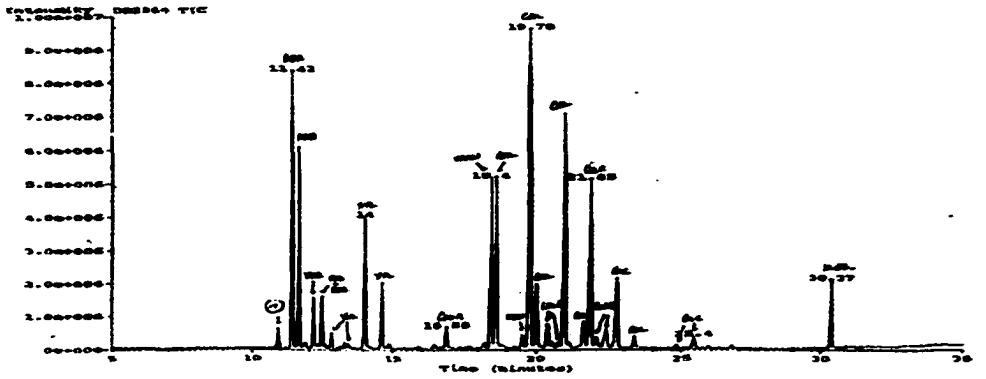


Figure 30:

GC-MS Chromatograph of alkaline hydrolyzed and affinity purified JIM 101 sugar compotion analysis. The Tri-Sil derivatized samples were run on GC for 35 min. using Supelco column. Myo-inositol was also added (20μg) as an internal standard.

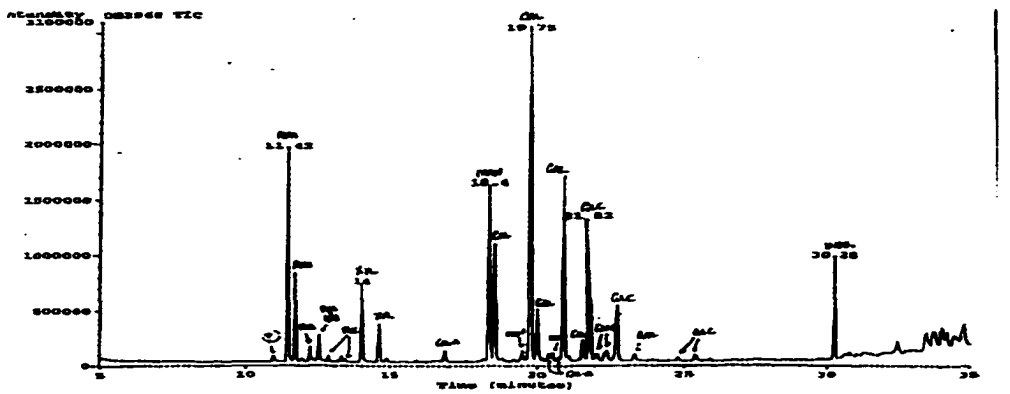


Figure 31:

GC-MS Chromatograph of alkaline hydrolyzed and immunoaffinity purified JIM 103 sugar composition analysis. The Tri-Sil derivatized samples were run on GC for 35 min using Supelco column. Myo-inositol was also added (20μg) as an internal standard.

3. Carbohydrates Linkage Analysis of JIM 101 and JIM 103:

The results of linkage analyses performed on the two JIM-specified oligosaccharides given in Table 10 show that both contain a substantial number of β-D (1-3) and β-D (1-6) Gal residues. Because "The defining structural features of the glycan chains of AGPs are a (1-3)-β-D-galactan backbone with (1-6)-β-D-galactan sidechains that in turn carry numerous α-L-arabinosyl residues..." (Nothnagel 1997), it is highly probable that the selected epitopes are from *bon fide* AGPs. Also characteristic of AGPs is a predominance of D- galactosyl and L-arabinosyl residues. Moreover, the wide variation with respect to other sugar present in the two epitopes analyzed is also to be expected (Nothnagel 1997).

Table 10:

Sugar Linkage Analysis using GC-MS of the oligosaccharides containing the epitopes of JIM 101 and JIM 103.

SUGAR	JIM 101 Mol. %	JIM 103 Mol. %
t Ara (f)	22.30	25.00
t Ara (p)	3.60	6.10
t Gal (p)	15.50	14.70
4 Ara or 5 Ara	1.80	4.80
3 Gal	13.00	16.50
4 Glu	3.65	1.60
6 Gal	10.50	6.50
4, 6 Glu	6.50	0.0
4, 6 Gal	6.60	1.15
3, 6 Gal	14.90	19.65
3 Ara	1.00	1.75

The analysis revealed that, as is the case with the composition analysis, the neutral monosaccharides Gal and Ara were the most abundant residues, with Gal higher in concentration.

The linkage composition appears to be of AGP type II (Aspinall, 1973). Both JIM 101 and IM 103 have substantial amounts of (1,3)-Gal and (1,6)Gal residues (Table 10).

Although type II arabinogalactan proteins show great structural diversity, they have in common a main chain of β-D (1,6)Gal residues.

Gymnocolea inflata share these features with a number of other plants, such as AGPs from sycamore suspension culture (Aspinall et al., 1969), cell wall & plasma membrane of Rose cells (Komalavilas et al., 1991, Serpe and Nothngel, 1995), and stigma/style of Nicotiana alata. In Gymnocolea inflata the side branching, however, is not limited to (3,6) Gal. The (4,6) Gal and (4,6)Glu residues were present in JIM 101 but only a small amount of (4,6) was present in JIM 103. In addition, terminal galatrose and arabinosyl are detected in two forms furanosyl (f) and pyranosyl (p). The Ara (f) is found to be more abundant than the (p). Other residues were also detected but in a smaller amounts; tGlu , traces of 3, 4, or 5 Ara. The two acidic sugars, Glucuronic Acid and Galacturonic Acid, although detected in composition analysis were not detected in the linkage analysis. Rhamnose and fucose were shown as minor residues too spare to be identified.

In Conclusion..

All the analyses performed during the course of this investigation into the nature of the Yariv β-glucosyl reagent-positive molecules specified by the McAbs JIM 101 and JIM 103 support the hypothesis that they are specific for AGPs.

Since the AGPs of the two McAbs specify were only secreted into the culture medium after ammonium ion induced the "desupression" of cell proliferation relative to leaf and branch development, these results are also supporting the hypothesis that there is a causal relationship between the ammonium-ion induced desuppression and the secretion of the McAb-specified AGPs (Basile et al. 1999).

How the presence of ammonium ion in the culture medium could cause such a profound change in the pattern of development of *Gymnocolea inflata* plants can only be partially understood from these data. The partial explanation is related to the finding that the amino acid composition of the core proteins that bind to JIMs 101 and 103 are distinctly different from the plants when they are expressing their normal pattern of (suppressed) leaf and branch development.

In series of experiments, antagonists of hydroxyproline-protein synthesis had the same effect on four other species of leafy liverworts as ammonium ion had on *G. inflata*. Based on the results obtained by using these antagonists, it was hypothesized that the observed desuppressed leaf and branch development was a consequence of the antagonists altering the structure and thereby the function of some hydroxyproline-containing morphoregulatory molecules, probably AGPs (Basile 1980, 1990; Basile and Basile 1990, 1993).

Before this study was undertaken, the only evidence to support this hypothesis were differences in Yariv β-glucosyl regent-positive material that were revealed by comparative electrophoretic, CsCl density gradient (Basile and Basile 1993), and immunochemical analyses (Basile et al. 1999).

Now, we can be more confidently stating that the Yariv's β-glucosyl reagent-positive macromolecules released by *Gymnocolea inflata* when leaf and branch development were "desuppressed" are actually AGPs. These Two groups of released AGPs showed two different epitopes to JIM 101 and JIM 103. The amino acid compositions are significantly different, with Hyp higher in the released molecules than the ones stayed bound. Both whole molecules and deglycosylated are present in repeated units of monomers, dimers, trimers or tetramers.

The challenge facing investigators is to distinguish among the mechanisms by which ammonium ions cause the release of the higher hydroxyproline containing AGPs. Possible mechanisms include breaking bonding, altering structures of some AGPs on the expense of others, moving molecules or altering localized electric charges.

APPENDIX I

Knop Macronutrients Media

KNO_3	0.125 g/l
$MgSO_4 . 7H_2O$	0.125 g/l
$Ca(NO_3)_2 . 4H_2O$	0.5 g/l
KH_2PO_4	0.125 g/l
Dextrose	1% w/v
Vitamine B12 (cyanocobalamine)	0.1% v/v (74 μM)
Hutner's Metals "49"micronutrient	1% w/v

- Ingredients were dissolved in 700 CC deionized water
- pH adjusted to 4.6 with N NaOH and N HCl.
- total volume was increased to 1000 CC using distilled water.
- Media were autoclaved at 121° C and 15 psi.

APPENDIX II

Knudson Macronutrients Media

$(NH_4)_2SO_4$	0.5 g/l
$MgSO_4 . 7H_2O$	0.25 g/l
$Ca(NO_3)_2 . 4H_2O$	1.0 g/l
KH_2PO_4	0.25 g/l
Dextrose	1% w/v
Vitamine B12	0.1% (74μM)
(cyanocobalamine)	
Hutner's Metal "49"	1 ml

- The ingredients were dissolved in 700 CC distilled water
- pH adjusted to 4.6 with N NaOH and N HCl.
- total volume was increased to 1000 CC using distilled water.
- Media were autoclaved at 121° C and 15 psi.

APPENDIX III

Extraction of AGP from Dried Plant

Material:

- 400 mg of freeze dried plant.
- Barbitol extraction buffer, 10-15 ml, pH 8.6 (Sigman Barbitol Buffer) containing: 0.2 M $CaCl_2$, 5mM ethylenediamine tetracetic acid (EDTA), 3.2 mM DL-dithiothretol (DTT), 0.05% w/v 3- [(3-chloramidopropyl) dimethlamonio] 1-propanesulfonate (CHAPS, and 0.02% w/v NaN_3.
- Miracloth (Visking Corp., Chicago, IL).
- Grinding Sand.

Procedures:

1. Samples of 400 mg dry weight were grinded in 10 ml of ice cold extraction buffer using a pestle and mortar to which a small amount of acid washed sand was added.
2. After grinding, the extract was filter-centrifuged at 3,000 RPM for 15 minutes through two layer of Miracloth.
3. The material retained by the Miracloth was grinded once again in an additional 10 ml of extraction buffer and filter- centrifuged.
4. The two extracts were pooled and centrifuged at 25,000x *g* for one hour at 4° C.

5. The protein in the supernatants (10 ml) was precipitated by diluting the suspension (v/ v) 80% with 95% ethyl alcohol, incubating at 4° C for 2 hours or overnight, then centrifuging at 3,000x *g* for 30 min.
6. The pellet was re-dissolved in deionized water, the solution brought again to 80% with ethanol (95%), and precipitated twice more. After the third precipitation the sample was finally dissolved in deionized water.
7. The approximate concentration of AGPs was determined by radial diffusion assay (van Holst & Clarke 1985), and the sample freeze-dried for later use.

Appendix IV

Isolation of AGPs from Media

Material & Equipments:

Same as Appendix III

Method:

1. Culture media containing AGPs was pooled and concentrated to approximately 10% of the original volume using an Amicon HIP 30-20 hollow fiber cartridge.
2. Follow steps from 6 to 9 under Appendix III

Appendix V

Radial Diffusion Assay with Yariv's Reagent

This follows a method described by van Holst & Clarke, 1985.

Materials:

- Agarose
- NaCl (o.15 M) with 1% NaN_3
- Yariv's Reagent, β-glycosyl

Procedure:

1. The required amounts of:	NaCl (0.15 M) with 1% NaN_3	agarose	Yariv's
Amount per large Plate	25 ml	0.25 g	500 µl
Amount per small Plate	3.5 ml	0.033 g	70 µl

are mixed together.

2. Heated for 30 sec. with 10 sec. at a time to prevent solution from boiling.
3. Yariv's reagent was then added. All the components were mixed well.
4. A 10 ml pipette size was used to dispense the gel into plates.
5. Gel was left to polymerize on a flat even surface, then wells were made each with a capacity of 8 µl.

Appendix VI

Dot-Blot Immuno-binding Assay

This assay was performed according to Smith et al. (1984).

Materials:

- *Tris Buffered Saline (TBS) 10x stock:*

  80 g NaCl, 4 g KCl, and 30 g Triazma Base (THAM) all in 800 ml distilled H_2O then pH was adjusted to 7.5 (using HCl) finally the whole volume was brought to 1000ml and 0.02% NaN_3 was added.

- *TBS Working Solution:*

  100 ml of TBS 10x stock was brought up to 1000 ml with distilled water.

- *TBS with 3% Bovine Serum Albumin (BSA) w/v (Blocking Solution):*

  Add 3% of BSA to the prepared solution of TBS (W/V).

- *Alkaline Phosphatase Buffer:*

  Per one liter total volume: 5.54 g NaCl (100 mM), 12.1 g Tris (100 mM), and 1.0 g MgCl. 6 H_2O (5 mM). All in 800 ml dist. , pH was adjusted to 9.5, and then the final volume was brought up to 1000 ml.

- *Nitro Blue Tetrazolium (NBT):*

  0.5 g/ 10 ml dimethylformamide (70% w/v) [enzyme specific for antigen - antibody complex).

- *Bromochloroindo ---phosphate (BCIP):*

  0.5 g/ 10ml dimethylformamide (100%)[enzyme substrate]

- *Nitrocellulose Sheets:*

  Each sheet of nitrocellulose was ruled into 1-cm squares

- Using a paper template and a light box.

Procedures:

1. One microliter drops containing 0.2-0.3 mg /ml AGP as determine by radial diffusion assay (van Holst & Clarke, 1985) was applied to each square using a P-10 Pipette and allowed to dry.
2. Right before performing Dot Blot Assay, the nitrocellulose paper was soaked in water in a petri dish for a couple of min. The water was then discarded.
3. TBS with 3% BSA was added (about 10 ml/ plate) then left on a "tippy" shaker at room temperature (RT) for I hour to block areas which were not covered with the antigen (AGP).
4. The primary monoclonal antibody (20 µl)[specific in its binding] was added to the TBS with 3% BSA in each plate then placed back on the "tippy" shaker at RT for half an hour.
5. To wash excess antibody, the solution in plate was poured off, then a 10 ml of TBS working solution was added & left to wash excess antibody for 5 min.
6. The previous step in repeated 4 times each for 5 min.
7. The secondary antibody (alkaline phosphate conjugate-goat anti-rat antibody) was added in a 10 ml of Blocking Buffer & plate was put back on the "tippy" shaker.

8. Excess secondary antibody was rinsed off for 5 times each for 5 min. using TBS working solution. Then the 6th wash was done also for 5min. using alkaline phosphate buffer (pH 9.5).
9. Then the solution was discarded and another 10 ml of alkaline phosphate buffer to which 66 μl of NBT and 33μl of BCIP were added.
10. Nitrocellulose sheets were left on shaker until color develops (about 10 minute or less). Then finally rinsed with H_2O.

Appendix VII

Alkaline Hydrolysis with $Ba(OH)_2$ of Knudson Media AGPs for the Release of Oligosaccharides

This method causes the cleavage of polysaccharides at O-linkage to release the Oligosaccharide.

Materials:

- $Ba(OH)_2$ (0.2 M) in dist. Water.
- Heating Block
- 1 N H_2SO_4

Procedure:

-Samples were hydrolyzed in 0.2 M $Ba(OH)_2$ for 18 h at 105° C, neutralized with 1 N H_2SO_4, and freez-dried as described by Lamport and Miller, 1971.

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